Methods in Molecular Biology 1393

Springer Protocols

Attilio Bondanza
Monica Casucci *Editors*

Tumor Immunology

Methods and Protocols

Humana Press

METHODS IN MOLECULAR BIOLOGY

Series Editor
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ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-4939-3336-5 ISBN 978-1-4939-3338-9 (eBook)
DOI 10.1007/978-1-4939-3338-9

Library of Congress Control Number: 2015958743

Springer New York Heidelberg Dordrecht London

Printed on acid-free paper

Humana Press is a brand of Springer
Springer Science+Business Media LLC New York is part of Springer Science+Business Media (www.springer.com)

Preface

Tumor immunology is the branch of immunology that studies the interactions between the immune system and tumor cells. Closely related to tumor immunology, cancer immunotherapy is a growing field of therapeutic intervention aimed at exploiting immune cells and molecules to fight human tumors. Given the extremely high human and economical burden that cancer brings to our industrial societies, it is understandable how, from its very beginnings at the end of the nineteenth century, tumor immunology was shaped around the promise of effective cancer immunotherapy. Conversely, passed a phase of profound skepticism witnessed in the 1970–1990s, it took some decades before tumor immunology/cancer immunotherapy, after marking crucial scientific and clinical milestones, affirmed itself as a therapeutically viable form of treatment. With the recent successes of immune checkpoint inhibitors and chimeric antigen receptor (CAR) T cells, nowadays the field has even become fashionable, and "hot," as the generalized press likes to recognize it.

Despite this enthusiasm, it is clear that there is definitively "more than meets the eye," and that, in the near future, superiorly effective and/or less toxic cancer immunotherapies will only follow a better understanding of the rules governing tumor immunology, as well as innovations in the technology of the immunological "smart bullets" (monoclonal antibodies, vaccines, tumor-reactive T cells) used to specifically target cancer cells. All these aspects are covered in this book, whose explicit aim is to open the doors of the laboratories of world-renowned experts in the field and exhaustively gaze into the methods they routinely use to unravel the still undiscovered secrets of tumor immunology/cancer immunotherapy. Our deepest wish is that anyone approaching this book, scientist, clinician, or industry professional, would appreciate its wide breadth of subject coverage and find the information he/she needs for further achievements.

Milano, Italy

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Contents

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Chapter 1

ATMPs for Cancer Immunotherapy: A Regulatory Overview

Maria Cristina Galli

Abstract

This chapter discusses European regulatory requirements for development of advanced therapy medicinal products (ATMP) for cancer immunotherapy approaches, describing the framework for clinical trials and for marketing authorization.

Regulatory critical issues and challenges for developing ATMP are also discussed, with focus on potency determination, long-term follow-up, comparability, and insertional mutagenesis issues. Some of the most critical features of GMP application to ATMP are also described.

Key words Advanced therapy medicinal products, European regulations, Cancer immunotherapy, Clinical trials, Translation

1 Introduction

New promising perspectives have been recently opened for cancer patients with development of advanced therapy medicinal products (ATMP), a new class of medicines defined in Europe by EU Regulation 1394/2007 [1]. ATMP include medicinal products for gene therapy, cell therapy, and tissue engineering (GTMP, CTMP, TEP, respectively). GTMP and CTMP may potentially have a great beneficial impact on cancer treatment, while TEP might help in restoring/replacing tissues damaged by cancer.

Cancer therapy has been one of the most popular ATMP application, either by means of GTMP or by means of CTMP, i.e., "cancer vaccines," now more appropriately termed "cancer immune therapeutics."

A wide variety of GTMP have been used in clinical trials in a wide variety of tumours; as few examples, adenoviral vectors were used to express p53 in head and neck carcinoma [2] or to express CD40L in bladder carcinoma [3]; oncolytic herpes virus encoding GM-CSF was used in patients with melanoma [4]; T cells engineered with chimeric antigen receptors (CARs) redirected against various tumor-associated antigens have been recently used [5].

Attilio Bondanza and Monica Casucci (eds.), *Tumor Immunology: Methods and Protocols*, Methods in Molecular Biology, vol. 1393, DOI 10.1007/978-1-4939-3338-9_1,

Among cell therapy approaches to cancer treatment, use of autologous dendritic cells (DCs) loaded with tumor antigens has been pursued actively [6], in the attempt to elicit clinically relevant immune responses. In 2010, FDA approved a DC-based therapeutic vaccine for prostate cancer [7].

This chapter aims at giving a regulatory overview of the issues linked with development of ATMP for cancer immunotherapy, in an EU perspective.

2 European Regulatory Frame for ATMP

In Europe ATMP are considered as medicines; therefore they are covered by the European legal frame for medicinal products through the EU Regulation 1394/2007 [1]. This regulation contains the first legal definition of TEP, while the current EU definition of GTMP and CTMP is contained in the new Annex 1 recently issued amending Directive 2001/83/EC [8].

GTMP definition includes the concept of a recombinant nucleic acid being used in patients, either ex vivo or in vivo, for regulating, repairing, replacing, adding, or deleting a genetic sequence, whose expression is directly related to the intended clinical effect. In general, a wide variety of products such as viral or nonviral vectors, plasmid or bacterial vectors, recombinant oncolytic viruses, and genetically modified cells can be considered GTMP. It should be noted that, according to new Annex 1, vaccines for infectious diseases no longer fall under GTMP definition [8].

CTMP and TEP definition is based on two concepts: (1) cells or tissues substantially manipulated so that to alter their properties or (2) cells or tissues intended to be used in the recipient for an essential function different from the donor original one. The difference between CTMP and TEP lays in the mechanism of action: while CTMP act through pharmacological, immunological, or metabolic action [8], TEP are aimed at regenerating, repairing or replacing a human tissue [1].

EU Regulation 1394/2007 puts ATMP within the frame of EU Regulation 726/2004 that stipulates no medicinal product can be marketed in Europe without an approval. For biological medicines, such as ATMP, EU-wide market authorization is granted (or denied) by the European Commission through the centralised procedure taking place at the European Medicine Agency (EMA) [9].

EU Regulation 1394/2007 has also created a specific Committee for Advanced Therapy (CAT) that provides EMA with scientific expertise in ATMP relevant areas. Scientific scrutiny of ATMP marketing authorisation applications is a CAT task, but the Committee for Human Medicinal Products (CHMP) is still responsible for giving EMA final opinion. After EU Regulation

1235/2010 came into force [10], the ATMP marketing application evaluation procedure requires the interplay of three Committees: CAT, Pharmacovigilance Risk Assessment Committee (PRAC) and CHMP, while the marketing application for a non-ATMP drug requires only PRAC and CHMP. Since the timetable for giving final recommendation is anyway 210 days, in order to accommodate the three Committee actions within the deadline the time frame for ATMP evaluation by CAT is shortened as compared to a less complex drug, e.g., a new chemical compound being evaluated by CHMP.

Two new procedures specific for CAT, namely ATMP classification and certification of quality and non-clinical data, have been set by EU Reg. 1394/2007.

ATMP classification procedure is useful when a medicinal product might be considered borderline with other types such as a transplant. Even though not legally binding, CAT recommendation on classification may help to direct development along the correct pathway. CAT issued a reflection paper on this procedure [11]. It should be noted that EU Reg. 1394/2007 stipulates that a product which may fall within the definitions of somatic cell therapy/tissue engineered product and of gene therapy product shall be considered as a gene therapy product [1].

Certification of quality and non-clinical data is available for SME only; it is considered an incentive to developers who wish to sell their product at an early stage to another developer. This procedure does not envisage an early approval, rather it determines (in a non-legally binding way) whether the data obtained at a given developmental stage are in line with requirements for a full application dossier. The minimum quality and non-clinical data needed for certification are described in a guideline recently issued by CAT [12].

European Regulation 1394/2007 sets a legal requirement for an active traceability system that ensures to track ATMP to the patient who received it and vice versa, for 30 years after product expiry date. It is a long-term goal, connected with the need to increase our knowledge on ATMP long-term effects.

For the same purpose, another requirement is specifically set in the Regulation: long-term follow-up of ATMP safety and efficacy. A pharmacovigilance plan, with risk management measures, for which guidance is given in a specific EMA document [13] is required in the ATMP market application dossier.

3 Guidance for ATMP Clinical Development

Drug development includes basically two steps: preclinical and clinical development. The first part includes designing the substance that represents the medicine, proving its activity and testing its safety in a preclinical model, while the latter part includes translating those results into human subjects by means of clinical trials.

As for any other medicine, clinical trials with ATMP are covered by the Directive 2001/20/EC [14] that stipulates that for all medicines clinical trial approval is the responsibility of Competent Authority in each European Member State (MS). Therefore clinical development takes place at national level. For any given clinical trial to be performed in a given MS, a separate evaluation and authorization procedure are carried out. This is also the case for an European multinational trial, which may represent a difficult task if reviewers have divergent opinion in the different EU MS on the same clinical trial proposal. Procedures and initiatives have been put in place by EMA and national Competent Authorities to decrease the chance that such divergences occur and to facilitate an efficient translation of research discoveries into effective ATMP.

A number of guidance documents describing quality, preclinical, and clinical requirements for ATMP are available through the EMA website [15], to help applicants in developing their products.

A risk-based approach is applicable during the ATMP life cycle (clinical development, market authorisation application, postmarketing), as described in the current legislation [8] and in a specific guideline recently issued by the European Medicine Agency (EMA) [16].

The guideline on human cell-based medicinal products [17] and the guideline on quality, preclinical, and clinical aspects of gene transfer medicinal products [18] are the main guidance documents for CTMP/TEP and for GTMP, respectively, covering in general all issues faced during development.

In addition, other guidance documents further elaborate on specific aspects such as chondrocyte-based CTMP [19], potency testing of cell-based immunotherapy medicinal products for the treatment of cancer [20], clinical aspects related to TEP [21], genetically modified cells [22], risk of germ-line transmission for GTMP [23], long term follow up for GTMP [24], environmental risk assessment for GTMP [25], non-clinical studies required before first clinical use of GTMP [26], reflection paper on stem cell products [27], reflection paper on design modifications of GTMP during development [28], and reflection paper on management of clinical risks deriving from insertional mutagenesis [29].

4 Critical Issues for ATMP in Cancer Immunotherapy

Potency (i.e., the quantitative measure of biological activity) is a required quality parameter for a biological medicinal product. Determining the biological activity of cell-based immunotherapy products is not an easy task, since the active ingredient is composed of whole cells and their activity cannot be generally attributed only to a single cellular feature. EMA has issued a guideline that discusses this issue [20]. Potency of cell-based immunotherapy products is

measured using appropriately validated in vivo or in vitro tests that should be based on a defined biological effect as close as possible to the mechanism(s) of action/clinical response. Several assays are based on the principle that cellular immunity ultimately should lead to tumor cell destruction; however the mechanism of action may be more complex involving immune mechanisms which are still only partially understood. In addition, these assays may be complicated by multi-antigen formulations and starting material inherent variability, particularly for autologous cells. It is also possible that different assays may be used depending on the purpose, e.g., for characterisation or for validation purposes. If feasible, a suitable potency assay should be in place when the first clinical trial is carried out.

Changes in product and/or in process are frequently made during development, generally aiming at increasing product safety/efficacy profile. For cancer immunotherapy products based on genetically modified cells, guidance given in a recently issued EMA guideline [24] may be useful. Changes may be related to the vector used to transduce the cells, e.g., a different promoter, or may relate to the cells themselves. In any case, evaluation of change impact on safety/efficacy profile should address for instance if modified product has different characteristics (e.g., expression level, tissue tropism) that might have altered previously established toxicity or biodistribution. Further non-clinical or clinical studies may be needed (e.g., toxicity if expression level is increased, biodistribution if new tissue tropism has arisen). In some cases, such an exercise might conclude that the changes have resulted into a different product. Similar general concepts are applicable to CTMP used in cancer therapy.

Monitoring for short- or long-term safety is a regular activity in the clinical use of any medicine. Given ATMP complexity, follow-up of efficacy in addition to safety is a crucial aspect, acknowledged also in the legislation. EU Reg. 1394/2007 [1] requires that the dossier for an ATMP marketing authorization application contains a risk management plan describing a patient monitoring plan to ensure safety as well as efficacy long-term follow-up. As this is a relatively new field, a general guideline was developed by EMA describing aspects of pharmacovigilance, risk management planning, safety, and efficacy follow-up for ATMP [13]. GTMP-specific characteristics should drive risk management plan design. Thus a guideline on follow-up of patients administered with GTMP [24] has been issued giving recommendations for clinical monitoring and follow-up. A clinical monitoring plan should aim at detecting, at different time points after therapy, signals of adverse reactions or of declining efficacy in order to prevent their clinical consequences and to ensure timely treatment. The monitoring plan should take into consideration product risk profile, disease, comorbidity, and patient population characteristics.

In order to fulfil its goals, the clinical follow up protocol should have very clear objectives and be hypothesis driven, while at the same time being feasible both for patients and clinicians. Risk is linked to potential for adverse events as well as to potential for declined/lack of efficacy. While in cancer therapy long term efficacy is related to tumour relapse, the type of gene therapy approach should be taken into account when profiling risk with regard to safety. In the case of gene therapy aimed at stimulating immune responses against tumor, local, and systemic antigen-specific responses have been reported [4]. Immune-toxicity as a result of enhanced persistence or modulation of regulatory cell subpopulations should thus be considered in the follow-up plan. When using recombinant oncolytic viruses for cancer therapy, selective replication in tumour tissue is exploited to destroy the tumour without damaging normal tissues. The risk of virus spreading to and acting on nontarget cells should be taken into account in the follow-up plan.

For cancer immunotherapy products based on genetically modified cells, also the clinical risk deriving from insertional mutagenesis of integrating vectors should be taken into account. Integrating vectors are able to permanently insert into the cell genome. Serious adverse events may be caused by insertional mutagenesis, that actually has been reported in 12 patients treated with transduced hematopoietic stem cells containing gammaretroviral vectors. In all cases, transformed cell clones harbored vector insertions next to proto-oncogenes leading to their activation; cells subsequently progressed into malignancy [30]. Relevant guidance on this matter is given in a recently issued EMA reflection paper [25]. It discusses the factors contributing to genotoxicity, the strategies to reduce risks associated with insertional mutagenesis, the methods to evaluate vector oncogenicity. Risk factors are related to vector (backbone and regulatory elements, insertion profile, dose), to transgene (biological activity of its product), and to target cell population. Depending on the available results from previous studies with the vector, integration data may be required at preclinical level to assess toxicology and/or biodistribution as well as at clinical level for monitoring potential safety issues identified in non-clinical studies. Immunological data and clinical observation are needed to complete molecular data.

Development of ATMP has also opened new scenarios at production process level, as ATMP are new pharmaceutical entities. While viral vectors may be compared to vaccines, cell-based products cannot be compared to any other classical drug. ATMP are biological [8] and injectable medicinal products: thus they must be sterile and apirogenic and their production must comply with applicable requirements: good manufacturing practice (GMP). EU GMP Annex 2 [31] gives guidance for biological medicinal products production: it has been recently revised to incorporate specific guidance for ATMP. Like all biological ATMP

may potentially carry pathogens, in addition they are optimal substrate for contaminating microorganism growth such as viruses, mycoplasm, and bacteria. As a consequence, not only aseptic production is always needed for cells/tissues containing ATMP but also a tight control on cell/tissue donor status is required [32]. A general approach to viral safety requirement is also described in the European Pharmacopoiea [33], requiring that any reagent or material of animal origin entering the production process is assessed for viral safety. ATMP production process also carries greater variability than classical pharmaceutical entities, because ATMP contain in the final product a population of living entities. Such variability is inherent in the product, due to donor variability and to process variability, since ATMP all are produced in cell culture systems.

Cell-based ATMP may be patient specific, so each one product is different from the others in the same lot, even though containing the same type of cells. Limitations in cell quantity might arise at sourcing level, when for clinical reasons cell donation can be obtained only once (e.g., infants or very severe diseases) and/or at processing level, because normal cell growth cannot be pushed further its physiological limit without serious risks. When harvested cells are barely sufficient for therapy, there may be a problem with "wasting" cells for quality control (QC) testing. Difficulties in sourcing from donor may also pose an ethical problem with rejecting a product lot, because this would delay therapy. Cell-based ATMP may have a very short shelf life, impacting on starting material/product storage and time for delivering them to patients. For all those reasons, ATMP are very frequently custom-made.

The above-described features have important consequences for many GMP issues: prevention of cross contamination, facility segregation, cleaning validation, QC program, environmental monitoring, and retention samples. Cross contamination prevention measures are critical: one patient at a time should be handled in one production suite, between two successive patients clearing and cleaning procedures should be equal to those for two different products. For cells from infected patients and for viral vectors, segregated areas and dedicated equipment should be used, cleaning procedures used at changeover should be validated to molecular level (e.g., able to detect transgene and/or vector). When final product has a short shelf life and/or limited cell number, traditional GMP procedure should be adapted: results of some QC testing and environmental microbiological monitoring are only available after lot release, retention samples are not possible or are not held in the same conditions as the product, a full QC testing program (i.e., identity, purity, quantity, activity) is not possible unless putting at risk the clinical dose. This has been taken on board in GMP annex 2 revision (ref. [31]) that recognizes the possibility for a modified testing and sample retention strategy and requires a

suitable control strategy be in place, built on enhanced understanding of product and process performance. GMP annex 2 also describes a stepwise batch release procedure, before and after QC results are available. It is thus evident that a robust quality assurance system is of paramount importance and that production process control should be maximized.

5 Conclusion

Expanding our knowledge on ATMP efficacy and safety profile, during development, at marketing level and long term after therapy will allow to develop safer and more efficacious ATMP in the overall strategy against cancer while contributing in the continuous effort to protect European patients.

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Chapter 2

Natural Antibodies to Tumor-Associated Antigens

Sheila María Álvarez-Fernández, Lucia De Monte, and Massimo Alessio

Abstract

Natural autoantibodies raised by humoral immune response to cancer can be exploited to identify potential tumor-associated antigens (TAAs), and might constitute new putative prognostic and/or diagnostic biomarkers. Here we describe how sera from tumor patients can be used to identify TAAs by screening antibody immunoreactivity against the cancer proteome resolved by two-dimensional gel electrophoresis.

Key words 2D-electrophoresis, Immunoproteomics, Tumor-associated antigens, Autoantibodies, Western blot

1 Introduction

The identification of autoantibodies directed against tumor antigens as humoral immune response to cancer has been documented since long time [1]. The recognition by the immune system of self-proteins in cancer is directed against qualitatively and/or quantitatively aberrant proteins defined as tumor-associated antigens (TAAs). Autoantibodies can be used as probes in cancer immunoproteomics to identify potential TAAs, and may be considered as prognostic, diagnostic biomarkers [2–5]. Among the immunoproteomics approaches, the SERological Proteome Analysis (SERPA) [6, 7] combines the separation of tumor proteins on two-dimensional gel electrophoresis (2DE), Western blotting with patients and healthy subjects sera, image analysis, and antigenic protein identification by mass spectrometry (MS) analysis (*see* Fig. 1). This approach allows the screening of a large number of patient sera and to establish the frequency of the reactivity against relevant autoantigens [5]. The high-throughput screening can be done on available tumor cell lines being the serological reactivity comparable to that obtained on the autologous tumor proteome (*see* Fig. 2). In addition, SERPA enables to distinguish immunoreactivity toward protein isoforms and/or directed against posttranslational modifications (PTM), which is of central importance, being the protein immunogenicity frequently

Attilio Bondanza and Monica Casucci (eds.), *Tumor Immunology: Methods and Protocols*, Methods in Molecular Biology, vol. 1393, DOI 10.1007/978-1-4939-3338-9_2,

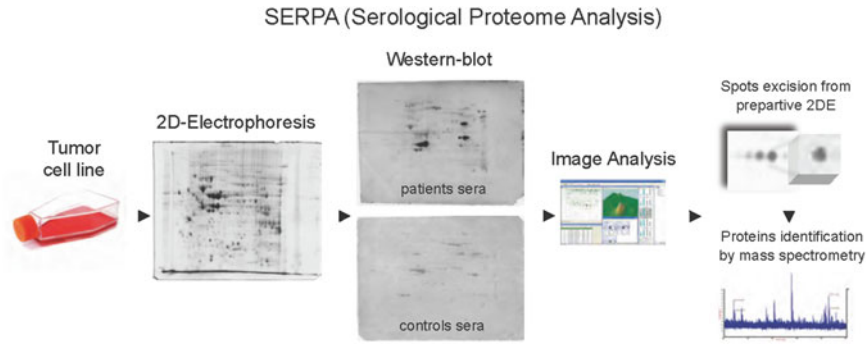

Fig. 1 Schematic representation of the workflow for tumor-associated antigen identification by SERPA that exploits the presence of autoantibodies directed against tumoral proteins in the sera of patients

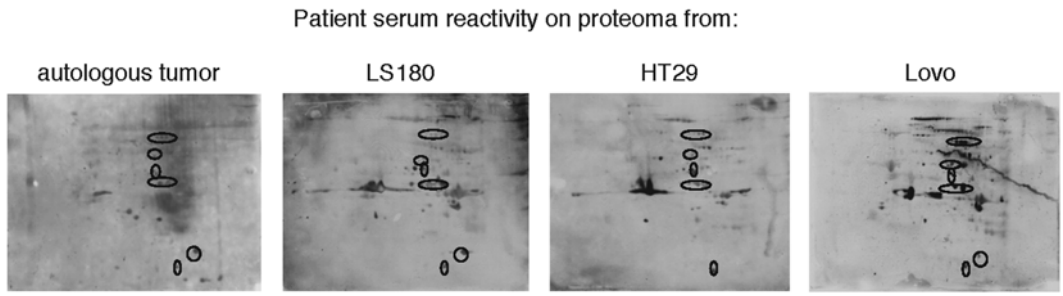

Fig. 2 Proteome obtained from tumor cell lines is representative of the primary tumor proteome. Colon cancer patient serum reactivity on the autologous tumor proteome and on the proteome from three different colon cancer cell lines (LS180, HT29, and Lovo). Shared reactivities are underlined by *circles*

associated to unusual isoforms or to different PTMs occurring during tumoral transformation [8–10].

Furthermore, it has been reported that the antibody response to TAAs changes upon tumor progression, revealing the possible modulation of B cell responses [8]. The TAAs expressed at different stages of a disease can become useful biomarkers for prognosis and/or diagnosis of the cancer. Therefore, with SERPA, it is possible to identify several target antigens in one time, and have a complete view of the B cell response against the tumor in each patient at any given stage of the disease. Nevertheless, which is the role of humoral immune response raised in cancer is still under discussion [4, 11].

The SERPA-based approach has been successfully applied to different types of cancer [4] such us renal cell carcinoma [7, 12–14], neuroblastoma [15], acute leukemia [16], lymphoma [17], melanoma [18], hepatocellular carcinoma [19], lung [20–22], breast [23, 24], pancreatic [9, 25, 26], and gastric and colorectal cancers [8, 27–29]. Several of the antigenic proteins identified by immunoproteomics have already provided novel cancer

biomarker candidates that need to undergo rigorous validation schemes [4, 5].

2 Materials

2.1 Sera Collection and Preparation, Cell Line Culture

1. Human blood samples are obtained from patients and control subjects following informed consent after approval by the Local Research Ethical Committee.
2. Blood collection tubes (BD-Vacutainer) containing no anticoagulant.
3. Sterile cryo vials (NUNC).
4. Tumor cell lines obtained from American Type Culture Collection (ATCC) or similar biological resource agency.
5. Cell culture medium: RPMI 1640 with l-glutamine (Lonza) supplemented with 10 % fetal calf serum (FCS) (Lonza), penicillin (1:100 from 10,000 U/ml stock)/streptomycin (1:100 from 10,000 μg/ml).
6. Phosphate-buffered saline (PBS) w/o Ca^{2+} Mg^{2+} (Gibco).
7. Sterile cell scraper (Euroclone).
8. Cell culture flasks: T25, T75, T162 (Corning or similar products).
9. Polypropylene 15 ml tubes (Falcon).
10. Eppendorf 1.5 ml tubes (Eppendorf).

2.2 Sample Preparation

1. Ultrasonic homogenizer Sonoplus HD 2070 (Bandelin).
2. Rehydratation buffer (RB): 8 M Urea, 4 % CHAPS (3-[(3-cholamidopropyl).dimethylammonio]-1-propanesulfonate) prepared in deionized H_2O (*see* **Notes 1** and **2**). Prepare 1 ml aliquots and store at –20 °C.
3. Dithiothreitol (DTT) 1 M: Dissolved 77.1 mg of DTT in 500 μl of deionized H_2O. Prepare just prior to use.

2.3 Protein Precipitation

1. Acetone 100 %. Store at –20 °C.
2. Acetone 80 % solution in deionized H_2O. Store at –20 °C.

2.4 Protein Quantitation by Bradford Protein Assay

1. BioRad Protein Assay Standard II Lyophilized. Bovine serum albumin (BSA) (*see* **Note 3**).
2. BioRad Protein Assay. Dye reagent concentrate (5×).
3. 5 ml polystyrene round-bottom tube.
4. Polystyrene spectrophotometry cuvettes.
5. Smart Spec Plus Spectrophotometer (BioRad).

2.5 IPG Equilibration and First Dimension

1. Drystrip Tray (Immobiline drystrip reswelling tray from GE Healthcare or similar).
2. Equilibration buffer (EB): Urea 6 M, glycerol 30 %, SDS 2 %, Tris–HCl 1.5 M pH 8.8 (*see* **Notes 4** and **5**). Store at 4 °C.
3. Dithiothreitol (DTT) 130 mM in EB (*see* **Notes 6** and **7**). Prepare just prior to use.
4. Iodoacetamide (IAA) 130 mM in EB (*see* **Notes 7** and **8**). Prepare just prior to use.
5. Bromophenol blue 0.05 %. Store at room temperature.
6. Immobilized pH gradient (IPG) buffer: Carrier ampholytes pH 3–10 NL (GE Healthcare).
7. Mineral oil (BioRad).
8. IPG strips 3–10 NL 7 cm (GE Healthcare). Store at –20 °C (*see* **Note 9**).
9. Ettan IPGphor Ceramic Strip Holder 7 cm (GE Healthcare).
10. Ettan IPGphor Isoelectric Focusing System (GE Healthcare).
11. Strip Holder Cleaning Solution (GE Healthcare).

2.6 Second Dimension: SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Mini-Protean 3 Dodeca Cell apparatus (BioRad) or similar multigel apparatus.
2. Casting frame and glass plate sandwich (BioRad).
3. Casting stand (BioRad).
4. Resolving buffer: Tris–HCl 1.5 M pH 8.8 (*see* **Note 10**). Store at 4 °C.
5. BioRad preweighed 30 % acrylamide/bis 29:1 solution (150 g) (*see* **Note 11**). Store at 4 °C.
6. Sodium dodecyl sulfate (SDS) 20 % (BioRad). Store at room temperature.
7. Ammonium persulfate (APS) 10 % solution in deionized H_2O. Prepare just prior to use.
8. Tris-glycine 10× (*see* **Note 12**). Store at room temperature.
9. N,N,N′,N′-tetramethylethane-1,2-diamine (TEMED). Store at room temperature.
10. SDS electrophoresis buffer (running buffer): 10 % Tris-glycine, 0.1 % SDS (*see* **Note 13**).
11. Agarose 1 % containing bromophenol blue (BBF) and SDS 0.1 % (*see* **Note 14**).
12. Protein marker 10–250 kDa. Precision Plus Protein Standards. Dual color (BioRad).

2.7 Western Blot

1. Mini Trans-Blot Electrophoretic Transfer Cell (BioRad).
2. Nitrocellulose membranes (GE Healthcare Amersham Hybond ECL).
3. Transfer buffer: 10 % Glycine and 10 % methanol (*see* **Note 15**).
4. Whatman 3 MM Blotting papers.
5. Tris-buffered saline (TBS) 10×: 1.5 M NaCl, 0.2 M Tris, 0.02 M KCl, pH 8. Store at 4 °C.
6. Ponceau S Solution (Sigma).
7. Washing buffer: TBS 1× containing 0.2 % Tween-20 (TBST). Mix 100 ml of TBS 10× with 898 ml of deionized H_2O. Add 2 ml of Tween-20. Store at 4 °C.
8. Blocking buffer: 5 % of skim powdered milk in TBS 1× containing 0.2 % Tween-20 (*see* **Note 16**).
9. Dilution buffer: 2.5 % of skim powdered milk in TBS 1× containing 0.2 % Tween-20 (*see* **Note 17**).
10. Enhanced chemiluminescence ECL reagent (GE Healthcare).
11. Chemiluminescence films (GE Healthcare or similar dedicated products)

2.8 Antibodies

1. Sera from patients.
2. Secondary antibody goat anti-human IgG conjugated with horseradish peroxidase (HRP) (SouthernBiotech).

2.9 Colloidal Coomassie Blue Staining

1. Fixing solution: 40 % Methanol and 10 % acetic acid.
2. Staining solution: Brilliant Blue G-Colloidal concentrate (Sigma) (*see* **Note 18**) and methanol in proportion 1:4 (*see* **Note 19**). Mix the solution before each use. Store at 4 °C.
3. Destaining solution A: 25 % Methanol and 10 % acetic acid in deionized H_2O.
4. Destaining B: 25 % Methanol in deionized H_2O.
5. Storage solution: 5 % Acetic acid in deionized H_2O.
6. Nalgene staining box (Sigma L × W × H 12.5 × 12.5 × 5 cm).

2.10 Image Analysis

1. Progenesis SameSpots software (Nonlinear Dynamics).

3 Methods

Two-dimensional electrophoresis is carried out in two steps: the first dimension to separate the proteins according to their isoelectric points (pI) and, after that, the second dimension where the proteins are separated by their relative mass (Mr). Then the resolved proteome is transferred to nitrocellulose by Western blot and challenged for serological reactivity.

3.1 First-Dimension Step: Isoelectric Focusing (IEF)

All procedures are explained for a single sample/IPG strip/gel. Collect an appropriate number of patient and control sera according to the study design. Sample selection must ensure that age and gender distributions are homogeneous between the groups (*see* **Note 20**).

3.1.1 Sera Collection and Preparation

1. Collect 5–10 ml of blood samples in the vacutainer tubes, and incubate at room temperature for 30 min to allow clotting (*see* **Note 21**).
2. Centrifuge for 15 min at 1500 rcf at room temperature.
3. Collect the supernatant corresponding to the serum fraction leaving the clot undisturbed (*see* **Note 22**).
4. Aliquot the serum into sterile cryovials and store at −80 °C.

3.1.2 Cell Line Culture

1. Culture tumor cell lines under standard conditions (5 % CO_2, 37 °C and 99 % humidity) until they reach 80–90 % confluence (*see* **Note 23**).
2. Wash the cells (2×) with cold PBS to eliminate FBS-derived proteins, adding sufficient amount of cold PBS to cover the cells.
3. Detach the cells using a cell scraper and transfer cell suspension into a 15 ml tube (*see* **Note 24**).
4. Pellet cells by centrifugation (10 min, 1400 rcf, 4 °C) and resuspend the cells in 1 ml of cold PBS.
5. Transfer the cell solution to a 1.5 ml Eppendorf tube and centrifuge (10 min, 1400 rcf, 4 °C), discard carefully all the supernatant, and proceed to the solubilization step; otherwise, the cell pellet could be stored at −80 °C.

3.1.3 Sample Solubilization

IEF must be carried out under low-ionic-strength conditions to avoid interference with protein focusing. Cell lysis and protein solubilization are important in order to denature and break non-covalent interactions and disulfide bonds and must be preferentially carried out under conditions that maintain very low salt concentration and limit the presence of charged compounds.

1. Wash (3×) cells with PBS and resuspend the pellet in RB with the addition of 1:10 v/v DTT 1 M (0.1 M final concentration) (*see* **Notes 25**–**27**).
2. Incubate for 2 h at room temperature in order to allow complete solubilization (*see* **Note 28**).
3. To fragment the DNA and reduce the solution viscosity, sample is sonicated with 3 × 20-s stroke at 200 W of power using Bandelin Sonopuls or similar instruments (*see* **Note 29**).

3.1.4 Protein Precipitation with Acetone

After solubilization, if the protein concentration in the sample is low or if the sample contains impurities, such as salts (above 10 mM), lipids, and/or charged compounds, that could interfere

during the IEF step, it is advisable to carry out an additional step of protein precipitation with acetone to remove impurities and to concentrate proteins, and then resuspend the proteins in RB buffer.

1. Add 3× volumes of acetone 100 % (kept at –20 °C) to 1 volume of sample, vortex the mix, and incubate at –20 °C from 30 min to overnight (*see* **Note 30**). Following the incubation, all steps are performed at 4 °C.
2. Spin at 14,000 × g for 30 min.
3. Discard the supernatant and fragment the pellet with a tip.
4. Wash (2×) with 1 ml of acetone 80 % (kept at –20 °C) repeating **steps 2** and **3**.
5. Allow the pellet to dry at room temperature for 10–15 min.
6. Resuspend the pellet with 111 µl of RB.

3.1.5 Protein Quantitation by Bradford Protein Assay

For protein concentration quantitation is mandatory to use the Bradford method since it is not affected by the urea present in RB. We use the BioRad Protein Assay but similar reagents can be used (*see* **Note 31**).

1. Generate a standard curve with a BSA stock solution (1 mg/ml).
2. Prepare BioRad Protein Assay 1×. Note that standards and each unknown sample should be tested in duplicate or triplicate. Mix into a polystyrene round-bottom tube 800 µl of ultrapure H_2O and 200 µl of BioRad Protein Assay buffer 5× for each sample and for the standard curve points.
3. Two duplicates of BioRad Protein Assay 1× will be used as blank to set the spectrophotometer to 0 absorbance.
4. Add 1 µl, 3 µl, 5 µl, and 9 µl of the BSA stock (1 mg/ml) into the polystyrene round-bottom tube containing 1 ml of BioRad Protein Assay buffer 1× in order to have the Protein Standards 1 mg/ml, 3 mg/ml, 5 mg/ml, and 9 mg/ml, respectively.
5. Add 1 µl of the unknown sample into the polystyrene round-bottom tube containing 1 ml of BioRad Protein Assay 1× buffer (*see* **Note 32**).
6. Vortex all samples and incubate at room temperature for 10 min.
7. Read each sample by using polystyrene spectrophotometry cuvettes.
8. Determine the absorbance at 595 λ (wavelength) by using a spectrophotometer.

3.1.6 IEF

1. Use 250 µg of total protein for each IPG strip adjusting the final volume to 111 µl with RB, and then add 10 µl of DTT 1 M, 2 µl of BBF 0.05 %, and 2 µl of IPG buffer to reach a final volume of 125 µl.

2. Clean the ceramic strip holders with dedicated soap Strip Holder Cleaning Solution (GE Healthcare, or similar dedicated products) and deionized water, respectively. Dry thoroughly (*see* **Note 33**).
3. After incubation apply the sample (125 μl per IPGstrip) homogeneously distributed into the strip holder being sure that no dry areas are left.
4. Take the IPG strip 3–10NL 7 cm from the package and remove carefully the protective cover with forceps. Position the IPG strip with the gel side down in contact with the slot making sure that the acidic end corresponds to the pointed end of the ceramic strip holder, and situate the strip against this end of the slot. Take care not to trap bubbles under the IPG strip. Cover the IPG strip with mineral oil to avoid evaporation and urea crystallization and close it with the protective lid.
5. Place the ceramic strip holder in the Ettan IPGphor Isoelectric Focusing System with the built-in platinum electrodes on the down side in contact with the Ettan IPGphor unit platform according to the guide mark. Put the pointed end over the cathode and the blunt end over the anode. Close the ceramic strip holder with the plastic lid.
6. Set the running protocol that will be carried out automatically and begin IEF (*see* **Notes 34** and **35**).
7. At the end of IEF run, wash the IPG strip with deionized water on the plastic holder (opposite side of the gel) in order to eliminate any residual mineral oil (*see* **Note 36**).
8. Proceed to the second dimension immediately; otherwise, place the IPG strip into a Drystrip tray with the gel up, close with the plastic lid, and store at −80 °C.

3.2 Second-Dimension Step: SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.2.1 IPG Strip Equilibration

1. Place the IPG strip on a Drystrip Tray.
2. Add 2 ml of the equilibration buffer (EB) containing DTT 130 mM to each strip. Equilibrate for 15 min at room temperature with gentle agitation.
3. Wash with 2 ml of EB.
4. Add 2 ml of the EB containing IAA 130 mM to each strip. Equilibrate for 15 min at room temperature with gentle agitation.

3.2.2 Performing SDS-PAGE

1. Wash the glass plates with soap and deionized water, dry, and finally clean with ethanol 70 %. Dry thoroughly.
2. Place the casting frame and glass plate sandwich in the casting stand in the correct mode to pour the gel.
3. Mix in a Falcon of 50 ml: 4 ml of deionized water, 2.5 ml of resolving buffer, 50 μl of SDS 20 %, and 3.3 ml of acrylamide.

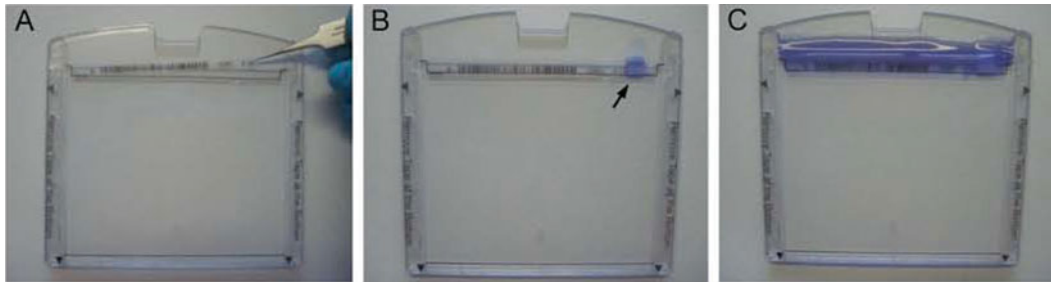

Fig. 3 IPG strip positioning procedure and sealing for the 2-DE second dimension. (a) The IPG strip is placed with the positive end in contact with the left end of the gel; (b) on the right, in contact with the basic end of the IPG strip, is placed a piece of 3 MM for the protein marker loading; (c) strip and markers are sealed with liquid agarose

Then add 50 µl of ammonium persulfate (APS) 10 % and 5 µl of TEMED to catalyze the polymerization reaction. Gently mix and immediately pour the gel into the glass-plate sandwich. Finally overlay the gel solution with 1 ml of isopropanol to exclude oxygen that inhibits acrylamide polymerization.

4. Allow the gel to polymerize for 1 h. After that, remove the isopropanol from the top of the gel (*see* **Note 37**).
5. Following the IPG strip equilibration, cut the two plastic sides of the IPG strip (about 0.5 cm) without gel.
6. Place the gel cassette with the longer glass plate down and insert the IPG strip with the gel surface up and the acidic end on the left.
7. Dissolve agarose 1 % by heating.
8. Cut 0.5 × 0.5 cm of 3MM, add 6 µl of molecular weight protein marker, rinse in agarose 1 %, and place it attached to the basic end of the IPG strip (*see* Fig. 3).
9. Finally, seal the IPG strip in place overlaying the gel with agarose 1 %. Allow agarose to became solid (*see* Fig. 3).
10. Start the electrophoresis at 20 mA until the dye front enters the gel. Then, 30 mA until the front reaches the end of the gel.

3.3 Western Blot

3.3.1 Electrophoretic Transfer

1. Use the Mini Trans-Blot Electrophoretic Transfer Cell. Cut two 3MM blotting paper sheets and the nitrocellulose membrane off a size 1 cm wider than the gel. Use the smaller glass plate as template. Immerse into transfer buffer the fiber pads, the paper filters, and the nitrocellulose membrane.
2. Once the electrophoresis is finished, separate the glass plates with the aid of a spatula and rinse carefully the gel into the transfer buffer.
3. Place on the transparent side of the gel holder cassette in this order: fiber pad, paper filter, nitrocellulose membrane, gel, paper filter, and fiber pad. Be careful not to incorporate any air

bubble between the gel and the membrane and between all the other layers. Add few milliliters of transfer buffer to every layer (*see* **Note 38**). Close the sandwich within the gel holder cassette and place it into the electrophoresis box (*see* **Note 39**).

4. Fill in the electrophoresis box with transfer buffer including an ice container.
5. Perform the electrophoretic transfer at 80 V for 2 h (*see* **Note 40**).

3.3.2 Immunoblot Transferred Proteins

1. Following the electrophoretic transfer, the nitrocellulose membrane is immediately rinsed in a Ponceau S solution in order to check the correct transfer of the proteins to the membrane. Incubate at room temperature for 10 min with gentle shaking (*see* **Note 41**).
2. Wash the membrane 3–4 times with deionized H_2O.
3. Insert the membrane between two transparencies and acquire the image with a scanner.
4. Place membrane in blocking buffer and incubate for 1 h at room temperature under gentle shaking.
5. Incubate the membrane under gentle agitation with the sera (5 ml, 1:200 in dilution buffer) overnight at 4 °C.
6. Rinse quickly with TBST and wash 3× with TBST 10 min each time.
7. Incubate the membrane with anti-human IgG HRP secondary antibody (5 ml, 1:2000 in dilution buffer) for 1 h at room temperature with gentle shaking (*see* **Note 42**).
8. Rinse with TBST and wash 3× with TBST 10 min each time.
9. Develop by chemiluminescence substrate reaction by using ECL or similar, according to the manufacturer's procedures.
10. Acquire the images by autoradiography with film exposure or by using a dedicated image acquisition system (G-Box or equivalent) equipped for chemiluminescence detection.

3.4 Staining of Preparative 2D Gel by Colloidal Coomassie Blue Staining

Following the 2D electrophoresis, one of the gels that have been run on Mini-Protean 3 Dodeca Cell apparatus is stained and used as preparative gel for successive spot/s excision and protein/s identification by mass spectrometry.

1. At the end of the 2D electrophoresis, separate the glass plates with the aid of a spatula and place the gel into the Nalgene staining box.
2. Add 100 ml of fixing solution and incubate for 1 h at room temperature with gentle agitation.
3. Discard the solution.

4. Incubate overnight with 100 ml of colloidal Coomassie Blue staining solution at room temperature with gentle agitation.
5. Discard the solution.
6. Add destaining solution A and shake gently replacing the solution several times until acrylamide background staining disappears.
7. Discard the solution.
8. Add destaining solution B for 10–15 s and shake gently.
9. Immerse gel in storage solution at 4 °C.
10. Insert the gel in a plastic transparent envelop and seal for long storage.
11. Acquire images of the enveloped gel by using an image acquisition system.

3.5 2D Image Analysis

Each serum should be tested twice on 2-DE map and the corresponding images analyzed. Sera pattern reactivity of patients is compared to that of control subjects. Images of the 2D serological reactivity patterns are analyzed using Progenesis SameSpots software (Nonlinear Dynamics), or similar dedicated software, under automatic default conditions. Spot signals are considered relevant if the fold change of serological reactivity is >0.5 (based on the spots' normalized volume of optical density) and the p value for the one-way ANOVA analysis is <0.05 (*see* **Note 43**).

Spot signals considered relevant must be identified by image software analysis on the preparative gel (*see* Subheading 3.4), and then the corresponding protein spots are excised with a scalpel and analyzed by mass spectrometry [8] for protein identification (*see* **Note 44** and **45**).

4 Notes

1. Dissolve 96 g of urea and 8 g of CHAPS in deionized H_2O and adjust the volume to 200 ml.
2. Since urea is highly concentrated the weighted powder occupies a large volume and the solution gets very viscous; thus it is advisable to add 100 ml of deionized H_2O into a 500 ml graduated cylinder, mix gently the urea and CHAPS, and rotate with a magnetic stir bar until almost completely dissolved. Finally adjust the volume to 200 ml with deionized H_2O.
3. Rehydrate with 13.51 ml of ultrapure water to obtain a stock solution of 1 mg/ml. Aliquot and store at −20 °C.
4. Mix 72 g of urea, 60 ml of glycerol 99 %, and 20 ml of SDS 20 % and adjust to 200 ml with Tris–HCl.

5. *See* **Note 2**; it is advisable to dissolve urea powder in 100 ml of Tris–HCl 1.5 M into a 500 ml graduated cylinder, mix gently, add glycerol and SDS, and rotate with a magnetic stir bar until almost completely dissolved. Finally adjust the volume to 200 ml with Tris–HCl.
6. Add 40 mg DTT to 2 ml of EB and stir up until dissolved.
7. Allow the buffer equilibrate at room temperature before use.
8. Add 50 mg of IAA to 2 ml of EB and stir up until dissolved.
9. We recommend using 3–10 NL when it is intended to have a general view of the whole proteome. The overall separation range is high and provides an optimal spread of the proteins.
10. Weigh 181.65 g of Tris base and dissolve in 600 ml of deionized H_2O. Adjust to pH 8.8 with HCl and adjust to 1 L with H_2O.
11. Add to the bottle of acrylamide/bis powder 362 ml of deionized H_2O and stir up until completely dissolved; filtering is recommended.
12. Weigh 144 g of glycine and 30.3 g of Tris base and bring it to 1 L with deionized H_2O.
13. Mix 100 ml of Tris-glycine 10× and 5 ml of SDS 20 % and add deionized H_2O to a volume of 1 L.
14. Dissolve 1 g of agarose with 100 ml of running buffer. Add a pinch of powdered BBF until the whole turns blue. Rotate with a magnetic stir and heat until it boils. Prepare 5 ml aliquots and store at 4 °C.
15. Mix 100 ml of methanol 100 % and 100 ml of glycine 10×. Adjust with deionized H_2O to 1 L.
16. Add 5 g of skim milk into a graduated cylinder and add TBS 1× to adjust volume to 100 ml, and add 200 μl of Tween-20.
17. Dissolve 2.5 g of skim powdered milk in 100 ml of TBS 1×, and add 200 μl of Tween-20.
18. Dilute concentrated Brilliant Blue G to 1 L with 800 mL of deionized H_2O; the final suspension will contain 0.1 % (w/v) Brilliant Blue G, 0.29 M phosphoric acid, and 16 % saturated ammonium sulfate.
19. Mix 250 ml of methanol 100 % with the reconstituted Brilliant Blue G-Colloidal and rotate with a magnetic stir.
20. Control group may include healthy subjects and/or patients affected by non-tumoral pathologies in order to discriminate between tumor-specific serological reactivity and that dependent from generic inflammatory status.
21. Incubation should be no longer than 1 h and without agitation to avoid hemolysis.
22. The serum appears as a limpid yellow solution; if it is still turbid repeat the centrifugation step.

23. The number of cells necessary to obtain the required amount of proteins depends on the cell type used; for example for an adherent epithelial tumor cell line 1×10^6 cells give rise to about 250 μg of proteins necessary for a single 2D-electrophoresis.
24. Do not use trypsin treatment for cell detachment in order to avoid loss of membrane proteins.
25. Solubilization volume depends on the cell type; on average 20–100 μl of RB for 1×10^6 cells should give rise to a protein concentration of about 4–5 μg/μl.
26. Cell solubilization can be performed with different lysis buffers containing different detergents (nonionic, zwitterionic, ionic); however, if it is possible, avoid the presence of the negatively charged SDS detergent. If ionic detergent is necessary for solubilization of specific proteins, bring its final concentration lower than 0.1 %. Similarly the final salt concentration must be lower than 10 mM.
27. If RB is used to lyse the cells, protease inhibitor cocktail is facultative since 8 M urea concentration inhibits protease activity.
28. Keep the temperature above 15 °C; otherwise urea crystallizes.
29. Keep the temperature controlled in a range of 15–40 °C immersing the sample tube in ice for 10 s at the end of each sonication stroke.
30. Incubation time depends on the protein concentration and sample volume: high protein concentration and/or small sample volume require short time of incubation.
31. Protein concentration in the sample must be quantified after sample resuspension in RB, because if acetone precipitation step has been performed, the protein recovery varies from 50 to 80 %.
32. It is essential to maintain the sample concentration between the concentration range of the standard curve.
33. We noticed that washing the ceramic strip holders with normal soap generated artifacts in protein focusing.
34. The first part of the 3–10 NL running protocol allows the IPG strip rehydration and sample loading into the strip, while the second part includes several steps in which focusing occurs.
    - *Rehydration 1 h at 18 °C.*
    - *S1 Step and hold: 30 V for 8 h.*
    - *S2 Gradient from 30 to 300 V for 1 h.*
    - *S3 Step and hold: 300 V for 1:30 h.*
    - *S4 Gradient from 300 to 3500 V for 2:30 h.*
    - *S5 Step and hold: 3500 V 2 h.*
    - *S6 Gradient from 3500 to 5000 V for 2 h.*

- *S7 Step and hold 5000 V until arrival to 15,000V/hr.*
- *S8 step and hold: 30 V for 20:00 h.*

35. Protein focusing is considered finished at the end of S7. S8 is a step added to maintain the IPG strip at a constant voltage until the next step will be performed.
36. A correct run is recognized by the correct trend of voltage increase during the selected protocol and because the BBF migrates toward the acidic end and turns yellow.
37. Take the IPG strip by using forceps from one end of the strip and keep it vertically while deionized water is spilled over the opposite side of the gel; dry carefully the excess of water with a paper towel, avoiding the contact with gel side of the strip.
38. Precast 4–20 % acrylamide gradient gels, which allow obtaining a good separation for a wide range of protein size, are currently distributed by BioRad (Mini-PROTEAN TGX 4–20 %).
39. A 10 ml pipette can be used in order to roll over the sandwich and allow adhesion of the gel to the nitrocellulose membrane.
40. Pay attention to place the nitrocellulose oriented to the positive charge and the gel oriented to the negative charge of the electrophoretic system, respectively.
41. Use cold transfer buffer refrigerated at 4 °C.
42. Before the removal of the gel, mark with a pen on the nitrocellulose the position of the gel corners.
43. Incubation must be done in the dark; thus the box can be wrapped with an aluminum foil.
44. The stringency for criteria of relevance may change according to the study design.
45. After mass spectrometry analysis, protein/s identity must be validated performing a Western blot analysis using protein-specific commercial antibodies on the selected proteome resolved by 2-DE. Specific commercial antibodies can be used to identify whether serological reactivity is directed against specific isoforms as shown [8]. If available, a purified protein corresponding to the putative tumoral antigen can be used to confirm the specificity of the serological reactivity. Alternatively, specificity can be assessed by antigen immunodepletion from the proteome using the commercially available antibodies.

Acknowledgments

M.A. is supported by AIRC. Special Program Molecular Clinical Oncology 5 per mille n.9965, and IG-10538.

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Chapter 3

Generation and Cryopreservation of Clinical Grade *Wilms' Tumor 1* mRNA-Loaded Dendritic Cell Vaccines for Cancer Immunotherapy

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Abstract

First described in the 1970s, dendritic cells (DC) are currently subjects of intense investigation to exploit their unique antigen-presenting and immunoregulatory capacities. In cancer, DC show promise to elicit or amplify immune responses directed against cancer cells by activating natural killer (NK) cells and tumor antigen-specific T cells. Wilms' tumor 1 (WT1) protein is a tumor-associated antigen that is expressed in a majority of cancer types and has been designated as an antigen of major interest to be targeted in clinical cancer immunotherapy trials. In this chapter, we describe the generation, cryopreservation, and thawing of clinical grade autologous monocyte-derived DC vaccines that are loaded with WT1 by messenger RNA (mRNA) electroporation. This in-house-developed transfection method gives rise to presentation of multiple antigen epitopes and can be used for all patients without restriction of human leukocyte antigen (HLA) type.

Key words Dendritic cells, Electroporation, RNA, Clinical grade, Wilms' tumor 1, WT1

1 Introduction

Dendritic cells (DC) are currently being investigated in clinical trials to treat cancer patients, due to their unique capacity to direct immune responses toward tumor cells by activating natural killer (NK) cells and tumor-specific T cells [1–3]. Presentation of tumor-associated antigens by DC in an active state can license T cells to attack tumor cells.

Because of their low numbers in vivo, most DC used in clinical vaccination trials are obtained by ex vivo differentiation of monocytes in the presence of interleukin (IL)-4 and granulocyte macrophage colony-stimulating factor (GM-CSF) [2, 4]. Alternative protocols to generate immunostimulatory monocyte-derived DC include differentiation of monocytes in the presence of IL-15 [5–8] or interferon-α [9].

Attilio Bondanza and Monica Casucci (eds.), *Tumor Immunology: Methods and Protocols*, Methods in Molecular Biology, vol. 1393, DOI 10.1007/978-1-4939-3338-9_3,

In our laboratory, a safe and efficient method was developed to load DC in vitro with tumor-associated antigens by means of messenger RNA (mRNA) electroporation [10, 11]. This non-viral gene transfer technique results in high, transient expression of the encoded protein(s) without the risk of insertional mutagenesis, in contrast to DNA electroporation, and is now widely implemented in immunotherapy to load DC with antigens and/or immunomodulatory molecules [7, 12–17].

Our main tumor-associated antigen of interest is the Wilms' tumor protein 1 (WT1). WT1 has been evaluated as having top priority as target antigen in tumor immunotherapy trials because it fulfills several criteria, including expression in a variety of cancer types, high immunogenicity, and involvement in oncogenesis [18–20]. Loading of DC with the WT1 protein by mRNA electroporation leads to presentation of multiple epitopes and can be performed for all patients, without the need to take the human leukocyte antigen (HLA) type into consideration [12, 14, 21].

For logistical reasons, for each patient we generate several DC vaccines out of one leukapheresis product [22]. DC vaccines are then stored in liquid nitrogen and thawed two hours before injection. Here we will describe the generation, cryopreservation, and thawing of clinical-grade autologous monocyte-derived DC vaccines loaded with WT1 by mRNA electroporation. These DC have been successfully used to prevent or postpone relapse in patients with acute myeloid leukemia (AML) [16], are currently tested in a multicenter randomized controlled phase II trial in AML patients, and are also being investigated in patients with different types of solid tumors [23].

2 Materials

2.1 Generation of Human Monocyte-Derived DC

1. Monocytes, obtained from leukapheresis products after CD14 magnetic bead labeling and isolation with the CliniMACS system (Miltenyi Biotec), according to the manufacturer's protocol.
2. Cell culture medium consisting of CellGro DC Medium (CellGenix) supplemented with 1 % human AB serum (PAA), 250 U/mL recombinant human (rh)IL-4 (Miltenyi), and 800 U/mL rhGM-CSF (Gentaur).
3. Opti-MEM Reduced Serum Medium, phenol red-free (Gibco).
4. Prostaglandin E2 (PGE_2; Prostin E2, Pfizer).
5. Rh tumor necrosis factor (TNF)-α (Miltenyi).
6. Keyhole limpet hemocyanin (KLH, Immucothel).
7. T175 flasks (BD Falcon).
8. Centrifuge.

9. 50-mL tubes.
10. 5-, 10-, 25-, 50-mL pipettes.

2.2 mRNA Electroporation of Mature DC

1. Gene Pulser XCell Electroporation System for eukaryotic cells, including main unit, CE unit, and ShockPod cuvette chamber (BioRad).
2. 4-mm electroporation cuvettes.
3. Cell culture medium at room temperature consisting of CellGro DC Medium (CellGenix) supplemented with 1 % human AB serum (PAA), 250 U/mL rhIL-4 (Miltenyi) and 800 U/mL rhGM-CSF (Gentaur).
4. Opti-MEM Reduced Serum Medium (Gibco).
5. 50-mL tubes.
6. Transfer pipettes.
7. Micropipettes and filter tips for volumes from 5 to 1000 μL.
8. 6-Well low adherence plates (Corning).
9. 0.9 % NaCl.
10. 20 μg of 1 μg/μL mRNA encoding WT1 (Curevac) per electroporation.
11. 5-, 10-, 25-, 50-mL pipettes.

2.3 Cryopreservation of Electroporated Mature DC

1. Cold cryopreservation medium consisting of human AB serum (PAA) supplemented with 10 % dimethyl sulfoxide (DMSO; Cryosure) and 2 % glucose (Glucosteril, S.A. Laboratories).
2. 1.5-mL cryopreservation tubes.
3. Cryofreezing container (Nalgene Cryo 1 °C Freezing Container, with a rate of cooling of –1 °C/min).

2.4 Thawing of Electroporated Mature DC and Preparation of the Vaccine

1. 37 °C water bath.
2. Small zip lock bags.
3. 6-Well low adherence plate (Corning).
4. Cold thawing medium consisting of CellGro DC Medium (CellGenix) and 1 % human AB serum (PAA).
5. Warm incubation medium consisting of CellGro DC Medium (CellGenix), supplemented with 1 % human AB serum (PAA), 250 U/mL rhIL-4, 800 U/mL rhGM-CSF, 2.5 μg/mL PGE_2, and 600 U/mL TNFα.
6. 50-mL tubes.
7. 0.9 % NaCl.
8. 1-mL syringe.
9. 23G×1" (0.6×25 mm) needle.
10. Transport box.

3 Methods

Carry out all procedures at room temperature unless otherwise specified. In order to generate clinical grade DC vaccines, all procedures should be carried out in a clean room, separately patient per patient (*see* **Note 1**).

3.1 Generation of Human Monocyte-Derived DC

1. Transfer the monocytes from the CliniMACS cell collection bag to 50-mL tubes and determine the cell volume, concentration, and viability.
2. Centrifuge at 480 × *g* for 6 min.
3. Culture the monocytes in T175 flasks at a minimum of 50×10^6 and a maximum of 70×10^6 total cells per flask in 50 mL culture medium.
4. Resuspend and pool the cell pellet(s) in culture medium (50 mL × number of T175 flasks required) and distribute the cell suspension into the T175 flasks using a 50-mL pipette.
5. Keep the flasks in a horizontal position at 37 °C and 5 % CO_2 in a humidified incubator for 8 days (start of the culture is day 0).
6. At day 3 or 4, add 250 U/mL rhIL-4 and 800 U/mL rhGM-CSF to the culture medium.
7. At day 6, add 2.5 μg/mL PGE_2 and 600 U/mL TNFα to the culture medium for DC maturation and 10 μg/mL KLH as an adjuvant.
8. At day 8, harvest the mature DC from the T175 flasks:
   - Tap the flask on the bottom and the sides to loosen the cells.
   - Resuspend the cells with a 25-mL pipette and pipette the cell suspension up and down to detach as many cells as possible.
   - Transfer the cell suspension to a 50-mL tube.
   - Rinse the bottom of the flask with 25-mL Opti-MEM by pipetting up and down with a 25-mL pipette.
   - Transfer the cell suspension to a 50-mL tube.
   - Harvest, collect, and pool the cells from all flasks into 50-mL tubes.
   - Centrifuge at 480 × *g* for 6 min.
   - Remove the supernatant using a pipette.

3.2 mRNA Electroporation of Mature DC (See *Note 2*)

1. Resuspend the cell pellets in Opti-MEM and pool the cell pellets into 100 mL in two 50-mL tubes (*see* **Note 3**).
2. Centrifuge at 480 × *g* for 6 min.
3. Repeat the washing step with Opti-MEM. Resuspend the cell pellets in one 50-mL tube in a volume of Opti-MEM suitable for accurate cell counting (*see* **Note 4**).

4. Determine cell number and viability.
5. Calculate the number of electroporations required. Each electroporation is performed in a minimum of 200 µL and a maximum of 500 µL Opti-MEM, with a maximum total cell concentration of 50×10^6/500 µL.
6. Centrifuge the cell suspension at 480 × *g* for 6 min.
7. During centrifugation, prepare 50-mL tubes with 10 mL culture medium per electroporation, as well as empty 50-mL tubes.
8. After centrifugation, remove the majority of the supernatant with a pipette.
9. Remove the remaining supernatant using a filter tip.
10. Resuspend the cell pellet in Opti-MEM (500 µL × number of electroporations required), taking the cell pellet volume into account.
11. Distribute the cell suspension into the cuvettes using a filter tip.
12. Adjust the electroporation settings on the gene pulser: time constant protocol, time 7 ms; voltage 300 V; cuvette 4 mm.
13. Add 20 µg of mRNA to the cell suspension, tap the cuvette to disperse the mRNA throughout the suspension (*see* **Note 5**).
14. Insert the cuvette into the ShockPod cuvette chamber inside the laminar flow, close the chamber, and trigger the pulse.
15. Immediately after electroporation, add culture medium to the cell suspension with a transfer pipette and transfer this mixture of medium and cell suspension to a second empty 50-mL tube.
16. Rinse the cuvette twice with the remaining fresh culture medium and transfer the rinse suspension and the rest of the remaining medium to the second 50-mL tube, to obtain a cell concentration of about 5×10^6/mL.
17. Repeat this for each electroporation.
18. Distribute the cell suspension into 6-well low adherence plates at 3–4 mL/well.
19. Incubate for 2 h at 37 °C and 5 % CO_2 in a humidified incubator.
20. Harvest the electroporated mature DC with a transfer pipette and transfer cell suspension to 50-mL tubes.
21. Rinse the wells with 0.9 % NaCl, and add to the rest of the cell suspension to the 50-mL tubes.
22. Centrifuge at 480 × *g* for 6 min.
23. Remove the supernatant with a pipette.
24. Resuspend the cell pellets in 0.9 % NaCl and pool them in one 50-mL tube in a volume suitable for accurate cell counting (*see* **Note 4**).
25. Determine cell number and viability.

3.3 Cryopreservation of Electroporated Mature DC (See Note 6)

1. Centrifuge the cell suspension at 480 × *g* for 6 min.
2. Remove the supernatant with a pipette.
3. Resuspend the cell pellet in cold cryopreservation medium at a viable cell concentration of $10–20 \times 10^6$/mL (*see* **Note 7**).
4. Distribute the cell suspension into labeled cryovials (*see* **Note 8**).
5. Transfer the vials to a freezing container and place it immediately in a –80 °C freezer.
6. After 48 h, transfer the cryovials to the gas phase of liquid nitrogen.

3.4 Thawing of Electroporated Mature DC and Preparation of the Vaccine

1. Thaw a vial of electroporated mature DC in a 37 °C water bath with constant, moderate agitation until there is still a small clump of frozen suspension left in the vial.
2. Dilute the thawed cells in 30 mL cold thawing medium in a 50-mL tube as soon as possible.
3. Rinse the vial with the cold thawing medium and transfer the rinsing suspension back to the 50-mL tube.
4. Centrifuge at 480 × *g* for 6 min.
5. Remove the supernatant with a pipette and resuspend the cell pellet in 6 mL warm incubation medium.
6. Distribute the cell suspension at 3 mL per well in a low adherence 6-well plate.
7. Incubate for 2 h at 37 °C and 5 % CO_2 in a humidified incubator.
8. Harvest the cells with a transfer pipette and transfer the cell suspension to a 50-mL tube.
9. Rinse the wells with 0.9 % NaCl, and add to the rest of the cell suspension in the 50-mL tube. Fill up to 30 mL with 0.9 % NaCl.
10. Centrifuge at 480 × *g* for 6 min, and remove supernatant with a pipette.
11. Resuspend the cell pellet in 30 mL 0.9 % NaCl in order to wash the cells.
12. Centrifuge at 480 × *g* for 6 min, and remove supernatant with a pipette.
13. Repeat washing step.
14. Resuspend the cell pellet in 10 mL 0.9 % NaCl.
15. Determine cell number and viability.
16. Fill up to 30 mL with 0.9 % NaCl, centrifuge at 480 × *g* for 6 min, and remove supernatant with a pipette.
17. Resuspend the cell pellet with 0.9 % NaCl to obtain the appropriate concentration required for vaccination (i.e., 10×10^6/500 μL), taking the cell pellet volume into account.

18. Aspirate 600–700 μL of the cell suspension with a labeled 1-mL syringe and 23 G needle. This will allow to administer 500 μL of the DC suspension to the patient, following the loss of volume during purging of the air from the dead volume of the syringe and needle.
19. The vaccine is now ready for administration. Transport the vaccine in a labeled zip lock bag inside a sealed transport box.

4 Notes

1. The entire procedure for the manufacturing of DC vaccines, from isolation of the monocytes to preparation of the vaccines, must be performed in a clean room facility grade A in B, following the guidelines of Good Manufacturing Practice (GMP). All reagents used must be of GMP grade and the materials must be certified for use in a GMP-grade facility. Traceability of all used products and of the DC vaccines produced must be ensured. Quality control of the working environment must be ensured by daily sterility measurements and continuous monitoring of the air quality. Batch quality control of the cell products must be ensured by performing sterility controls at different time points during production, as well as at the end point, which is the thawed DC vaccine before administration. Next to sterility controls, extra quality controls must be performed on the cell products, including cell recovery, concentration, viability, phenotypic markers, protein expression (quality control of the electroporation efficiency) and migratory capacity.
2. Working with RNA requires an RNase-free environment. It is important to wear gloves and use filter tips throughout the procedure.
3. The three washing steps of the mature DC with Opti-MEM prior to electroporation ensures serum-free and thus RNAse-free conditions required for mRNA electroporation.
4. In order to determine a correct volume for cell counting, the detection limits of the counting device must be taken into account. Using a HORIBA ABX MICROS 60 OT Hematology Analyzer (Horiba) with the detection limit maximum at 20×10^6 cells per mL, we resuspend the cells in 50 mL for counting.
5. When electroporating 20×10^6 cells or less, 10 μg of mRNA per electroporation is sufficient. It is important to work quickly during the electroporation procedure, in order to avoid mRNA degradation after it has been added to the cell suspension and to reduce the exposure time of cells to serum-free conditions.

After pulsing, the cells must be resuspended as quickly as possible in serum-containing medium to speed up the resealing process of the membrane pores.

6. When working with DMSO, it is important to perform freezing of the cells as quickly as possible and in cold DMSO solution, since the cryoprotectant DMSO is toxic for cells at room temperature.
7. The concentration and the total number of cells to be cryopreserved per vial depends on the number of viable cells required for vaccine administration as specified in the trial protocol. After thawing, the possibility of a loss of 50 % of the cells should be taken into account. Thus for the preparation of 1 vaccine dose of e.g. 10×10^6 DC, ideally 20×10^6 DC should be cryopreserved in 1 cryopreservation vial.
8. In order to ensure traceability of the vaccines produced, labeling of all cell products is very important. Labels should mention product number, patient ID, production date, expiry date, and storage conditions.

Acknowledgements

This work was supported in part by research grants of the Research Foundation Flanders (FWO Vlaanderen), Foundation against Cancer (Stichting tegen Kanker), Vlaamse Liga tegen Kanker, National Cancer Plan Action 29, King Baudouin Foundation, and the Methusalem program of the Flemish Government. E.S. is a postdoctoral fellow of the Research Foundation Flanders. Y.W. is holder of a predoctoral fellowship of the Agency for Innovation by Science and Technology (IWT) and S.A. of an Emmanuel Van der Schueren fellowship of the Vlaamse Liga tegen Kanker.

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Chapter 4

Quantitative and Qualitative Analysis of Tumor-Associated CD4+ T Cells

Giulia Di Lullo, Lucia De Monte, Claudio Doglioni, and Maria Pia Protti

Abstract

CD4+ T cells comprise a significant portion of tumor-infiltrating lymphocytes. Different subsets of CD4+ T cells exist and they exert different effector functions in tumor immunity depending on the cytokines produced going from antitumor to pro-tumor. Methods that use small aliquots of cells to identify ex vivo the frequency and functional orientation of tumor-specific CD4+ T cells in the blood and visualization of the presence of different CD4+ T cell subsets and their localization at the tumor site are valuable tools to determine their clinical impact in neoplastic diseases.

Key words Tumor antigens, CD4+ T cells, Th subsets, Antigen-specific CD4+ T cell frequency, Antitumor cytokine, Pro-tumor cytokine, GATA-3, T-bet

Abbreviations

Ag	Antigen
APC	Antigen-presenting cell
BSA	Bovine serum albumin
FBS	Fetal bovine serum
HS	Human serum
IL	Interleukin
L	Liter
MHC	Major histocompatibility complex
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
RT	Room temperature
TCR	T cell receptor
Th	T helper
TSLP	Thymic stromal lymphopoietin

Attilio Bondanza and Monica Casucci (eds.), *Tumor Immunology: Methods and Protocols*, Methods in Molecular Biology, vol. 1393, DOI 10.1007/978-1-4939-3338-9_4,

1 Introduction

CD4+ T helper (Th) cells differentiate from naïve precursors into different subsets under the influence of the antigen-presenting cells (APCs) and the cytokine milieu present in the microenvironment [1]. CD4+ Th cell differentiation is initiated by T cell receptor (TCR) engagement with a peptide-major histocompatibility complex (MHC) class II complex at the surface of antigen (Ag)-loaded APCs and this stimulation induces expression of transcription factors specific for each subset [2, 3]. The two major CD4+ Th cell subsets are (1) Th1 cells that express T-bet, differentiate under the influence of interleukin (IL)-12 and IFN-γ, and secrete IFN-γ, TNF-α, and IL-2, and (2) Th2 cells that express GATA-3, differentiate under the influence of IL-4 and thymic stromal lymphopoietin (TSLP), and secrete IL-4, IL-5, IL-9, and IL-13 [1]. More recently, other subsets have been identified: (1) Th17 cells that express RORγt, differentiate under the influence of TGF-β and IL-6, and secrete IL-17 and IL-22 [4, 5], and (2) Th22 cells that express AHR, differentiate under the influence of TNF-α and IL-6, and secrete IL-22 in the absence of IL-17 [6, 7].

CD4+ T cells comprise a significant portion of tumor-infiltrating lymphocytes [8]. Tumor Ag-specific CD4+ T cells are mostly primed in tumor-draining lymph nodes (LNs) by tumor Ag-loaded APCs; then they exit the LNs and recirculate in the blood and eventually home to the tumor site under the influence of chemokines present in the tumor microenvironment. In this context, the nature of the APCs and the cytokine milieu present in the tumor and in draining LNs play an important role in determining which subset of tumor Ag-specific CD4+ T cells will be primed. On the other hand, tumor Ag-specific CD4+ T cells that home to the tumor may release either antitumor or pro-tumor cytokines and therefore dictate disease prognosis.

Studies in several tumors have now evaluated the association between tumor immunity and cancer prognosis and large-scale studies have revealed the prognostic and predictive impact of the tumor-infiltrating lymphocytes [9–11]. Indeed, features such as tumor-infiltrating lymphocyte distribution, density, and function dictate their antitumor versus pro-tumor activity [10]. The role of CD4+ T cells depends on the pattern of cytokines produced and the contribution of the different subsets in tumor immunity is still under debate [10, 12]. However, it is well established that Th1 cytokines exert antitumor activity, Th2 cytokines mostly promote tumor progression while Th17 cytokines exert antitumor or pro-tumor activity depending on the model [10, 13] (*see* Fig. 1).

The availability of methods to detect the frequency and functional orientation of tumor Ag-specific CD4+ T cells in the circulation and the proportion and localization of different Th subsets at the tumor site help in defining the role of the CD4+ Th subsets in tumor immunity.

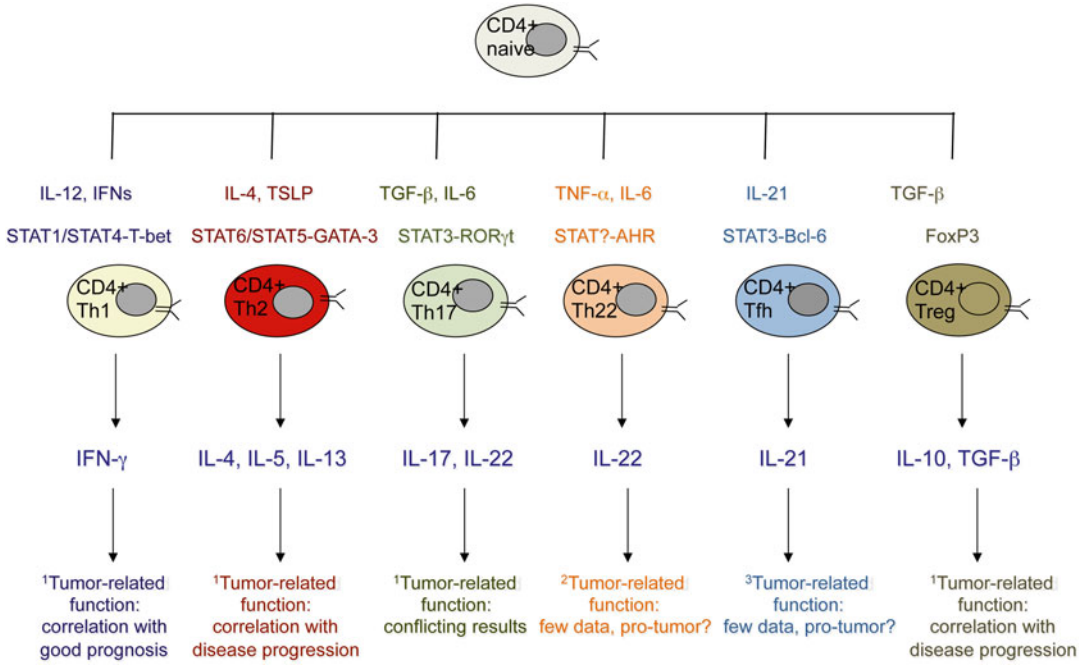

Fig. 1 Different CD4+ Th cell subsets exert different role in tumor immunity. Naïve CD4+ T cells differentiate into different Th subsets under the influence of cytokines present in the microenvironment and/or specifically secreted by specific subsets of APC responsible for priming. The cytokine milieu activates different transcription factors characteristic for each subset. Th1 cell differentiation is driven by IL-12 and IFN-γ that activate transcription factors STAT1 and STAT4, respectively. STAT1 and STAT4 drive T-bet activation. Th2 cell differentiation is driven by IL-4 and TSLP that activated STAT6 and STAT5, respectively. STAT6 drives GATA-3 activation. Th17 cell differentiation is driven by TGF-β and IL-6 that activate STAT3 and RORγt. Th22 cell differentiation is driven by TNF-α and IL-6 that should activate AHR (although it is still unclear how specifically). Tfh cell differentiation is driven by IL-21 that activates STAT3 and Bcl6. T regulatory cells (Tregs) are driven by TGF-β that activates FoxP3. Through the release of cytokines specific for each subset CD4+ Th cells exert different tumor-related functions. [1]data reviewed in [10], [2]data reported in [20, 21], [3]data reported in [22]

In this chapter we describe (1) an in vitro culture method that allows the calculation of the frequency of circulating tumor Ag-specific CD4+ T cells and their functional orientation and (2) immunohistochemical analysis methods for the enumeration and localization in the tumor of Th1 and Th2 cells, which are the best characterized Th subsets in tumor immunity.

2 Materials

2.1 Isolation of Peripheral Blood Mononuclear Cells (PBMCs) from Heparinized Blood

1. 25–50 mL heparinized human venous blood.
2. Saline or phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+}.
3. Lymphocyte separation medium or Ficoll-Paque (density 1.077 g/mL) (GE Healthcare Life Sciences).

4. 50 mL polypropylene Falcon tubes.
5. Trypan blue, hemocytometer, light microscope with phase contrast.

2.2 Isolation of CD4+ T Cells from PBMCs

1. CD4-MicroBeads (Miltenyi Biotec, *see* **Note 1**).
2. MS or LS separation columns (Miltenyi Biotec) (*see* **Note 2**).
3. Magnet for MS or LS columns (MACS separators, Miltenyi Biotec).
4. Bead separation medium (MACS buffer): Phosphate-buffered saline (PBS), without Ca^{2+} or Mg^{2+}, pH 7.2, 2 % fetal bovine serum (FBS). Alternatively, replace 2 % FBS with 0.5 % bovine serum albumin (BSA). Keep buffer cold (2–8 °C). Degas before use, as air bubbles could block the column.
5. (Optional) Pre-Separation Filters, 30 μm (Miltenyi Biotec), to remove cell clumps to obtain a single-cell suspension before magnetic separation.
6. Refrigerator (2–8 °C) and/or dry ice.
7. 15 mL polypropylene Falcon tubes.
8. Trypan blue, hemocytometer, light microscope with phase contrast.

2.3 Selection of Promiscuous MHC Class II Epitopes within Tumor Ags of Interest for Synthetic Peptide Synthesis

1. Promiscuous MHC class II epitopes (i.e., sequence regions predicted to bind several MHC class II alleles) to be used as tumor Ags are selected by computational analysis [14]. The use of promiscuous epitope peptides allows testing in a large part of the population (i.e., no MHC class II restriction is applied).
2. Web site for predictions is found at http://www.imtech.res.in/raghava/propred/ [15] and instructions are easy to be followed. Set threshold (i.e., the percentage of best scoring natural peptides) at 5 % and select either an HLA-DR allele of interest for allele-specific prediction (i.e., patient specific) or ALL for selection of promiscuous epitopes (i.e., large population screening).
3. Design peptide sequences with length up to 15–25 amino acids, eventually containing more than one epitope core frame (i.e., amino acids 1–9). Prediction of amino acid at position 1 is identified by red font.

2.4 Ex Vivo Re-stimulation Assays for Detection and Enumeration of Tumor Ag-Specific CD4+ T Cells

1. Tissue culture-treated 96-well round-bottom plates.
2. T cell medium: X-VIVO 15 (Lonza), penicillin (100 U/mL), streptomycin (50 mg/mL), 3 % heat-inactivated pooled human serum (HS).
3. Recombinant cytokine IL-2 (Novartis) (*see* **Note 3**).

4. Synthetic peptides (stocks: 1 μg/mL in sterile DMSO).
5. Leucoagglutinin (PHA-L) (*see* **Note 4**).
6. Autologous APCs (i.e., total PBMCs or CD4 fraction obtained from CD4 magnetic bead separation of PBMCs).
7. 15–50 mL polypropylene Falcon tubes.
8. Trypan blue, hemocytometer, light microscope with phase contrast.
9. γ-Irradiator.
10. 37 °C incubator.

2.5 Immunohistochemistry for Expression of GATA-3 (Transcription Factor for Th2) and T-bet (Transcription Factor for Th1)

1. Slides from paraffin-embedded tissues from patient tumor or normal tissue samples.
2. Chemical hood.
3. Xylene (Bioclear).
4. Rehydration solutions: Absolute ethanol, ultrapure deionized H_2O. Prepare: 95 % ethanol in ultrapure deionized H_2O and 70 % ethanol in ultrapure deionized H_2O.
5. Ag retrieval solution: Tris-EDTA pH 9.0. For 1 liter (L) weigh 6.1 g Tris base, 0.372 g EDTA and adjust volume to 1 L with ultrapure deionized H_2O (H_2O_d).
6. Pap-pen (Biocare Medical or similar).
7. Peroxidase blocking solution: For 3 % final solution add 10 mL of 30 % H_2O_2 to 90 mL of H_2O_d.
8. Wash buffer: PBS-T. Prepare 10× PBS: 80 g NaCl, 2 g KCl, 14,4 g Na_2HPO_4, 2,4 g KH_2PO_4, up to 1 L with H_2O_d. Make 1× solution diluting 1:10 with H_2O_d and add 1 mL Tween-20 or 100 μL Triton-X (Fluka) to 1 L of PBS.
9. Protein block solution: BSA solution. 3 g BSA (bovine serum albumin Merck or similar), 100 mL PBS 1×, 100 μL Tween-20, store at 4 °C.
10. Antibody diluent: Primary antibody diluting buffer with surfactants (Bioptica).
11. Primary antibodies: (i) GATA-3 mouse IgG1 (BD Biosciences), 1:300 in antibody diluent; (ii) T-bet (4B10 clone) mouse monoclonal antibody (Santa Cruz Biotechnology), 1:100 in antibody diluent
12. Secondary antibody: See universal secondary antibody from Ultravision Quanto Detection system HRP-polymer kit (Thermo Scientific).
13. DAB Quanto kit # TA-125 QHDH (Thermo Scientific).
14. Papanicolau Harris hematoxylin (Bioptica) filtered on Whatman filter paper.

15. Dehydration solutions. Prepare 70 % ethanol in H_2O_d and 95 % ethanol in H_2O_d.
16. Xylene (Bioclear). Mounting solution: Permanent mounting medium (Bioptica).

2.6 Quantification of GATA-3 and T-bet-Positive Cells

1. Aperio slide scanner.
2. Spectrum Plus software (Aperio).

3 Methods

3.1 Isolation of PBMCs from Heparinized Blood

1. Distribute the heparinized blood volume (25–50 mL) into one to two 50 mL polypropylene Falcon tubes, up to 25 mL per Falcon (*see* **Note 5**).
2. Dilute the blood 2× with PBS/saline and mix well.
3. Pipet 12–13 mL of Ficoll-Paque on the bottom of two to four 50 mL Falcon tubes.
4. Carefully layer 25 mL of the diluted blood over the Ficoll-Paque phase, to a final blood:Ficoll-Paque volume ratio of 2:1. Try to minimize the mixing of blood with Ficoll-Paque (*see* **Note 6**).
5. Centrifuge at 800 × *g* for 30 min at room temperature (RT) in a swinging-bucket rotor without brake.
6. Recover the PBMCs that accumulate in the white layer (ring) on top of the Ficoll-Paque layer (*see* **Note 7**).
7. Wash PBMCs 3× with 50 mL PBS/saline:
   - First wash: centrifuge at 650 × *g* for 10 min, RT. Gently decant supernatant.
   - Second wash: centrifuge at 300 × *g* for 10 min, RT. Gently decant supernatant.
   - Third wash: centrifuge at 170–200 × *g* for 10 min, RT. Gently decant supernatant.
8. (Optional) Repeat the third wash in order to remove most of the platelets.
9. Resuspend PBMCs in PBS/saline and count leukocytes with trypan blue in the hemocytometer under microscope (*see* **Note 8**).

3.2 Isolation of CD4+ T Cells from PBMCs

$CD4^+$ T cells are isolated by positive selection after magnetic bead labeling and magnetic separation with MACS (Miltenyi) reagents. Following the manufacturer's instructions is highly recommended to obtain maximal cell recovery and purity. The manufacturer's protocol is here enlisted:

1. Preparation of samples:
   - Determine the amount of PBMCs needed to obtain the desired number of CD4+ T cells ($1–4 \times 10^7$ cells/mL) (*see* **Note 9**).
   - Centrifuge cell suspension at $300 \times g$ for 10 min. Aspirate supernatant completely.
   - Resuspend PBMCs in MACS buffer at 1.25×10^8/mL (80 µL per 1×10^7 cells).
2. Magnetic labeling (protocol for 1×10^7 PBMCs) (*see* **Note 10**):
   - Add 20 µL of CD4-MicroBeads per 10^7 total cells.
   - Mix well and incubate for 15 min in the refrigerator (2–8 °C) or on ice for a longer time (*see* **Note 11**).
   - Wash cells by adding 1–2 mL of buffer per 10^7 cells and centrifuge at $300 \times g$ for 10 min. Aspirate supernatant completely.
   - Resuspend up to 10^8 cells in 500 µL of buffer.
3. Magnetic separation (MS columns) (*see* **Note 10**):
   - Place column in the magnetic field of a suitable MACS separator.
   - Prepare column by rinsing with the appropriate amount of MACS buffer (500 µL for MS columns).
   - (Optional) Pass cells through a 30 µm nylon mesh to remove cell clumps, which may clog the column.
   - Apply cell suspension onto the column.
   - Collect in a 15 mL polypropylene Falcon tube the unlabeled cells that pass through. Wash three times the column by adding the appropriate amount of buffer (3×500 µL for MS columns). Only add new buffer when the column reservoir is empty. Collect total effluent cells; this is the unlabeled cell fraction (CD4 PBMCs) (*see* **Note 12**).
   - Remove column from the separator and place it on a 15 mL polypropylene Falcon tube.
   - Pipette the appropriate amount of buffer onto the column (1 mL for MS columns) and flush out the magnetically labeled cells by firmly pushing the plunger into the column (*see* **Note 13**).

3.3 Peptide Synthesis

Synthetic peptides corresponding to the tumor Ag sequences are purchased based on the selection of promiscuous sequence regions on the tumor Ag of interest (*see* **Note 14**).

3.4 Ex Vivo Re-stimulation Assays for Detection and Enumeration of Tumor Ag-Specific CD4+ T Cells

3.4.1 Short-Term Cultures of CD4+ T Cells for Identification of Responsive Donors (Ex Vivo Re-stimulation Assay-Type A)

The methods described here are based on a strategy developed and described in [16, 17].

1. Count cells of the positive fraction obtained from the magnetic selection ($CD4^+$ T cells).
2. Irradiate at 3000 rad the cells of the negative fraction obtained from the magnetic selection (CD4 PBMCs). They are used as APCs for re-stimulation of $CD4^+$ T cells (*see* **Note 12**).
3. Count irradiated $CD4^-$ cells.
4. Determine the number of $CD4^+$ T cells and APCs needed for the culture, considering that the T:APC ratio in the cultures must be 1:3 (*see* **Note 15**).
5. Resuspend $CD4^+$ T cells in T cell medium at a concentration of 0.5×10^6 cells/mL.
6. Resuspend APCs in T cell medium at a concentration of 1.5×10^6 cells/mL.
7. Dilute the peptide stocks in T cell medium to 100 μg/mL (10× dilution) and PHA-L to 100 μg/mL (10× dilution).
8. Plate cells and peptides in 96-well round-bottom plates as follows, for a final volume of 220 μL/well:
   - 0.5×10^4 $CD4^+$ T cells (100 μL/well of cell suspension).
   - 1.5×10^4 APCs (100 μL/well of cell suspension).
   - 10 μg/mL single peptide or PHA-L (20 μL of the 10× dilution).

   Plate 6 replicate wells per stimulation condition, i.e.:
   - No stimulation (unstimulated) as baseline.
   - PHA-L (10 μL/mL) as positive control
   - Each single peptide (10 μg/mL) (*see* **Note 16**)
9. Incubate at 37 °C.
10. At day 7 (d7), remove half medium (100 μL) from each well and replenish with fresh medium containing IL-2 (25 IU/mL) without any further peptide stimulation.
11. At day 14 (d14), collect 100 mL of supernatant from each of the 6 replicate wells/condition and pool them (*see* **Note 17**).
12. Use 6-well pooled supernatants to measure IFN-γ (for Th1 cells) and IL-5 (for Th2 cells) secretion by ELISA (Mabtech), according to the manufacturer's instructions (*see* **Note 18**). Samples from peptide-stimulated $CD4^+$ T cells (pooled supernatants of stimulated wells) are considered positive if at least double of the values from unstimulated cells (pooled supernatants of unstimulated wells) and above 50 pg/mL.

13. (Optional) To test peptide-specific CD4+ T cell proliferation, pulse single d14 wells for 16–18 h with [^{3}H]TdR (add 50 mL/well T cell medium supplemented with 1 mCi [^{3}H]TdR, corresponding to 6.7 Ci/mol; Amersham Corp.). Collect cells with a FilterMate Universal Harvester (Packard Instruments) in specific plates (Unifilter GF/C; Packard) for measurement of thymidine incorporation by a liquid scintillation counter (TopCount NXT; Packard). Responses are considered positive when significantly higher than the unstimulated wells, as determined by unpaired, one-tailed Student's *t* test. Alternatively, pool CD4+ T cells from d14 replicated cultures and test them in ex vivo re-stimulation assay type C (*see* below).
14. (Optional) Add 100 μL/mL fresh medium containing IL-2 (25 IU/mL) without any further peptide stimulation to prolong T cell cultures for additional 7 days. At day 21 (d21) repeat **steps 11–13**.

3.4.2 Short-Term Cultures for Frequency Estimation of Ag-Specific CD4+ T Cells (Ex Vivo Re-stimulation Assay-Type B)

Repeat **steps 1–12** from protocol A with the following modifications:

1. Select the peptide of interest to use for determining the frequency of peptide-specific CD4+ T cells.
2. Plate a total number of 1.8×10^5 CD4+ T cells in 60 wells, 3×10^4 CD4+ T cells/well. Add 9×10^4 APCs/well (*see* **Note 19**).
3. Add no peptide to half wells *(30)*, in order to have 30 replicates of the "unstimulated" condition (–Ag).
4. Add the selected peptide to half wells *(30)*, in order to have 30 replicates of the "stimulated" condition (+ Ag) (*see* **Note 19**).
5. At day 14, collect 100 μL of supernatant from each of the 60 wells without pooling them.
6. Measure IFN-γ and IL-5, or any other relevant cytokine, release by ELISA on single-well supernatants.

3.4.3 Calculation of Peptide-Specific CD4+ T Cell Frequency

Use Table S9 from [16], which can be downloaded at http://www.plosone.org/article/info%3Adoi%2 F10.1371%2Fjournal.pone.0042340#s5 and follow the instructions:

1. Insert the number of seeded cells/well in Subheading 3.4.2 (*see* **Note 18**).
2. Set the confidence level of interval estimates (usually alpha = 0.05).
3. Insert in the appropriate column ("Un-stimulated wells" or "Ag-stimulated wells") the concentrations of the assayed cytokine, measured for each well (1 well = 1 excel-cell).
4. Point and interval frequencies of Ag-specific CD4+ T cells are automatically calculated and are referred to the cytokine tested (*see* **Note 20**).

3.4.4 Short-Term Cultures for Validation of the Ag Specificity of Expanded CD4+ T Cells (Ex Vivo Re-stimulation Assay-Type C)

1. Collect cells from replicate wells of d14- or d21-peptide-stimulated $CD4^+$ T cells in 15 mL Falcon tubes.
2. Centrifuge at $400 \times g$ for 5 min, and discard the supernatants.
3. Wash once with T cell medium. Centrifuge at $400 \times g$ for 5 min and discard the supernatants.
4. Resuspend $CD4^+$ T cells in T cell medium (without IL-2) at 1×10^5 cells/mL.
5. Thaw autologous PBMCs (or use fresh, if available) and irradiate them at 4000 rad. Count irradiated PBMCs.
6. Centrifuge at $400 \times g$ for 5 min, discard the supernatants and resuspend PBMCs at 5×10^5 cells/mL. They are used as APCs in a T:APC ratio of 1:5 (*see* **Note 21**).
7. Resuspend $CD4^+$ T cells in T cell medium at a concentration of 0.5×10^6 cells/mL.
8. Dilute the peptide stocks in T cell medium to 100 μg/mL (10× dilution).
9. For every pool of d14- (or d21-) peptide-stimulated $CD4^+$ T cells, plate in 4–6 wells in 96-well round-bottom plates:
   - 1×10^4 $CD4^+$ T cells (100 μL/well of cell suspension).
   - 5×10^4 APCs (100 μL/well of cell suspension).
   - No peptide in half wells.
   - The relevant peptide at 10 μg/mL (20 μL of the 10× dilution) in half wells.
10. Incubate at 37 °C.
11. After 48 h, collect 100 μL of supernatant from each of the 2–3 replicate wells/condition and pool them.
12. Use pooled supernatants to measure IFN-γ and IL-5, or any other relevant cytokine, release by ELISA (*see* **Note 18**).
13. (Optional) To test peptide-specific $CD4^+$ T cell proliferation, repeat **step 13** of Subheading 3.4.1.

3.5 Immunohistochemistry for Expression of GATA-3 (Transcription Factor for Th2) and T-bet (Transcription Factor for Th1)

Examples of the use of this method for detection of GATA-3 and T-bet-positive tumor-infiltrating lymphoid cells are reported in [17–19].

1. Under a chemical hood, de-wax slides from paraffin-embedded tissues from patient tumor or normal tissue samples in xylene (Bioclear or similar) for 30 min and then rehydrate them sequentially for:
   - 2 min in absolute ethanol.
   - 2 min 95 % ethanol in $H_2O_{dd.}$
   - 2 min 70 % ethanol in $H_2O_{dd.}$
   - 2 min $H_2O_{dd.}$

2. For Ag retrieval treat the slides in Tris-EDTA pH 9.0 for 30 min in a water bath set at 97 °C (*see* **Note 22**).
3. Let the slides cool to RT for 15 min.
4. Wash with tap water.
5. With a pap-pen circle the area to be stained on the slide.
6. Block sections with peroxidase blocking solution for 5 min sharp.
7. Wash with PBS-T.
8. Leave sections in protein block solution for 10 min at RT.
9. Add on sequential slides anti-GATA-3 or anti-T-bet primary antibodies diluted as indicated and incubate for 1 h at RT.
10. Wash with PBS-T.
11. Apply the universal secondary antibody from Ultravision Quanto detection system HRP kit (Thermo Scientific) for 30 min at RT and wash with PBS-T.
12. Apply HRP polymer and incubate for 30 min.
13. Wash with PBS-T thoroughly.
14. Add 1 drop (30 μL) of DAB chromogen to 1 mL of DAB substrate, mix, and apply to tissue for 5 min.
15. Wash in H_2O_{dd}.
16. Counterstain the sections with hematoxylin for 5 s and rinse in running tap water until clear (10 min) (*see* **Note 23**).
17. Apply ascending alcohol scale for dehydration to the sections:
    - 2 min 70 % ethanol.
    - 2 min 95 % ethanol.
    - 2 min Absolute ethanol.
    - 2 min Xylene (Bioclear).
18. Under a chemical hood mount the slides by pipetting 1–2 (5 μL) drops of permanent mounting medium, cover with a glass cover slip, and observe by light microscopy.

3.6 Quantification of GATA-3 and T-bet-Positive Cells

1. GATA-3 and T-bet immunostained slides are scanned at 20× magnification with the Aperio Scanscope instrument utilizing ImageScope V10 and stored in the Spectrum database.
2. Intertumoral areas, rich in lymphoid infiltrates, are selected in digitalized slides with the pen tool of the ImageScope software. Multiple areas accounting for at least 2 mm^2 area are selected. It is important to exclude from the area to be analyzed the cellular component not related to the immune infiltrate: indeed, neoplastic cells can display GATA-3 nuclear and cytoplasmic reactivity.

3. GATA-3 and T-bet immunostained nuclei are enumerated with the Nuclear V9 algorithm. This software permits the identification and automatic counting of all nuclear stained structures in the selected area as either absolute number or the percentage of the total cell number. This software identifies also the intensity of immunostaining in each cell with a 3 grade scale.
4. The results obtained can be exported in an Excel file for statistical analysis.

4 Notes

1. $CD4^+$ T cell isolation kits are also distributed by other vendors, like Dynal Inc., and provided with their tailored manufacturer's protocol.
2. The choice of MS or LS columns depends on the maximum number of labeled cells (10^7 and 10^8 for MS and LS, respectively) and on the maximum number of total cells used for the separation (2×10^8 and 2×10^9 for MS and LS, respectively).
3. Recombinant human IL-2 is available from a number of vendors, including Novartis, Peprotech, and R&D Systems. Different vendors sell cytokines and growth factors by weight (expressed in μg) or by activity (expressed in IU). The stocks are often sold with an indicative activity of $\geq 1 \times 10^6$ IU/mg cytokine. The World Health Organization and the National Institute for Biological Standards and Control (UK) produce batches of cytokines with assigned biological activity against which the activity of various cytokine preparations can be tested, in order to calculate their IU. This allows standardization of the measurement of many cytokines and growth factors.
4. PHA-L is one of the two variants of phytohemagglutinin (the other is PHA-E) and it is a lectin extracted from the *Phaseolus vulgaris* (red kidney bean). It is commonly used as a mitogen to trigger T-lymphocyte polyclonal activation and cell division.
5. Take necessary precautions when working with human blood products. Assume that all blood-derived products are to be considered potential hazards, including culture supernatants, which are tested by ELISA for cytokines.
6. Instead of layering the blood on the Ficoll-Paque phase the latter can be added on the bottom of the Falcon tube containing the diluted blood by using a needle and a syringe. This does not affect the efficacy of the gradient separation.
7. For easiest recovery of the PBMC ring from the Ficoll-Paque interface aspirate the platelet-containing supernatant to around 5 mL above the PBMC ring, and then pipet PBMCs out.

8. The expected yield of PBMCs from venous blood is $0.5–1 \times 10^6$ cells/mL.
9. The expected yield of CD4+ T cells is 30–40 % of total PBMCs (higher percentages are obtained from frozen samples, as after thawing T cells recover better compared with other cells present in total PBMCs).
10. Volumes for magnetic labeling and magnetic separation are for up to 10^7 total cells. When working with fewer than 10^7 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes, accordingly.
11. Work fast, keep cells cold, and use precooled solutions. This will prevent capping of antibodies on the cell surface and nonspecific cell labeling. Working on ice may require increased incubation times. Higher temperatures and/or longer incubation times may lead to nonspecific cell labeling.
12. The CD4− PBMCs are recovered to be used as APCs in ex vivo re-stimulation assays. This fraction mainly contains monocytes, but also B cells and rare dendritic cells, which are all able to activate memory CD4+ T cells. The ex vivo re-stimulation assays described here (both types A and B) only detect memory CD4+ T cell responses and do not induce in vitro priming of naïve CD4+ T cells, as demonstrated in [16, 17].
13. To increase the purity of CD4+ T cells, the eluted fraction can undergo a second round of magnetic separation over a new column, but this also reduces the yield of cells, which may be trapped in the column. The purity of CD4+ T cells can be determined by flow cytometric analysis with directly labeled antibodies to CD4. Kits usually yield more than 90 % purity already after the first column separation and this is sufficient for the use of CD4+ T cells in the re-stimulation assays.
14. Peptides to be used in these experiments should have been tested prior to their use for the capacity to elicit a CD4+ T cell response.
15. The same protocol can be carried out with frozen PBMCs (or even frozen CD4+ T cells and the autologous CD4− counterpart). It should be considered that after thawing most T cells are alive, whereas in the CD4− fraction a higher cell loss (i.e., monocytes) is present. To guarantee the appropriate T:APC ratio, the starting amount of PBMCs must be higher for frozen samples, owing to the reduced fraction of APC vitality after thawing.
16. Peptides can be used in cultures as single or in small pools of 2–5 peptides. In this case it is suggested lowering the single peptide concentration to 5 μg/mL, in order to maintain the activity while avoiding cell toxicity.

17. Protein integrity may be compromised by repeated cycles of freezing and thawing. For this reason, freezing small volumes of supernatant is recommended. Alternatively, a number of tests on the same supernatants should be carried out in a short time span. Meanwhile, storing supernatants at 4 °C is recommended.
18. The test of choice to determine the Th profile of the activated $CD4^+$ T cells is the measurement of cytokines secreted in culture supernatants by ELISA. As reported, IL-5 is a Th2-associated cytokine (IL-4 and IL-13 can also be measured), and IFN-γ a Th1-associated cytokine. It is recommended to measure additional cytokines (i.e., GM-CSF, IL-10, IL-17, IL-21, IL-22, TGF-β, and TNF-α to detect Th subsets different from Th1 and Th2 (*see* Fig. 1 for multiple subset characteristics and cytokine released). Depending on the cytokine tested, highly specific and sensitive ELISA kits can be chosen from Mabtech, or R&D Systems. Alternatively, to reduce the amount of supernatant used for one test, multiple simultaneous cytokine measurements are possible using multiparametric assays such as cytometric bead array (CBA) kits (BD Biosciences) or Luminex Multiplex kits (R&D system).
19. The total number of seeded wells sets the detection limits of the method for frequency calculation. For 60 wells (half unstimulated and half Ag-stimulated), the lower detection limit is $m = 1.13 \times 10^{-6}$ and the highest limit is $M = 1.13 \times 10^{-4}$. The number of $CD4^+$ T cells seeded/well has been set at 3×10^4/well, but it can be modified whenever needed. For more details, refer to [16].
20. The calculation of point and interval frequencies, provided by Table S9 [16], is based on the implementation of the Poisson distribution with other statistical parameters (outlier exclusion, exceptionality threshold, etc.), extensively described in [16]. The experimental design of ex vivo re-stimulation assay type B allows considering the presence/activation of an Ag-specific $CD4^+$ T cell in each single well as a "rare event" and thus the Poisson distribution can be applied to the assay.
21. When using total PBMCs as APCs the T:APC ratio increases in favor of PBMCs (1:5) because the proportion of monocytes, B cells, and dendritic cells is lower than in $CD4^-$ fraction of PBMCs.
22. Shorter or longer incubation time may affect the staining result.
23. Hematoxylin can be reused several times.

Acknowledgements

This work was supported by the Italian Association for Cancer Research (AIRC, IG-4510, and IG-11353) and the International Association for Cancer Research (AICR).

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Chapter 5

Detection and Functional Analysis of Tumor-Derived LXR Ligands

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Abstract

There is growing evidence highlighting the ability of nuclear receptors to control not only metabolism, but also inflammation and cancer progression. In particular liver X receptors (LXRs), the nuclear receptors physiologically involved in cholesterol homeostasis, have been shown to regulate innate and adaptive immune responses in many pathological conditions, including cancer.

We have recently demonstrated that LXR ligands (oxysterols) released by tumor cells may have an immunomodulatory role, affecting the immune cells involved in the antitumor immune response. Indeed, oxysterols inhibit the expression of the chemokine receptor CCR7 on dendritic cells (DC) in an LXR-dependent manner, thus impairing DC migration to secondary lymphoid organs, and therefore dampening the induction of successful antitumor responses.

We have resorted to direct (i.e., luciferase-based LXR activation assay) and indirect (i.e., activation of LXR target genes in dendritic cells) methods in order to assess the presence of LXR ligands (oxysterols) in tumor-conditioned media.

These two methods are also suitable to study strategies to block oxysterol release by tumor cells.

Key words Liver X receptors, Oxysterols, CCR7, Dendritic cells, Tumor

1 Introduction

Liver X receptors (LXR) α (also known as NR1H3) and β (NR1H2) belong to a superfamily of 48 ligand-dependent transcription factors, which mainly regulate development and homeostasis [1]. LXRα and LXRβ receptors are involved in lipid and cholesterol homeostasis through the induction of the target gene products ABCA1, ABCG1, SREBP-1c, etc., which regulate cholesterol efflux and fatty acid synthesis [2]. LXRβ is expressed ubiquitously, while LXRα is expressed by liver, adipose tissue, adrenal glands, intestine, lungs, and cells of myelomonocytic lineage [3]. The main ligands of LXRs are oxysterols [4], which may form through two alternative pathways: (1) enzymatically by means of cholesterol 24-hydroxylase (24S-HC), sterol 27-hydroxylase (27-HC), cholesterol 25-hydroxylase (25-HC),

Attilio Bondanza and Monica Casucci (eds.), *Tumor Immunology: Methods and Protocols*, Methods in Molecular Biology, vol. 1393,
DOI 10.1007/978-1-4939-3338-9_5,

and Cyp11A1 (22R-HC) [5–7], and (2) nonenzymatically through reactions initiated by non-radical reactive oxygen species such as singlet O_2, HOCl, and ozone (O_3) or by inorganic free radical species derived from nitric oxide, superoxide, and hydrogen peroxide [6]. 7β-HC and 7KC are exclusively generated by nonenzymatic cholesterol oxidation. 7α-HC, 4β-HC, and 25-HC can be produced by both pathways, whereas 24S-HC and 27-HC can be generated only by cholesterol hydroxylases [5, 6]. LXRα and β isoforms function as heterodimers with the retinoid X receptor (RXR), a common partner for several nuclear receptors. The LXR/RXR complex binds to an LXR response element (LXRE) in the promoter region of target genes and regulates gene expression through different mechanisms, such as direct activation, ligand-independent and ligand-dependent repression, and trans-repression [8, 9].

These receptors have been shown to modulate both innate and adaptive immune responses in infectious, inflammatory, and autoimmune diseases [10]. In particular LXRs have been shown to play an important role in macrophages, by protecting them from the apoptosis induced by some pathogens [11, 12]. Moreover, LXRs have been shown to promote the clearance of apoptotic cells by macrophages and dendritic cells, thus maintaining immune tolerance [13]. Also, T and B cells are influenced by LXR activation. The engagement of LXRs blocks the proliferation of T- and B-cells undergoing activation in physiologic conditions [14]. On the other hand, LXRs have been shown to inhibit Th1 and Th17 differentiation [15, 16].

We have recently reported a molecular link between LXR ligands and tumor-infiltrating DCs [17]. Indeed, we have shown that LXR ligands are released by tumors and inhibit the functional expression of the chemokine receptor CCR7 on maturing DCs through the engagement of LXRα [17]. The up-regulation of CCR7 is mandatory for DC migration to secondary lymphoid organs where they present antigens to naïve T- and B-cells. In our tumor models, the genetic or pharmacological inactivation of the LXR/LXR ligand pathway restored DC migration to draining lymph nodes and, therefore, the antitumor immune response. The relevance of this pathway is further highlighted by immunohistochemical studies showing the presence of CCR7-negative mature DCs in human tumors of various histological types (melanoma, pancreatic cancer, NSCLC, colon and breast cancers) [17]. The inhibition of CCR7 on maturing DCs treated with LXR agonists has been observed also by other groups [18, 19].

We set two methods to test the presence of LXR ligands in tumor-conditioned medium. Tumor-conditioned medium was directly tested for the presence of LXR ligands by using a luciferase-based LXR activation assay, or their presence was indirectly evaluated by measuring the up-regulation of the LXR target gene *ABCG1* by qRT-PCR assay, in DC undergoing activation [17, 20]. Importantly, these two tests were also used to identify possible strategies to block

the production or the functionality of LXR ligands. Indeed, the treatment of LXR ligand-releasing tumor cells with the squalene synthase inhibitor zaragozic acid [21] partly abrogated luciferase-based LXR activation and *ABCG1* up-regulation in DC undergoing activation [17]. A more potent effect was observed by taking advantage of the enzyme sulfotransferase 2B1b (SULT2B1b) [22], which inactivates LXR ligands through a specific sulfation process. Indeed, conditioned medium from tumors engineered to express SULT2B1b failed to activate luciferase-based LXR, as well as to induce *ABCG1* mRNA up-regulation in DC undergoing activation [17].

2 Materials

2.1 Tumor-Conditioned Medium Testing by Luciferase-Based LXR Activation Assay

2.1.1 HeK 293 Seeding

1. Hek 293 (ATCC Manassas, USA) cell suspension (*see* **Note 1**).
2. Dulbecco's phosphate-buffered saline (D-PBS) without Ca^{2+} and Mg^{2+} (Euroclone).
3. 5 % Trypsin-EDTA 10× (GIBCO).
4. Culture medium: 500 mL Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose (BioWhittaker), 5 mL l-glutamine 200 mM 100× (GIBCO), 50 mL fetal bovine serum (FBS), 5 mL penicillin (10,000 U/mL)/streptomycin (10,000 μg/mL) (GIBCO).
5. 15 mL polypropylene tubes.
6. Tissue culture-treated 24-well plates.
7. Trypan blue, phase-contrast light microscope.

2.1.2 HeK 293 Transfection

1. DNA mini or maxi prep grade (*see* **Note 2**) of the following plasmids:
   - Nuclear receptor ligand-binding domain - Gal 4 (pCMV-Gal4-LXRα).
   - Tk-Luciferase (pMH100X4-Tk-Luc).
   - β-Galactosidase.
2. FuGENE HD transfection Reagent (Roche) (*see* **Note 3**).
3. Demineralized water.
4. Iscove's Modified Dulbecco's Medium (IMDM) with l-glutamine (BioWhittaker) and 25 mM Hepes plus l-glutamine 200 mM 100× (GIBCO) plus 10 % FBS.
5. LXR-specific ligand (T0901317, 20 nM, Sigma) (*see* **Note 4**).
6. Eppendorf 1.5 mL.
7. Vortex.

2.1.3 HeK 293 Lysate Preparation and Luciferase Assay

1. Luciferase Assay Systems (Promega) (*see* **Note 5**).
2. Luciferase Assay Substrate (*see* **Note 5**).
3. Luciferase Assay Buffer.
4. Luciferase Cell Culture Lysis Reagent 5×.
5. Demineralized water.
6. Docking platform.
7. Opaque multiwall plates (COSTAR).
8. Luminometer (Mithras LB940).

2.1.4 β-Galactosidase Assay

1. Na_2HPO_4 35.61 g/l.
2. NaH_2PO_4 31.21 g/l.
3. NaP (sodium phosphate) 0.1 M pH 7.5: Na_2HPO_4 8.2 mL, NaH_2PO_4 1.8 mL, H_2O 10 mL.
4. Mg^{2+} 100×: $MgCl_2$ 1 M 100 µl, β-mercaptoethanol (ME) 315 µl, H_2O 585 µl.
5. ONPG 1× (o-nitrophenyl-β-D-galactoside) (*see* **Note 6**): ONPG 0.036 g, NaP 0.1 M pH 7.5.
6. ONPG MIX: ONPG 1 × 33 µl/well, Mg^{2+} 100 × 1.5 µl/well, NaP 0.1 M pH 7.5 105 µl/well.
7. 96-well Plate U bottom (COSTAR).
8. Microplate reader (BIO-RAD 680).
9. 15 mL polypropylene tubes.

2.2 Tumor-Conditioned Medium Testing by ABCG1 mRNA Expression in Maturing Dendritic Cells

1. 50 mL buffy coat of 1 unit of human blood.
2. D-PBS without Ca^{2+} and Mg^{2+} (Euroclone).
3. Lymphocyte separation medium or Ficoll (Sentinel Diagnostic). (Density 1.077 g/mL.)
4. 50 mL polypropylene conical tubes.
5. Trypan blue, hemocytometer, phase-contrast light microscope.

2.2.1 Isolation of PBMCs from Buffy Coat of Peripheral Blood

2.2.2 Differentiation of Dendritic Cells from Peripheral Blood Monocytes

1. T25 tissue culture flasks.
2. Culture medium:
   - 500 mL RPMI 1640 (BioWhittaker).
   - 50 mL Hyclone FBS.
   - 5 mL l-glutamine 200 mM 100× (GIBCO).
   - 5 mL Penicillin (10,000 U/mL)/streptomycin (10,000 µg/mL).

3. Recombinant human cytokines (*see* **Note 11**): GM-CSF (Leukine, Genzyme), IL-4 (Immunotools).
4. β-ME.
5. D-PBS.
6. 37 °C incubator.

2.2.3 Conditioning of Maturing Dendritic Cells with Tumor-Derived Medium

1. Tissue-culture treated 6-well plates.
2. 50 mL polypropylene conical tubes.
3. 15 mL polypropylene conical tubes.
4. Culture medium.
5. D-PBS.
6. Recombinant human cytokines: GM-CSF (Leukine, Genzyme), IL-4 (Immunotools).
7. β-ME.
8. LPS (Sigma).
9. 22R-Hydrocholesterol (Sigma or Avanti polar).
10. 37 °C incubator.
11. –80 °C freezer.
12. Trypan blue, hemacytometer, phase-contrast light microscope.

2.2.4 Total RNA Extraction

1. RNaesy minikit (Qiagen, Hilden, Germany) (*see* **Note 10**):
   - RNeasy Mini Spin Columns.
   - Collection tubes (1.5 mL).
   - Collection tubes (2 mL).
   - Buffer RLT.
   - Buffer RW1.
   - Buffer RPE (concentrate).
   - RNase-free water.
2. RNase-free syringes.
3. Blunt 20-gauge needles (0.9 mm diameter).
4. 70 % ethanol.
5. Microcentrifuge.
6. Nanospectrophotometer.
7. Vortex.
8. –80 °C freezer.

2.2.5 cDNA Synthesis

1. M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, USA) (*see* **Note 11**).
2. RNase inhibitor (Promega, Fitchburg, USA) (*see* **Note 12**).

3. 5× First-strand buffer [250 mM Tris–HCl (pH 8.3 at room temperature (RT)], 375 mM KCl, 15 mM $MgCl_2$.
4. 0.1 M DTT.
5. Oligo(dT)12–18 primers (500 μg/mL).
6. 25 mM dNTP mix (25 mM each dATP, dGTP, dCTP, and dTTP at neutral pH).
7. Nuclease-free water.
8. Nuclease-free microcentrifuge tubes.
9. Microcentrifuge.
10. Thermoblock.
11. –20 °C freezer.

2.2.6 QPCR Analysis to Evaluate LXR Activation

1. SYBR Green PCR Master Mix (Life Technologies, Carlsbad, USA) (*see* **Note 13**).
2. Nuclease-free water.
3. GAPDH primers:
   - Forward ACA TCC TCC CTG CCT CTA CTG
   - Reverse ACC ACC TGG TGC TCA GTG TA
4. ABCG1 primers:
   - Forward CCC AGC AGA TTT TGT CAT GGA
   - Reverse CCA GCC GAC TGT TCT GAT CA
5. Optical 96-well QPCR plates.
6. Optical adhesive films.
7. Real-time PCR instrument.

3 Methods

3.1 Tumor-Conditioned Medium Testing by Luciferase-Based LXR Activation Assay

3.1.1 HeK 293 Seeding

1. Harvest HeK 293 cells from T75 flask 1 day before cell transfection.
2. Remove supernatant.
3. Wash once with 2 mL of D-PBS without Ca^{2+} and Mg^{2+}.
4. Add 2 mL trypsin-EDTA 1×.
5. Incubate at 37 °C for 2′ until cells detach.
6. Add 8 mL of DMEM 10 % FBS.
7. Collect cells in 15 mL polypropylene tubes.
8. Count cells with trypan blue under light microscope.
9. Centrifuge cells at RT at 450 × *g*.
10. Resuspend cells in culture medium.

11. Plate cells in 24-well plate 1×10^5 cells/well (500 µl/well).
12. Incubate at 37 °C for 24 h.

3.1.2 HeK 293 Transfection

1. Prepare DNA MIX using the following plasmids:
   - Nuclear receptor ligand-binding domain—Gal4 (pCMV-Gal4-LXRα) 100 ng/well.
   - Tk-Luciferase plasmid (pMH100X4-Tk-Luc) 100 ng/well.
   - β-Galactosidase-expressing plasmid 30 ng/well.
   - H_2O to reach the volume of 5.7 µl/well.
2. Prepare the control DNA MIX using the following plasmids (*see* **Note 7**):
   - Without nuclear receptor ligand-binding domain—Gal4.
   - Tk-luciferase plasmid (pMH100X4-Tk-Luc) 100 ng/well.
   - β-Galactosidase-expressing plasmid 30 ng/well.
   - H_2O to reach the volume of 5.7 µl/well.
3. Dilute FUGENE in DMEM serum free (without P/S and G) to distribute 14.3 µl/well.
   - DMEM 13.65 µl/well.
   - FUGENE 0.65 µl/well.
4. Vortex 1″ the FUGENE MIX and incubate 5′ at RT.
5. Add the DNA MIX.
6. Vortex 1″ and incubate 15′ at RT.
7. Add 20 µl/well of the MIX containing DNA and FUGENE to each well.
8. Incubate at 37 °C for 4 h.
9. Remove the transfection medium.
10. Add 500 µl of the tumor-conditioned medium (*see* **Note 8**) or nuclear receptor ligands in IMDM 10 % FBS.
11. Incubate at 37 °C for 24 h.

3.1.3 HeK 293 Lysate Preparation

1. Dilute the lysis buffer.
2. Add 4 volumes of water to 1 volume of lysis buffer 5×.
3. Carefully remove the medium from each well.
4. Rinse cells with 100 µl/well D-PBS.
5. Remove D-PBS.
6. Add 100 µl/well of lysis buffer 1×.
7. Place the culture plate on a docking platform for 10′ at RT in the dark.
8. Perform a freeze-thaw cycle.
9. The cell lysate may be preserved at –20 °C for a month.

3.1.4 Luciferase Assay

1. Plate 10 μl/well of the cell lysate in Opaque multiwall plates.
2. Reconstitute the luciferase assay substrate with the luciferase assay buffer.
3. Read the plate at the luminometer.

3.1.5 β-Galactosidase Assay

1. Plate 10 μl/well of the cell lysate in a U-bottom 96-well plate.
2. Add 140 μl/well of ONPG MIX.
3. Read the plate at the microplate reader at 415 λ.

3.2 Tumor-Conditioned Medium Testing by ABCG1 mRNA Expression in Maturing Dendritic Cells

3.2.1 Isolation of PBMCs from Buffy Coat of Peripheral Blood

1. Distribute the buffy coat evenly (*see* **Note 9**) in four 50 mL conical tubes (about 12 mL per tube).
2. Add PBS to equalize volume in each tube at 35 mL.
3. Mix buffy coat and PBS well by pipetting up and down.
4. Layer 13 mL of Ficoll under the above-described mixture, being careful to minimize mixing of blood with Ficoll.
5. Centrifuge tubes for 25′ at RT, 580 × *g*, without brake.
6. Get rid of plasma-containing supernatant up to 5 mL above the dense PBMC layer at the interface with Ficoll.
7. Transfer the ring containing PBMCs into two new 50 mL conical tubes.
8. Wash PBMCs with 50 mL of D-PBS and centrifuge for 10′ at 450 × *g*.
9. Collect PBMCs in one 50 mL conical tube and wash two times with 50 mL of D-PBS. Centrifuge for 10′ at 240 × *g* to remove platelets.
10. Count PBMCs with trypan blue in hemacytometer under microscope (*see* **Note 10**).

3.2.2 Differentiation of Dendritic Cells from Peripheral Blood Monocytes

1. Resuspend PBMCs in culture medium (10×10^6 cells/mL) and plate 50×10^6 cells into T25 tissue culture flask.
2. After 1 h of plastic adherence at 37 °C, remove non-adherent cells by washing three times with D-PBS.
3. Add to the remaining adherent cells, culture medium supplemented with GM-CSF (1000 U/mL), IL-4 (500 U/mL), and β-ME (50 mmol/L) (*see* **Note 11**).
4. Incubate at 37 °C for 5 days.

3.2.3 Conditioning of Maturing Dendritic Cells with Tumor-Conditioned Medium

1. At d5 of culture, harvest dendritic cells from flasks by scraping them with a 5 mL pipette. Collect the media into a 50 mL conical tube.
2. Add 2 mL of PBS to each flask and wash once gently pipetting up and down.

3. Collect the mix of D-PBS and cells in the same 50 mL tube as in **step 1**.
4. Count cells with trypan blue in hemacytometer under light microscope.
5. Centrifuge at 450×*g* for 10′.
6. Resuspend the pellets in cell culture medium (7.5×10^6/mL) supplemented with GM-CSF (10,000 U/mL), IL-4 (5000 U/mL), β-ME (500 mmol/L), and LPS (1000 ng/mL) (*note that they are 10× concentrated*).
7. Add in different wells of a 6-well plate 1.8 mL of:
   - Culture medium (negative control).
   - Culture medium supplemented with 22R-hydrocholesterol at 5 μM/L (positive control).
   - Tumor-conditioned medium to be tested.
8. Plate in each well 200 μL of the dendritic cell suspension prepared as in **step 6** (corresponding to 1.5×10^6 cells/well).
9. Incubate at 37 °C for 8 h.
10. Harvest the cells by scraping with a P1000 pipette. Collect the samples in 15 mL conical tubes.
11. Wash with 13 mL of D-PBS and centrifuge for 10′ at 450×*g*.
12. Get rid of the supernatant and store the pellet at –80 °C.

3.2.4 Total RNA Extraction

1. Thaw the pellet of cells and disrupt it by adding 350 μl of RTL buffer. Vortex to mix (*see* **Note 12** and **13**).
2. Pass the lysate at least five times through a blunt 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe.
3. Add 1 volume of 70 % ethanol to the homogenized lysate, and mix well by pipetting.
4. Transfer the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 mL collection tube.
5. Centrifuge for 15″ at 8000×*g*. Discard the flow-through.
6. Add 700 μl Buffer RW1 to the RNeasy spin column.
7. Centrifuge for 15″ at 8000×*g* to wash the spin column membrane. Discard the flow-through.
8. Add 500 μl Buffer RPE to the RNeasy spin column.
9. Centrifuge for 15″ at 8000×*g* to wash the spin column membrane. Discard the flow-through.
10. Add 500 μl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2′ at 8000×*g* to wash the spin column membrane.

11. Optional: Place the RNeasy spin column in a new 2 mL collection tube, and discard the old collection tube with the flow-through.
12. Centrifuge at full speed for 1′.
13. Place the RNeasy spin column in a new 1.5 mL collection tube. Add 30 μl RNase-free water directly to the spin column membrane.
14. Centrifuge for 1′ at 8000 × *g* to elute the RNA.
15. Quantify RNA by reading at A260 and A280 wavelength by a nano spectrophotometer (*see* **Note 14**).
16. Store the RNA in H_2O at −80 °C.

3.2.5 cDNA Synthesis

1. Thaw RNA on ice.
2. Add the following components to a nuclease-free microcentrifuge tube:
   - 1 μl oligo (dT)12-18 (500 μg/mL).
   - 2 μg total RNA.
   - 1 μl 25 mM dNTP mix (10 mM each dATP, dGTP, dCTP, and dTTP at neutral pH).
   - Nuclease-free water to 12 μl.
3. Heat mixture at 65 °C for 5′ and chill on ice quickly.
4. Collect the contents of the tube by brief centrifugation and add:
   - 4 μl 5× First-strand buffer.
   - 2 μl 0.1 M DTT.
   - 0.5 μl RNase inhibitor (40 units/μl) (*see* **Note 15**).
5. Mix contents of the tube gently and incubate at 37 °C for 2′.
6. Add 1 μl (200 units) of M-MLV reverse transcriptase, and mix gently by pipetting up and down 1 μl M-MLV at 200 U/μL (*see* **Note 16**).
7. Incubate 50′ at 37 °C.
8. Inactivate the reaction by heating at 70 °C for 15′.
9. Add 80 μL of nuclease-free water in order to have a cDNA concentration of 20 ng/μL.
10. Store the cDNA at −20 °C.

3.2.6 QPCR analysis to evaluate LXR activation

1. Thaw cDNA on ice.
2. Set two different reactions for each sample (one for each couple of primers) as follows (perform each reaction in triplicate) (*see* **Note 17**):
   - 10 μL SYBR Green PCR Master Mix 2×.
   - 1 μL forward primer (10 μM).

- 1 μL reverse primer (10 μM).
- 4 μL nuclease-free water.
- 4 μL cDNA (20 ng/μL).

3. Load the reaction in an optical 96-well QPCR plate.
4. Cover the plate with an optical adhesive film (*see* **Note 18**).
5. Centrifuge the plate for 1′ at 450 × *g* and correct any adherent drop and bottom-bubble problem.
6. Run real-time PCR (*see* **Note 19**).

4 Notes

1. According to the vectors used to transiently transfect DNAs, different cell types are used to perform luciferase assay, such as HeK 293, NIH-3T3, HeLa, or CHO, in order to over-express gene-encoding recombinant proteins. We selected the HeK 293E, which has been transformed by the Epstein-Barr (EBNA-1) virus.
2. In this luciferase assay, the gene-encoded protein of the nuclear receptor ligand-binding domain fused to GAL-4 binds the upstream activation sequence (UAS) and activates the luciferase expression only in the presence of the nuclear receptor-specific ligand.
3. FuGENE HD is a lipid reagent that needs to be titrated according to the different cell types used for the transfection.
4. Different natural or synthetic specific ligands for LXRα have been identified. Each ligand needs to be titrated to reach the maximum effect by luciferase activation.
5. Different luciferase assays are available using firefly luciferase and *Renilla* luciferase. In this assay the firefly luciferase reporter is measured first to generate a "glow-type" luminescent signal. After quantifying the firefly luminescence, this reaction is quenched, and the *Renilla* luciferase reaction is initiated.
6. ONPG (o-nitrophenyl-ß-D-galactoside) is a chromogenic substrate hydrolyzed by β-galactosidase. The amount of β-galactosidase measures the transfection efficiency.
7. Each condition has been tested in triplicate and with control wells, containing all the reagents of the DNA mix, with the exception of the nuclear receptor ligand-binding domain-GAL4 DNA, in order to evaluate the background.
8. Tumor-conditioned media are prepared by plating $0.5–1 \times 10^6$ tumor cells/6 mL of medium and culturing them for 48/72 h, according to their features of growing. Conditioned medium was then collected and tested for LXR ligand content by luciferase assay and by *ABCG1* mRNA up-regulation on maturing DCs.

9. Recombinant human cytokines are available from a number of vendors, including Pharmingen (San Diego, USA) and Life Technologies (Bethesda, USA).
10. RNA extraction kits are available from a number of vendors, including Life Technologies (Bethesda, USA) and Promega (Fitchburg, USA).
11. M-MLV reverse transcriptase is available from a number of vendors, including New England Biolabs (Ipswich, USA) and Promega (Fitchburg, USA).
12. RNase inhibitor is available from a number of vendors, including Thermo scientific (Waltham, USA) and Sigma Aldrich (St. Louis, USA).
13. SYBR Green PCR Master Mix inhibitor is available from a number of vendors, including (Qiagen, Hilden, Germany) and Sigma Aldrich (St. Louis, USA).
14. Take necessary precautions when working with human blood products. Assume that all blood-derived products are to be considered potential hazards.
15. The expected yield of PBMCs from a 45–50 mL buffy coat ranges from 0.2 to 1×10^9 cells. Yield of dendritic cells is around 5–10 %.
16. Replace new gloves regularly, as skin and lab wares may have RNases. Use RNase-free filter pipette tips.
17. If the RNA is pure, the A_{260}:A_{280} ratio should be 1.8–2.2. Expect 6–12 μg of total RNA per sample.
18. Avoid touching both the plate and the adhesive film. This interferes with fluorescence excitation and reading. Avoid direct exposure of loaded plates to sunlight. This may compromise the fluorescent dyes in the mixture. If you need to transport a plate outside, wrap the plate in aluminum foil to protect it from sunlight.
19. Each system comes with different software to perform QPCR and analyze data. Refer to manufactory instructions.

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Chapter 6

In Vitro Generation of Antigen-Specific T Cells from Induced Pluripotent Stem Cells of Antigen-Specific T Cell Origin

Shin Kaneko

Abstract

Induced pluripotent stem (iPS) cells derived from T lymphocyte (T-iPS cells) preserve the T cell receptor (TCR) α and β gene rearrangements identical to the original T cell clone. Re-differentiated CD8 single positive αβ T cells from the T-iPS cells exhibited antigen-specific cytotoxicity, improved proliferative response, and elongation of telomere indicating rejuvenation of antigen specific T cell immunity in vitro. To regenerate antigen specific cytotoxic T lymphocytes (CTL), first, we have optimized a method for reprogramming-resistant CD8 T cell clones into T-iPS cells by using sendaiviral vectors. Second, we have optimized stepwise differentiation methods for inducing hematopoietic progenitor cells, T cell progenitors, and functionally matured CD8 single positive CTL. These protocols provide useful in vitro tools and models both for research of antigen-specific T cell immunotherapy and for research of normal and pathological thymopoiesis.

Key words Antigen-specific T cells derived from human iPS cells, In vitro thymopoiesis, Re-differentiation of antigen-specific T cells, T cell receptor rearrangement, T cell immunotherapy

1 Introduction

Peripheral T cells such as CD4 and CD8 single positive αβ T cells play a central role for protecting host from various types of pathogen. CD8 T cells especially exert cytotoxic effector functions to pathogenic cells in HLA-restricted and antigenic peptide-specific manner. The key molecule of T cell side for the recognition is αβ T cell receptor which is generated by recombination of V(D)J gene cluster regions. Although the mechanism permits T cells to have wide antigen specificity, only limited number of antigen-specific T cells exist in patients in chronic disease such as cancer or chronic viral infection. In addition to small pool of antigen-specific T cells, it is well known that continuous TCR stimulation by chronic antigens induces exhaustion of antigen specific T cells by overwork, and it resulted in gradual loss of antigen-specific T cell

Attilio Bondanza and Monica Casucci (eds.), *Tumor Immunology: Methods and Protocols*, Methods in Molecular Biology, vol. 1393, DOI 10.1007/978-1-4939-3338-9_6,

pool [1, 2]. That is one reason why sometime T cell immunity fails to eliminate chronic pathogens and why antigen-specific immunotherapies are desired to develop [3–5]. Very recently, we and another group have reported regeneration of antigen specific T cells via iPS cell ex vivo, as a new proof of concept for restoration of antigen specific T-cell immunity [6, 7]. In this chapter, the methods for in vitro regeneration of antigen-specific T cells from human iPS cells of antigen-specific T cell origin are described.

2 Materials

2.1 Reprogramming T Cells into Pluripotent Stem Cells

1. Antigen-specific T cell clone, T cell line, or tetramer-positive T cells.
2. T cell medium; RPMI1640 medium (Sigma-Aldrich) supplied with 10 % fetal bovine serum (FBS; Invitrogen) and 100 IU/ml of recombinant human interleukin-2 (Peprotech).
3. Anti-CD3/CD28 conjugated magnetic beads (Invitrogen).
4. Phosphate buffer saline Ca^{2+}- and Mg^{2+}- free (PBS; nacalai-tesque, Kyoto, Japan).
5. Retronectin™ (TAKARA, Otsu, Japan).
6. Protamine sulfate (Sigma-Aldrich).
7. Sendai viral vectors containing reprogramming factors (11, or Cytotune™, Dynavec, Tsukuba, Japan).
8. Rotator (TAITEC, Koshigaya, Japan).
9. MCP-1 magnet (Invitrogen).
10. Murine embryonic fibroblast (Japan SLC, Inc., Hamamatsu, Japan).
11. iPS cell medium; DMEM nutrient mixture F-12 HAM (Sigma-Aldrich) supplemented with 20 % knockout serum replacement (KSR; Invitrogen) and 2 mM l-glutamine (Invitrogen), 0.1 mM nonessential amino acid (Invitrogen), 0.1 mM 2-mercaptoethanol (Invitrogen), and 5 ng/ml basic fibroblast growth factor (b-FGF; Wako, Osaka, Japan).
12. Appropriate size of culture dishes, plates, and conical tubes (not specified).

2.2 Differentiation of Hematopoietic Stem/Progenitor Cells from T-iPS Cells [8]

1. T-iPS cells, cultured on irradiated MEF in iPS cell medium.
2. C3H10T1/2 murine stromal cell line (RIKEN Bio-Resource Center, Tsukuba, Japan).
3. Basal Eagle's medium (BME; Invitrogen) supplemented with 10 % FBS (Biological Industries, Kibbutz Beit Haemek, Israel) and 2 mM l-glutamine (Invitrogen).
4. 0.05 % Trypsin-EDTA (Sigma-Aldrich).

5. PBS (nacalai-tesque, Kyoto, Japan).
6. Dissociation solution; PBS supplemented with 0.25 % human trypsin (Invitrogen), 1 mM CaCl (nacalai-tesque, Kyoto, Japan), and 20 % KSR (Invitrogen).
7. Differentiation medium; IMDM (Sigma-Aldrich) with 15 % FBS (AusGeneX, Oxenford, Australia), 100 ng/ml human insulin, 55 ng/ml human transferrin, 50 pg/ml sodium selenite (ITS; Invitrogen), 2 mM l-glutamine (Invitrogen), 50 μg/ml ascorbic acid (nacalai-tesque, Kyoto, Japan), and 0.45 μM α-monothioglycerol (nacalai-tesque, Kyoto, Japan).
8. Human recombinant vascular endothelial growth factor (R&D systems).
9. Human recombinant interleukin-7 (Peprotech).
10. Human recombinant Flt3L (Peprotech).
11. Appropriate size of culture dishes, plates, and conical tubes (not specified).

2.3 Differentiation of Antigen-Specific Cytotoxic T Lymphocytes

1. Hematopoietic progenitor cells induced from T-iPSC.
2. OP9/DL1 murine stromal cell line (RIKEN Bio-Resource Center, Tsukuba, Japan).
3. Peripheral blood mononuclear cells.
4. α-MEM (Invitrogen) supplemented with 20 % FBS (BioWest, Nuaillé, France) and 2 mM l-glutamine (Invitrogen).
5. 0.05 % Trypsin-EDTA (Sigma-Aldrich).
6. PBS (nacalai-tesque, Kyoto, Japan).
7. Human recombinant Flt3L (Peprotech).
8. Human recombinant interleukin-7 (Peprotech).
9. Human recombinant interleukin-15 (Peprotech).
10. Phytohemagglutinin (PHA; Wako, Osaka, Japan).
11. Appropriate size of culture dishes, plates, and conical tubes.

2.4 Characterization of Re-differentiated T Cells by Flowcytometry

1. Re-differentiating or re-differentiated T cells.
2. Peripheral blood mononuclear cells (C.T.L.) for flow cytometry setting.
3. True count tube (BD Bioscience).
4. PBS (nacalai-tesque, Kyoto, Japan) supplemented with 2 % FBS (Invitrogen).
5. Anti-human antibodies CD45-V500 (BD Bioscience), CD5-PE/Cy7 (eBioscience), CD7-PE (Biolegend), CD3-Pacific Blue (eBioscience), CD4-APC/H7 (BD Bioscience), and CD8a-PerCP/Cy5.5 (Biolegend).
6. Propidium iodide (Sigma-Aldrich).

3 Methods

Carry out all culture procedures in appropriate sterile conditions unless otherwise specified.

3.1 Reprogramming of Antigen-Specific T Cells

1. Induce antigen-specific CD8 T cell clones or lines by any methods such as tetramer mediated cell separation and repeated antigen stimulation in vitro.
2. Collect the T cells at $0.5–2.0 \times 10^7$/ml of RPMI medium supplemented with 10 % FBS in 1.5 ml tube.
3. Prepare appropriate number of CD3/CD28 conjugated beads in another 1.5 ml tube. Wash them twice by PBS, then mixed them with collected T cells in the tube.
4. Rotate the tube for 45–60 min at 10 rpm. Appropriate stimulation allows T cell shape to change into blastic (*see* Fig. 1).
5. Transfer magnetically selected beads and T cell complex into appropriate size of plate and culture them for 48 h in 5 % CO2 incubator. Generally, $0.5–1 \times 10^6$/ml/cm^2 is recommended for T cell culture.

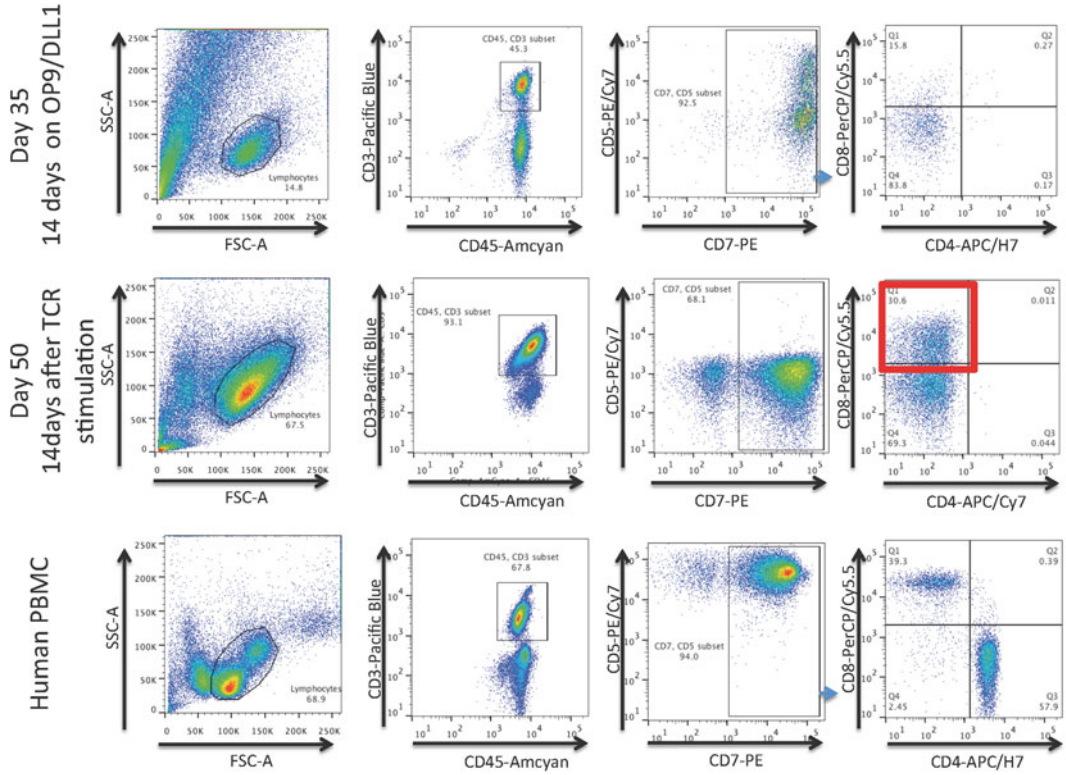

Fig. 1 Fully activated T lymphocytes show blastic shape soon after anti-human CD3/CD28 bead stimulation

6. Resuspend T cells in fresh T cell medium at 1×10^6/ml concentration then transfer them onto retronectin coated well of 24-well plate.
7. Add 10 μg/ml of protamine sulfate and reprogramming factors containing sendai viral vectors at appropriate multiplicity of infection (MOI) to the T cell culture.
8. Centrifuge the 24-well plate at $1000 \times g$ for 30 min at room temperature, and then incubate cells in 5 %CO2 incubator overnight.
9. Wash cells and culture them in fresh complete medium overnight.
10. Wash cells, remove residual beads by magnet, and transfer 1×10^6 T cells onto a 6 cm dish coated with pretreated MEF.
11. Discard half of T cell medium and add fresh pluripotent cell medium once a day till embryonic stem cell like colonies appear around day 21.
12. Isolate single cell-derived colonies and confirm their pluripotency by reported methods [9].

3.2 Induction of Hematopoietic Stem/Progenitor Cells from T-iPSCs [8]

1. Prepare pretreated 10 cm dishes containing confluent 10 T1/2 cell line.
2. Harvest T-iPS cells at confluent from the 6 cm dish. Cells should be recovered as small cell clusters by trypsinization with dissociation solution and pipetting. Transfer 1/10–20 volume of cell cluster onto a pretreated C3H10T1/2 cell layer of 10 cm dish and culture with differentiation medium containing 20 ng/ml VEGF. 30–50 sac-like structures in a 10 cm dish will appear at the final step of the section if they are spread appropriately.
3. Change differentiation medium containing 20 ng/ml VEGF every 2–3 days. The medium from day 7 up to day 14 of culture should contain 20 ng/ml VEGF, 10 ng/ml hFlt-3 L and 1 ng/ml hIL-7.
4. T-iPSC derived sac-like structures containing hematopoietic progenitor cells appear around day 10 of culture.

3.3 Induction of Antigen-Specific CD8 T Cells

The following method should be started around day 14 of the culture to have better recovery of hematopoietic progenitor cells.

1. Prepare pretreated 6 cm dishes containing confluent OP9/DL1 cell line.
2. Take out hematopoietic progenitor cell suspension by breaking the sac-like structures with mechanical stress, concretely, scratching them by cell scraper and pipetting.
3. Transfer hematopoietic cell containing medium into a new 50 ml tube through 40 μm cell strainer and centrifuge the cells at $400 \times g$ for 10 min.

4. Resuspend hematopoietic cells in fresh T cell differentiation medium supplemented with 10 ng/ml hFlt-3 L and 1 ng/ml hIL-7, and transfer them onto pre-irradiated 6 cm dishes of OP9/DL1 stromal cell line.
5. Change half of medium every 2–3 days. The culture medium is supplied with 10 ng/ml hFlt-3 L and 1 ng/ml hIL-7 up to day 21 of culture on OP9/DL1.
6. CD7 positive TCR-CD3 complex positive T-lineage cells appear until day 21 of culture. Expression level of CD5, CD4, and CD8 may vary among T-iPSC clone at this time point (*see* **Notes**).
7. Collect CD45 and TCR-CD3 complex positive cells from floating cells in culture by flow cytometry or magnetic bead selection.
8. Stimulate the TCR-CD3 complex positive T-lineage cells by 1–5 μg/ml PHA in the presence of 10 ng/ml IL-7, 10 ng/ml IL-15, and irradiated PBMCs. 5×10^6 irradiated PBMC in a well of 48-well plate is used to stimulate sorted cells up to 1×10^6. Size of cell culture could be modified according to number of sorted CD3 T-lineage cells.
9. CD8 single positive T cells appear until day 14 after stimulation. After this stage, antigen specificity and function of regenerated CD8 T cells can be evaluated by common methods for testing CTL clones (tetramer binding assay, ELISPOT, 51Cr release assay, and so on).

3.4 Characterization of Antigen-Specific CD8 T Lymphopoiesis by Flow Cytometry

1. Harvest 2×10^5 differentiating T cells in culture at desired time point.
2. Wash and suspend harvested cells in 200 μL of PBS supplemented with 2 % FBS.
3. Add indicated anti-human monoclonal antibody-cocktail to the cell suspension, and then incubate for 25 min on ice in the dark.
4. Wash cell twice by 1 ml of cold PBS supplemented with 2 % FBS.
5. Suspend labeled cells in PBS supplemented with 2 % FBS and 1 μg/ml propidium iodide.
6. Now labeled-T cells are ready for flow cytometoric analyses and cell sorting.

4 Notes

1. Freshly isolated polyclonal T cells can be reprogrammed as ease by both retroviral vectors or sendaiviral vectors containing Yamanaka factors [6]. SV40 large T antigen would be necessary for reprograming antigen-specific T cell clones that have been stimulated repeatedly in vitro by specific antigens.

2. Both low and high MOI of sendaiviral vectors result in low recovery of T-iPSC colonies by low transduction efficiency and low viability of cells, respectively [10].
3. Confluent MEF, C3H10T1/2, and OP9/DL1 layered dishes should be treated by 50 Gy irradiation or mitomycin C to inhibit further proliferation of those feeder cells in co-culturing steps.
4. Transferring various number of transduced T cells onto different MEF dishes would be helpful because induction efficiency of T-iPS cell may vary by status of T cell clones, transduction efficiencies, and many factors.
5. In our observation, T-iPS cells differentiate more effective into T-lineage cells than the other types of pluripotent stem cells without pre-rearrangement of TCR genes when they are cultured by the protocols.
6. In our observation, extensive culture of re-differentiating T cells on OP9/DL1 increase a chance of additional rearrangement of TCRA gene at CD4 and CD8 double-positive stage, which results in loss of antigen specificity of the TCR. Preliminary experiments using individual T-iPS cell clones are recommended to optimize the timing of TCR stimulation to induce functionally mature CD8 single positive T cells inhibiting RAG recombinase reactivation-mediated additional rearrangement of TCRA gene.

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Chapter 7

Chimeric Antigen Receptors for Cancer Immunotherapy

Claudia Geldres, Barbara Savoldo, and Gianpietro Dotti

Abstract

The adoptive transfer of T lymphocytes expressing chimeric antigen receptors (CARs) has emerged as a promising treatment for various lymphoid and solid malignancies. Patients treated with CAR-T cells have achieved dramatic responses and in some cases, complete tumor eradication. Given that CARs combine the specificity of a monoclonal antibody with the internal signaling domains of T cells, there is flexibility for choice of target antigen and strength of T-cell activation. This targeting mechanism also relinquishes the need for antigen processing and presentation via the major histocompatibility complex (MHC), making CARs a very attractive therapeutic option for the majority of patients. This review describes current methodological strategies used to generate CAR molecules, how to insert these molecules in T lymphocytes and how to evaluate the functionality of these CAR-T cells using various in vitro and in vivo experiments.

Key words Immunotherapy, Chimeric antigen receptor

1 Introduction

Significant progress has been made in the field of cancer immunotherapy, but no treatment has reached the extent of versatility and broad applicability as T cells redirected with chimeric antigen receptors (CARs) have. The main promise of cancer immunotherapy is the use of the patient's immune system to selectively eliminate cancerous cells. This can be achieved through immunization, the administration of antibodies, or various cell-based treatment modalities. The last approach encompasses dendritic cell vaccines, adoptive therapies based on the infusion of cytotoxic T lymphocytes (CTLs), tumor infiltrating lymphocytes (TILs), and natural killer cells (NK) or polyclonal T cells with redirected antigen specificity obtained through the expression of ectopic αβT-cell receptors (TCRs) [1] or CARs [2]. While each treatment has its own advantages, CARs have captured the attention of immunotherapists due to the MHC-independent function of CAR-modified T cells which makes them a curative option available for the majority of patients [3, 4].

Attilio Bondanza and Monica Casucci (eds.), *Tumor Immunology: Methods and Protocols*, Methods in Molecular Biology, vol. 1393,
DOI 10.1007/978-1-4939-3338-9_7,

CARs are recombinant fusion proteins that contain an antigen-binding domain with one or multiple intracellular signaling endodomains. The first chimeric receptors were fusion constructs between CD3-ζ and Fc receptor-γ [2]. These so-called "first generation CARs" primarily proved that fusion proteins could be expressed on T cells that had target specificity and activating potential. These proteins, however, did not generate robust cytokine responses and failed to support proper T-cell expansion [5, 6]. This was in part attributable to a lack of proper stimulation, as a T cell requires binding through its TCR as well as a secondary signal by an antigen-presenting cell in addition to growth cytokines. Understanding these limitations of first generation CARs led to the development of fusion constructs equipped with costimulatory endodomains derived from the intracytoplasmic portion of molecules like CD28, CD134, CD137, Lck, DAP10, and/or ICOS [7–10]. These fortified CARs were now capable of antigen-derived stimulation, proliferation, and cytotoxicity independent of external costimulation. Various second-generation CARs are currently being evaluated in clinical trials and have thus far proven to be superior to their prototypes [11–13].

Perhaps the most successful strategy is that of the CD19-targeted CAR. CD19 is a receptor restricted to the B-cell lineage compartment, retained by most B-cell-derived leukemias and lymphomas. Patients with refractory chronic lymphocytic leukemia (CLL) and acute lymphoblastic leukemia (ALL) treated with the anti-CD19 CAR achieved major objective clinical responses [14]. On the solid tumor front, T cells modified to express a diasialoganglioside (GD2)-specific CAR have also shown clinical responses in patients with relapsed/refractory neuroblastoma [3, 4].

This review will focus on the methodological description of the construction of a CAR, its insertion in T lymphocytes, and the assays used in vitro and in vivo to evaluate the functions of CAR-modified T cells.

2 Materials

2.1 Molecular Cloning of a CAR Molecule

1. cDNA encoding the variable regions of the heavy (VH) and light chains (VL) of a specific monoclonal antibody.
2. cDNA encoding the hinge-CH_2CH_3 fragment derived from the IgG1.
3. cDNA encoding costimulatory endodomain fragments.
4. Retroviral vector backbone.
5. High-fidelity DNA polymerase (Applied Bio Systems, Foster City, CA).
6. Primers.

7. Agarose (Sigma, St. Louis, MO).
8. LB Broth (Sigma, St. Louis, MO).
9. QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany).
10. GenElute HP Plasmid DNA Maxiprep Kit (Sigma, St. Louis, MO).
11. Restriction Enzymes (New England Bio Labs, Ipswich, MA).
12. Electrophoresis chamber.
13. 10× TBE (ThermoScientific, Pittsburgh, PA).
14. Clone Manager Software (Sci-Ed Software, Cary, NC).

2.2 Production of Retroviral Supernatant

1. 293 T cells.
2. 0.5 % Trypsin-EDTA 1× (Life Technologies, Grand Island, NY).
3. 10 cm tissue culture-treated dish (BD bioscience, San Jose, CA).
4. IMDM Complete: IMDM (Hyclone, Ogden, UT) supplemented with 10 % FBS, glutamine, and penicillin-streptomycin (Gibco/BRL, Bethesda, MD).
5. DMEM (Hyclone, Odgen, UT).
6. GeneJuice Transfection Reagent (EMD Millipore, Darmstadt, Germany).
7. 15 mL Falcon Tube (BD bioscience, San Jose, CA).
8. Acrodisc Syringe Filter 25 mm (Pall Life Sciences, Port Washington, NY).
9. 10 mL Syringe (BD bioscience, San Jose, CA).
10. Packaging plasmids.

2.3 Generation of T Lymphocytes Expressing a CAR

1. Peripheral blood mononuclear cells (PBMC).
2. T-cell medium: RPMI 1640 45 % (Hyclone, Odgen, UT), Click's medium (Irvine Scientific, Santa Ana, CA) 45 %, supplemented with 10 % FBS, glutamine, and penicillin-streptomycin (Gibco/BRL, Bethesda, MD).
3. Recombinant human interleukin-2 (IL-2) (Chiron, Emeryville, CA), reconstitute in T-cell medium at 200 U/μl, and store at −80 °C.
4. 24-well non-tissue culture-treated plates (BD bioscience, San Jose, CA).
5. 24-well tissue culture-treated plates (BD bioscience, San Jose, CA).
6. Recombinant fibronectin fragment (FN CH-296; Retronectin; Takara Shuzo, Otsu, Japan). Reconstitute in water at 1 mg/ml and store aliquots at −20 °C.

7. Trypan Blue (Sigma, St. Louis, MO).
8. Ficoll-Paque (Amersham Biosciences, Piscataway, N.J.).
9. Purified mouse anti-human CD28 antibody 1 mg/ml (BD Biosciences PharMingen, San Diego, CA).
10. OKT3 1 mg/ml produced from the hybridoma.

2.4 Phenotypic Analysis

1. 5 ml polystyrene tubes (Falcon).
2. FACS wash buffer: PBS + 1 % FBS.
3. Monoclonal antibodies conjugated with different fluorochromes: CD3-APC, CD4-PERCP, CD8-APC (BD Biosciences), and the IgG1-CH_2CH_3 fragment to detect the CAR (Jackson ImmunoResearch, West Grove PA) [15].
4. FACsCalibur system (Becton Dickinson) equipped with the filter set for quadruple fluorescence signals and CellQuest software.

2.5 Cytotoxicity Assay

1. ^{51}Cr (100 μcurie) (MP Biomedical, Solon, OH).
2. 96-well plate, V-bottom (Costar).
3. Target cells: Antigen-positive tumor cells, antigen-negative tumor cells, K562 (negative control to exclude NK cell activity). Cells are maintained in T75 flasks in appropriate medium, supplemented with 10 % FBS, PS/G at 37 °C, 5 %CO_2.
4. Triton-X (Sigma, St. Louis, MO): dilute in water for a final concentration of 1 %. Store at room temperature.
5. Gamma counter Packard Tri-CARB 4640 (Packard Instrument Company, Downers Grove, IL).
6. 1.5 mL microtubes (Costar).

2.6 Co-culture Assay

1. T-cell Medium.
2. 24-well tissue culture-treated plates (BD bioscience, San Jose, CA).
3. GFP^+ Tumor targets: antigen-positive and antigen-negative tumor cells.

2.7 Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) Assay

1. CFSE (Invitrogen) at 1.5 μmol/L.
2. Tumor targets: antigen-positive and antigen-negative tumor cells.
3. 24-well tissue culture-treated plates (BD bioscience, San Jose, CA).
4. Serum-free medium: AIM-V (Life Technologies, Grand Island, NY).
5. 1× PBS, 0.1 % FBS in 1× PBS, and 2 % FBS in 1× PBS.

2.8 Cytokine Release

1. Cytokine-specific ELISA kits (IL-2 and IFNγ) containing 96-well precoated microplates (R&D Systems, Minneapolis, MN).
2. Plate washer (Bioscan, Inc., Washington, DC).
3. Microplate Reader (Bioscan Inc., Washington, DC).

2.9 Tumor Cell Inoculation Preparation

1. Matrigel (BD Biosciences, San Jose, CA). Store at –20 °C. Thaw on ice and keep on ice during use.
2. 1.5 mL Eppendorf tubes.
3. 1× PBS.

2.10 T-Cell Infusion Preparation

1. 1× PBS.
2. 1.5 mL Eppendorf tubes.

2.11 In Vivo Imaging

1. Retroviral supernatant encoding Firefly luciferase (Vector production facility, Baylor College of Medicine, Houston, TX). Store at –80 °C.NSG Mice (Harlan-Sprague, Indianapolis, IN).
2. IVIS® Imaging System 100 Series equipment (Caliper Life Sciences, Hopkinton, MA).
3. d-luciferin, firefly potassium salt, 1.0 g/vial (Caliper Life Sciences, Hopkinton, MA). Reconstitute d-luciferin in PBS without Mg^{2+} and Ca^{2+} at 15 mg/ml. Filter using a 0.2 μm filter and store at –20 °C.

3 Methods

3.1 Molecular Cloning of a CAR Molecule

1. Using Clone Manager, design the scFv to align in frame with the leader sequence, VH, linker, VL, hinge-CH2CH3, costimulatory endodomain, and ζ chain [16].
2. Design specific primers to clone the CAR cassette by PCR using cDNAs as templates.
3. Perform PCRs using high-fidelity DNA polymerase.
4. Perform cloning using standard techniques.
5. Verify the sequence of the final cassette.
6. Prepare a maxiprep of the correct clone.

A representative CAR retroviral vector is shown in Fig. 1.

3.2 Production of Retroviral Supernatant

Day 1

1. Trypsinize 293T cells and plate 1.8×10^6 cells in 11 mL of IMDM-complete medium on a 10 cm dish. Incubate overnight at 37 °C.

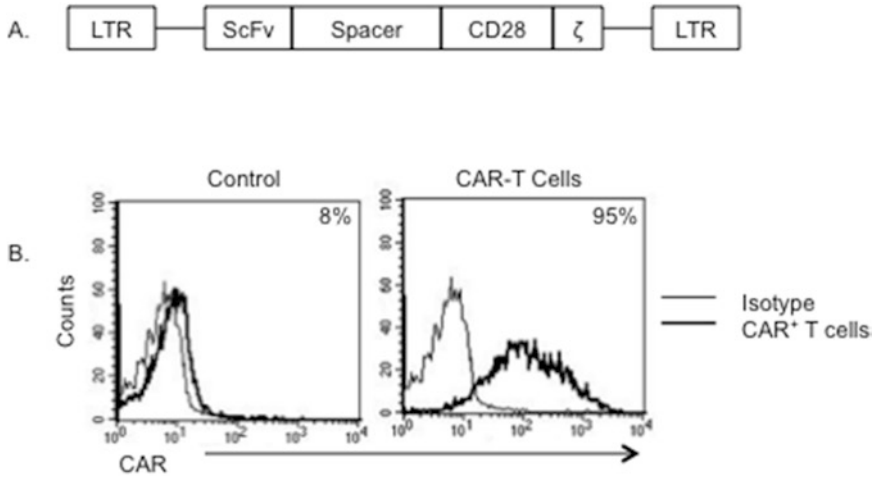

Fig. 1 Expression of a CAR in T lymphocytes**.** *Panel A***.** Schematic representation of a retroviral vector encoding a second-generation CAR incorporating the CD28 costimulatory endodomain. *Panel B***.** Representative histograms of the flow cytometry analysis showing the expression of CAR in T lymphocytes engineered with the retroviral vector encoding the CAR as compared to control T cells

Day 2

1. Add 470 μL of pure DMEM to a sterile 1.5 mL Eppendorf tube.
2. Add 30 μL of GeneJuice transfection reagent directly to the medium being careful not to touch the tube. Gently mix and incubate at room temperature for 5 min.
3. Prepare the DNA for the transfection: Add 10 μg of total DNA (3.75 μg plasmid encoding *gag/pol*, 2.5 μg plasmid encoding the envelope, 3.75 μg retroviral construct) into a separate 1.5 mL Eppendorf tube.
4. Dropwise, add the GeneJuice/DMEM mixture to the Eppendorf tube containing DNA, gently mix, and incubate for 15 min at RT.
5. Dropwise, add the GeneJuice/DMEM/DNA mixture to the 293 T cells.

Day 4

1. After 48 h, collect the medium from the 293 T-cell-containing dishes and filter the viral supernatant using a 0.45 μm filter.
2. Snap freeze the supernatant in 15 ml falcon tubes and store at −80 °C.
3. Replace 11 ml of fresh IMDM-complete medium into the 293 T cells and incubate at 37 °C.

Day 5

1. Collect the 72 h supernatant by filtering and snap freezing as described before.

3.3 Generation of CAR+ T Cells from Human PBMCs

Day 1

1. Coat a non-tissue culture-treated plate with 0.5 mL of sterile water containing OKT3 and anti-CD28 at 1 μg/ml. Incubate at 37 °C for 4 h or at 4 °C overnight.
2. Aspirate the antibody solution and block the plate with 2 ml/well of complete media for 30 min at 37 °C.
3. Aspirate the media and plate 1×10^6 PBMC/well in a final volume of 2 ml of complete media.

Day 2

1. Feed PBMC with 100 U/ml of IL-2.
2. Coat a 24-well, non-tissue culture-treated plate with retronectin at 7μl/well in 1 ml 1× PBS. Incubate at 4 °C overnight.

Day 3

1. Allow the retronectin plate to equilibrate to room temperature.
2. Aspirate the retronectin and block the plate by adding 1 ml of complete media and incubate at 37 °C for 20 min.
3. Add 1 mL of retroviral supernatant and spin at 2000G for 90 min (*see* **Note 1**), following TAKARA's protocol.
4. Aspirate the supernatant and add 0.5×10^6 OKT3/CD28 blasts/well in 2 ml of complete media containing 100 U/ml IL-2 (*see* **Note 2**).
5. Spin the plate at 500G for 10 min.

Day 5

1. Remove transduced cells from retronectin-coated plate using mechanical dislodgement and resuspend at 1×10^6 cells/mL in complete media containing 100 U/mL IL-2.
2. Plate 1×10^6 cells/well in a tissue culture-treated plate containing 2 ml of complete media. Feed with IL-2 (50-100 U/mL) every 3 days.

3.4 Evaluating Transduction Efficiency

1. Collect and count T cells to be evaluated.
2. Add 0.2×10^6 T cells into a polystyrene tube.
3. Wash with 1 mL of FACs buffer and centrifuge at 500G for 5 min.
4. Decant buffer and vortex to dislodge cell pellet.
5. Add CAR-specific antibody and corresponding isotype and incubate for 30 min at 4 °C in the dark.
6. Wash with 2 mL of FACs buffer, spin at 500G for 5 min, decant buffer, and resuspend T cells in 200 μL of FACs buffer.
7. Acquire using FACsCalibur.

The results of a representative transduction FACs analysis are shown in Fig. 1.

3.5 Cytotoxicity Assay

The cytotoxicity assay is performed to evaluate the short-term capacity of control versus CAR-transduced T cells to lyse target cells expressing the specific antigen but not cells lacking the antigen.

1. Collect and count effector cells (control and CAR-T cells).
2. Resuspend effector cells at a concentration of 2×10^6 cells/mL in complete medium.
3. Add 100 μL of complete medium to rows B, C, D, and F (spontaneous ^{51}Cr release control) of a 96-well V-bottom plate. Add 100 μL of 1 % Triton X to row H (Max ^{51}Cr release control).
4. Aliquot 200 μL of effector cells in triplicate into row A of the 96-well plate; perform three doubling dilutions into 100 μL medium. Discard 100 μL of what is left in row D.
5. Collect tumor targets and transfer 1–2×10^6 cells into a 15 mL centrifuge tube. Centrifuge and finger flick the pellet to dislodge the tumor cells.
6. Add 0.1 mCi ^{51}Cr sodium chromate in 100 μL medium. Incubate for 1 h at 37 °C (*see* **Note 3**).
7. Following the incubation, wash labeled tumor cells three times by centrifugation using complete medium.
8. Count labeled tumor cells and resuspend at 5×10^4 cells/ml in complete medium.
9. Add 100 μL of target cells to effectors in the 96-well plate. Row F should contain targets only, to assess spontaneous lysis of Target cells.
10. Centrifuge plates briefly, and then incubate for 4–6 h at 37 °C.
11. At the end of the incubation, centrifuge plate again, and then transfer 100 μL of supernatant from each well into microtubes arranged in a 96-well format.
12. Count the tubes for 2 min in the gamma counter.
13. Calculate percent (%) ^{51} Cr release as follow :

$$\%^{51}\text{Cr release} = \frac{\text{Experimental release} - \text{Background release}}{\text{Maximum release} - \text{Background release}} \times 100$$

Results of a representative cytotoxicity assay are shown in Fig 2.

3.6 Co-culture Assay

The co-culture assay is used to evaluate the long-term killing capacity of control versus CAR-transduced T cells against antigen-positive and antigen-negative tumor targets

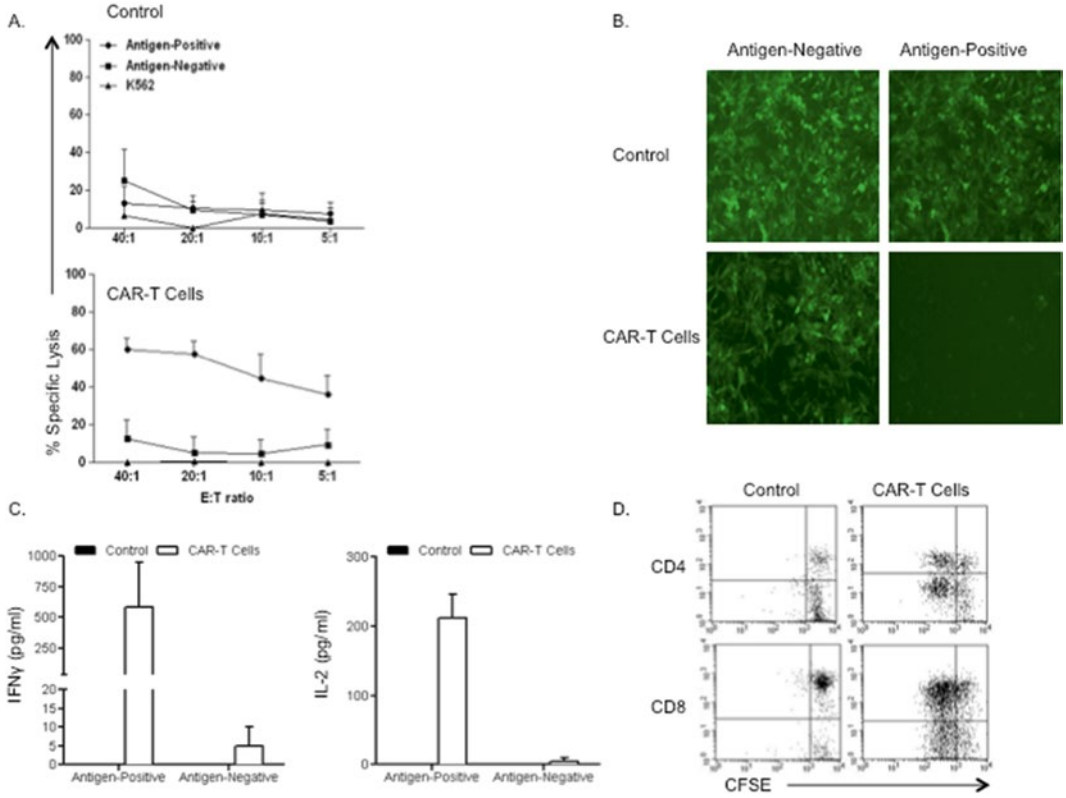

Fig. 2 Functional analysis of a CAR expressed in T lymphocytes**.** *Panel A***.** Control T cells and CAR-T cells are evaluated for cytotoxic activity in a 6-h ^{51}Cr release assay. Tumor targets include an antigen-positive tumor cell line, an antigen-negative tumor cell line, and the K562 cell line as a control for NK cell activity. *Panel B***.** Co-culture experiment in which control and CAR-T cells are cultured with GFP$^+$ antigen-positive and antigen-negative tumor cell lines. The panels illustrate the images on the fluorescence microscope 72 h after the initiation of the co-culture. Cells can also be collected and quantified by flow cytometry (data not shown). *Panel C***.** Quantification of IL-2 and IFNγ secreted by T cells co-cultured with tumor cells as measured by ELISA. *Panel D*. Carboxyfluorescein succinimidyl ester (CFSE) dilution in CD4 and CD8 control T cells and CAR-T cells as measured by flow cytometry after 96 h of co-culture with irradiated antigen-positive and antigen-negative tumor cells

1. Collect GFP$^+$ tumor targets from T75 flask and count cells.
2. Collect and count T cells, non-transduced and CAR$^+$, at least 2 days after last feeding at approximately 10–12 days old.
3. Plate T cells with tumor cells at desired effector-to-target ratio in 2 mL of complete media and incubate at 37 °C.
4. 24 h after the initiation of the co-culture, collect the supernatant for the detection of cytokine production (IL-2 and INFγ) by conventional ELISA.
5. Approximately 3–5 days after the initiation of the culture, collect T cells and tumor cells from each well and prepare for FACS analysis. Tumor cells and T cells are detected using GFP and CD3, respectively.

Results of a representative co-culture experiment are shown in Fig. 2.

3.7 CFSE Assay

The CFSE assay is performed to evaluate the proliferative potential of control versus CAR-transduced T cells when stimulated with their target antigen.

1. Collect and count control T cells, CAR$^+$ T cells, and tumor targets.
2. Irradiate tumor targets.
3. Wash T cells once with 1× PBS.
4. Wash T cells with 0.1 % FBS in 1× PBS.
5. For every 20×10^6 cells add 850 μl of 0.1 % FBS in 1× PBS and 150 μl of 1× CFSE labeling solution. Resuspend cells and incubate at RT for 10 min (*see* **Note 4**).
6. Add an equal volume of warmed FBS, resuspend, and incubate for 10 min at 37 °C in a water bath.
7. Wash twice with 2 % FBS in 1× PBS.
8. Count T cells and asses viability before use.
9. Plate tumor targets and T cells in AIM V medium at an effector-to-target ratio of approximately 4 to 1.
10. Incubate between 4 and 7 days at 37 °C.
11. Upon completion of the assay, collect the T cells and assess proliferative capacity using FACs analysis.

A representative CFSE experiment is shown in Fig. 2.

3.8 Tumor Cell Inoculation Preparation

1. Ensure that matrigel is properly thawed on ice before beginning the tumor cell collection.
2. Collect and count FF-Luciferase$^+$ tumor cells.
3. Wash tumor cells with 1× PBS.
4. Resuspend tumor cells in 1× PBS at desired concentration and aliquot 1 mL of tumor cells into 1.5 mL Eppendorf tubes (1 tube per mouse inoculation).
5. Pellet tumor cells, aspirate PBS, and resuspend tumor cells in 150 μl of matrigel.
6. Keep tumor cell suspension on ice until inoculation.

3.9 T-Cell Infusion Preparation

1. Collect and count T cells to be infused.
2. Wash the T cells with 1× PBS.
3. Resuspend the T cells in 1× PBS at desired concentration for mouse infusion. Usually it is recommended an injection volume of ~200 μl for IV tail injection.

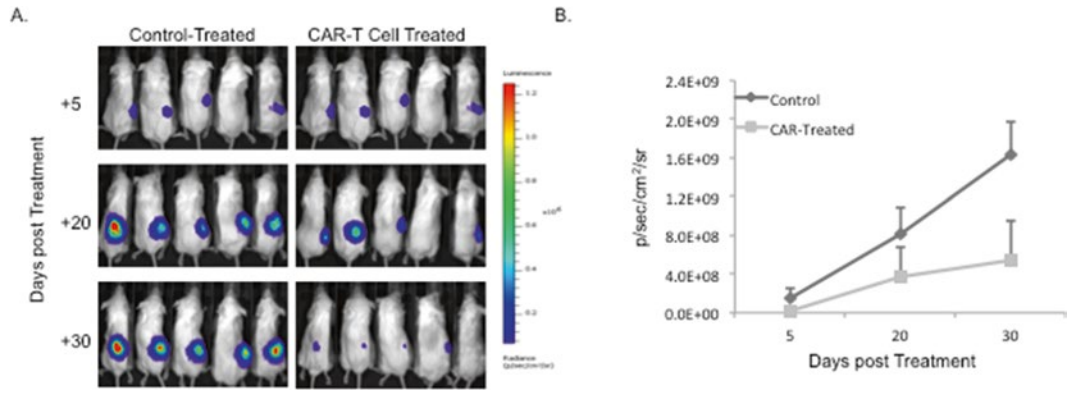

Fig. 3 In vivo anti-tumor activity of CAR-T cells. *Panel A*. NSG mice are engrafted subcutaneously with tumor cells labeled with Firefly luciferase. After tumor engraftment mice are infused intravenously with control or CAR-T cells. Tumor growth is then measured using the IVIS imaging system up to day 30 posttreatment. *Panel B*. Quantification of tumor growth. Data are representative of the average of five mice per group

3.10 In Vivo Imaging

1. Anesthetize mice in a clear anesthesia chamber containing 2.5 % isoflurane/air mixture.
2. Inject d-luciferin at 150 mg/kg body weight (10 μl/g of body weight) intraperitoneally.
3. Allow d-luciferin to biodistribute for 10 min.
4. Transfer anesthetized mice into the light-tight chamber making sure to place the snout in the isoflurane/air cones attached to the manifold. The position of the mouse may be dorsal or ventral depending on your experimental parameters.
5. Secure the chamber door and begin acquisition using the living image software.
6. Following acquisition, return animals to their cages and monitor until they are fully awake.

A representative in vivo experiment is shown in Fig. 3.

4 Notes

1. Do not refreeze unused viral supernatant.
2. Remember to plate control or non-transduced T cells at the same concentration in complete medium containing IL-2 (100 U/ml) in a 24-well tissue culture-treated plate.
3. When handling chromium, wear appropriate personal protective garments and monitor the work area with a survey meter. Label and dispose of the radioactive waste accordingly.
4. Do not expose CFSE to direct sunlight and keep in mind to incubate tubes with CFSE in the dark.

Acknowledgements

This work was supported in part by R01 CA142636 National Institutes of Health-NCI, W81XWH-10-10425 Department of Defence and Technology/Therapeutic Development Award.

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Chapter 8

Cancer and Chemokines

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Abstract

Chemokines are a large family of secreted cytokines whose main function is to mediate leukocyte directional migration. Most cancers contain chemokines and express chemokine receptors as a consequence of the activity of deregulated transcription factors or tumor-suppressor genes. Indeed chemokines expression at the tumor site dictates leukocyte infiltration and angiogenesis, while chemokine receptors expression by tumor cells promotes their growth and matastatization. Chemokines also have several indirect effects on tumor growth and are a relevant element in the cancer-related inflammation. In this chapter we will describe technical approaches available to study the role of chemokines in leukocyte infiltration and tumor metastatization in murine tumor models.

Key words Chemokines, Tumor-infiltrating leukocytes, Cancer-related inflammation, Metastasis

1 Introduction

Chemokines are small cytokines playing a fundamental role in directing leukocyte trafficking. The chemokine system is composed of 50 ligands classified according to the relative positioning of conserved cysteine residues in their N-terminal domain, and 19 G protein-coupled receptors [1]. Chemokines have been firstly identified in tumor supernatants [2], and now it is well known that they are the central component of cancer-related inflammation, because they are targets of genetic events causing neoplastic transformation and are also a component of chronic inflammatory conditions, which predispose to cancer [3]. Chemokines and their receptors affect in cell autonomous and non-autonomous ways multiple biological processes related to tumor progression [4]. In this chapter we will describe methods for studying the role of chemokines in determining tumor leukocyte infiltrate and metastasis in murine models.

Attilio Bondanza and Monica Casucci (eds.), *Tumor Immunology: Methods and Protocols*, Methods in Molecular Biology, vol. 1393, DOI 10.1007/978-1-4939-3338-9_8,

1.1 Chemokines and Tumor-Associated Leukocytes

Chemokine levels at tumor site and the subsequent leukocyte infiltration have a prognostic value in several types of tumor [5]. High levels of inflammatory chemokines such as CCL2 and CCL5 are correlated with infiltration of tumor-associated macrophages (TAM) that promote tumor growth by promoting angiogenesis and inhibiting T cell-mediated anti-tumor responses [6]. The CXC chemokines CXCL10 and CXCL9 are considered the main stimuli for attracting tumor-infiltrating lymphocytes (TIL), which express high levels of the cognate receptor CXCR3 that can elicit antitumoral responses [7]. On the contrary, CCL17 and CCL22 expression is often correlated with poor prognosis because these chemokines recruit Treg cells that suppress anti-tumor-specific immune responses [8]. A general characterization of the leukocyte infiltrate can be achieved by immunohistochemical staining of parallel tumor sections that allow the identification of the density and location of leukocytes into the tumor (invasive tumor margin or center). A more accurate quantification and phenotypic characterization of the leukocyte infiltrate in murine tumor models can be achieved by disaggregation of explanted tumor and subsequent flow cytometry analysis of leukocyte populations using specific antibodies.

1.2 Chemokines and Metastasis

Chemokines can drive organ-specific secondary localization of tumors because tumor cells upregulate or acquire expression of selected chemokine receptors unrelated to their tissue of origin [9]. Evidence indicates that in many tumors CXCR4 and its ligand CXCL12 are involved in metastatization to the lung [10], while CCR7 expression drives tumor metastatization to lymph nodes of melanoma and lymphomas [11] and to the brain of T cell acute lymphoblastic leukemia [12]. To study the role of chemokine receptor in tumor metastasis, besides retrospective studies conducted in human patients, two complementary murine models are generally used: genetically engineered models of cancer in which tissue-specific oncogenic mutations drive cancer progression, and transplantable tumors. The first model faithfully recapitulates important aspects of tumor initiation and progression, while syngeneic and xenograft transplantable tumor models can be more easily manipulated to understand cellular and molecular mechanisms [13]. Here we describe a classical histological technique and a more innovative imaging-based method for transplantable tumors for quantifying lung metastasis.

2 Materials

2.1 Chemokines Quantification in Organ Lysates

1. Capillary 100 µl (Alphalaboratories).
2. Syringes (0.5 ml) equipped with 26G needles.
3. Scissors and forceps.

4. Buffer: 1× phosphate-buffered saline (PBS) without Ca and Mg, 1× inhibitor cocktail (Complete Protease Inhibitor Cocktail Tablets; Roche).
5. Tissue lyser (Qiagen).
6. Sonicator H20 (Bioruptor).
7. ELISA kits.

2.2 Tumor Disaggregation for Leukocyte Staining

1. Collagenase type IV from *Clostridium histolyticum* (Sigma Aldrich) 1.6 mg/ml. Prepare a 10× solution (16 mg/ml) in order to add 300 µl of 10× to 2.7 ml of Medium (DMEM supplemented with 1 % FCS).
2. EDTA 0.5 M pH 8: weigh 18.61 g EDTA and transfer in a beaker with 100 ml of ultrapure water. Mix and adjust pH with NaOH (*see* **Note 1**).
3. ACK (red blood cell lysis buffer): 154.95 mM NH_4Cl, 9.99 mM $KHCO_3$, 0.0995 mM EDTA. Weigh 4.14 g NH_4Cl, 0.5 g $KHCO_3$, and 18.7 mg EDTA and dissolve them in 500 ml of ultrapure water. Mix and check the pH to be 7.
4. FACS buffer: 1× PBS, 1 % BSA (Bovine Serum Albumin) or FCS (Fetal Calf Serum), 3 ml NaOH 1 N, 0.01 % NaN_3 (*see* **Note 2**).
5. FACS Fix: 1× PBS, 1 % formaldehyde. Add 14 ml 37 % formaldehyde in 500 ml PBS.
6. FACS anti-mouse antibodies: CD45-APC and CD45-PercPCy5.5 (BD Biosciences), CD11b-PercPCy5.5 (BD Biosciences), CD11b-APC (Biolegend), F4/80-PB (AbDSerotec), Ly6C-PE (BD Biosciences), Ly6G-FITC (BD Biosciences), CD19-PE (BD Biosciences), CD3-FITC (BD Biosciences). In order to analyze only alive cells use also LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Invitrogen).

2.3 Lung Histology

1. 4T1 breast adenocarcinoma cell line (ATCC).
2. Ketamine (93.6 mg/kg Ketavet 100; Intervet).
3. Xylazine (7.2 mg/kg, Rompun; Bayer).
4. Fixative: 4 % paraformaldehyde (PFA) or 10 % neutral buffered formalin (NBF).
5. Syringes (0 5 ml) equipped with 26G needles.
6. Scissors, forceps.
7. Tissue cassettes.
8. 1× PBS.
9. 50, 70, 95, and 100 % ethanol.
10. Xylene.
11. Paraffin wax.
12. Rotary microtome.
13. Glass slides.

2.4 Determination of Lung Metastasis in Vivo by Luminescent Imaging of Luciferase-Transfected Tumor Cells

1. 4T1-luc2 (PerkinElmer).
2. Syringes (0.5 ml) equipped with 30G needles.
3. Ketamine (93.6 mg/kg, Ketavet 100; Intervet).
4. Xylazine (7.2 mg/kg, Rompun; Bayer).
5. Beetle luciferin, potassium salt (Promega).
6. IVIS Spectrum (Perkin Elmer).
7. Living Image 4.1 software (Perkin Elmer).

3 Methods

3.1 Chemokines Quantification in Serum and Organ Lysate

1. Collect blood in a microtube taking it with a capillary from peri-orbital venous sinus.
2. Centrifuge 371 × *g* 10 min. Transfer the serum in a microtube and store at −80°C until chemokine quantification.
3. Remove the tumor and the whole lung after heart perfusion with PBS. Weight organs.
4. Put up to 100 mg of tissue into a 2 ml microtube with 500 μl of buffer and cut with scissor in small pieces.
5. Maintain samples in ice in all passages.
6. Use consecutively a homogenizer (i.e., Qiagen TissueLyser based on vortexer bead-beating method, a cycle of 25 Hz 3 min) and a sonicator (30 sec 5 cycles) (*see* **Note 3**).
7. Centrifuge 371 × *g* 30 min. Transfer supernatant in a new microtube and store at −80°C.
8. Chemokines can be quantified with standard ELISA method with commercially available kits (i.e., R&D System) (*see* **Note 4**).

3.2 Tumor Disaggregation for Leukocyte Staining

1. Explant tumors from mice. If tumors are growing subcutaneously avoid keeping skin on them. Weigh tumors and put them in a 12-wells multiwall plate.
2. Prepare a 10× solution of collagenase type IV from *Clostridium histolyticum* in DMEM supplemented with 1 % FCS.
3. Cut tumors with scissor into small pieces, eventually removing the residual skin, and put them into a 15 ml tube with 2.7 ml of DMEM supplemented with 1 %FCS.
4. Add 300 μl of 10× collagenase solution to each tube.
5. Incubate the samples 30 min at 37 °C. Mix once in a while.
6. Place the samples at 4 °C and add EDTA to a final concentration of 10 mM. Incubate 10 min at 4 °C.
7. Filter samples through a 70 μm cell strainer mashing tumor pieces. Wash the cell strainer with DMEM + 1 % FCS and centrifuge 290 × *g* 10 min.

8. Discard the supernatant, resuspend the pellet in 1 ml of ACK for red blood cell lysis and incubate 5 min at 4 °C.
9. Add to each sample 10 ml of DMEM + 1 % FCS and centrifuge 290 × *g* 10 min.
10. Discard the supernatant and resuspend the pellet in FACS buffer. Count cell number and take 1×10^6 cells for each staining (*see* **Note 5** and **6**).
11. In order to identify the different infiltrating population use the antibodies listed below (*see* Fig. 1):

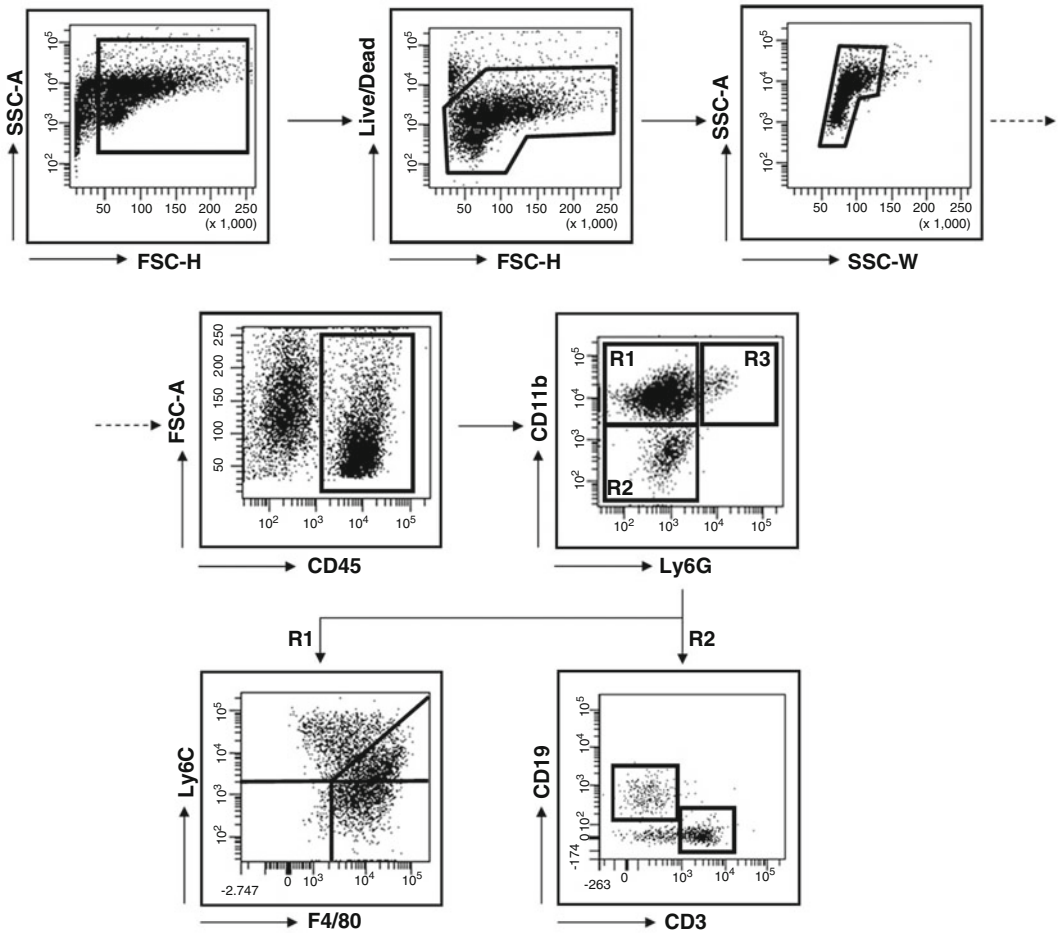

Fig. 1 Gating strategy for evaluating the percentage and absolute number of tumor-infiltrating leukocytes. Two parallel stainings were performed in accordance with the available fluorochromes. Dead cells, doublets, and CD45$^-$ cells were excluded for further analysis. Cell labeling with anti-CD11b and Ly6G allows the identification of myeloid cells (R1 + R3) and neutrophils (R3). Within the CD11b$^+$ Ly6G$^-$ population (R1) inflammatory monocytes (Ly6C^{high} F4/80^{low}), monocytes differentiating into macrophages (Ly6C^{int} F4/80^{high}), and macrophages (Ly6Cl^{ow} F4/80^{high}) were identified. Finally in the CD11b$^-$ population (R2) lymphocytes were distinguished as B cells (CD19$^+$ CD3$^-$) and T cells (CD19$^-$ CD3$^+$)

- CD45: total leukocytes
- CD11b: myeloid cells
- Ly6C and F4/80: monocytes and macrophages (monocytes are $Ly6C^+$ $F4/80^-$; monocytes differentiating into macrophages acquire F4/80 expression and will be displayed as $Ly6C^+$ $F4/80^+$; macrophages are $Ly6C^-$ $F4/80^+$)
- Ly6G: PMN
- CD19: B cells
- CD3: T cells

3.3 Histology

1. Anesthetize mouse injecting intraperitoneally 2 μl/g ketamine-xylazine solution.
2. Inoculate 5×10^5 4T1 cells resuspended in 50 ml of $PBS^{-/-}$ in the inguinal mammary fat pad of a female Balb/c mouse.
3. After 30 days euthanize the mouse according to standard procedures and in compliance with local regulations.
4. Remove the whole lung after tracheal instillation of fixative (*see* **Note 7**).
5. Place the whole lung with the ventral surface down in a histology cassette and immerse it into fixative using an approximate 20:1 fixative to tissue ratio for 24–48 h.
6. Process the lungs by dehydration through serial ethanol solutions and clearing with xylene followed by embedding in paraffin wax (*see* **Note 8**).
7. Cut three 4 μm sections of lung (at least 100 μm apart) with a standard rotary microtome, stain them with standard hematoxylin and eosin, and evaluate them for pulmonary metastases under a light microscope.
8. All lobes will be evaluated for the presence of metastases and the data from the three sections will be mediated (*see* Fig. 2). The metastases can be classified according to their size into: small (<30 neoplastic cells), medium (30–300 neoplastic cells), and large (>300 neoplastic cells). Calculate the total metastatic score for each lung, considering the size and total number of metastases detected throughout the lung section, as follows: total metastatic score = no. of small metastases × 1 + no. of medium metastases × 2 + no. of large metastases × 4.

3.4 Determination of Lung Metastasis in Vivo by Luminescent Imaging of Luciferase-Transfected Tumor Cells

1. Resuspend 0.5×10^6 4T1-luc2 in 50 μl of PBS and inoculate into the tail vein.
2. After 7–14 days anesthetize mice by intraperitoneally injection of 2 μl/g ketamine-xylazine solution (*see* **Note 9**).

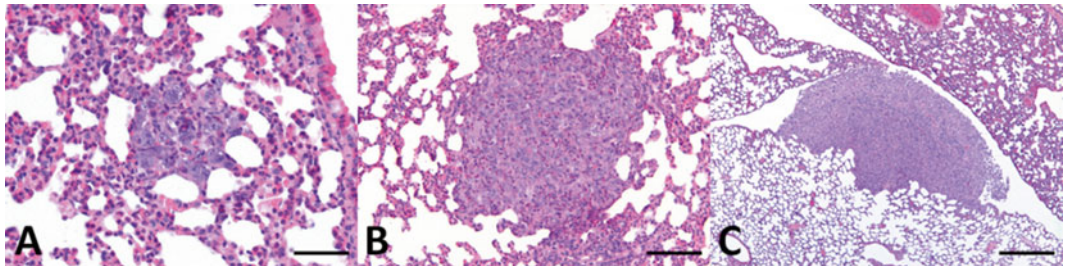

Fig. 2 Histopathological examination of pulmonary metastases. Histopathological examination of pulmonary metastases of 4T1 mammary carcinoma cells in female Balb/c mice at day 25 after inoculation. (**a**) Small pulmonary metastasis (composed of < 30 atypical cells) infiltrating the alveolar septa (H&E, scale bar = 50 μm). (**b**) Medium pulmonary metastasis (composed of 30–300 atypical cells) infiltrating and expanding the alveolar septa (H&E, scale bar = 100 μm). (**c**) Large pulmonary metastasis (composed of >300 atypical cells) infiltrating and expanding the subpleural lung parenchyma (H&E, scale bar = 400 μm)

3. During sedation inject intraperitoneally 150 mg/kg luciferin (*see* **Note 10**).
4. Place the mouse on its back in the imaging chamber and set the software for imaging parameters: FOV (C), binning (medium), f/stop [1] and exposure time (5 min) (*see* **Note 11**).
5. 10 min after luciferin injection, acquire the image using IVIS Spectrum in vivo imaging system (*see* **Note 12**).
6. After acquisition, repeat injection of luciferin in the same mouse and wait 10 min before sacrifice it by cervical dislocation.
7. Immediately dissect lung and put it in a Petri dish.
8. Acquire luminescence maintaining the same settings used for the live mouse.
9. Quantify the photon flux in the diverse samples/body areas (*see* **Note 13** and Fig. 3).

4 Notes

1. Dissolve the powder of EDTA by using a magnetic stirrer and by adjusting the pH with NaOH pellets. EDTA will dissolve only after reaching pH 8.
2. Animal serum proteins help minimizing nonspecific binding of antibodies; sodium azide inhibits metabolic activity and is used as a preservative. Do not add sodium azide to the buffer if you are concerned with recovering cell function.
3. To increase protein extraction from the tissues, a detergent could be added after homogenization (i.e., 0.1 % TritonX100).
4. Chemokine concentration obtained by ELISA has to be normalized with the sample weight or total protein content.

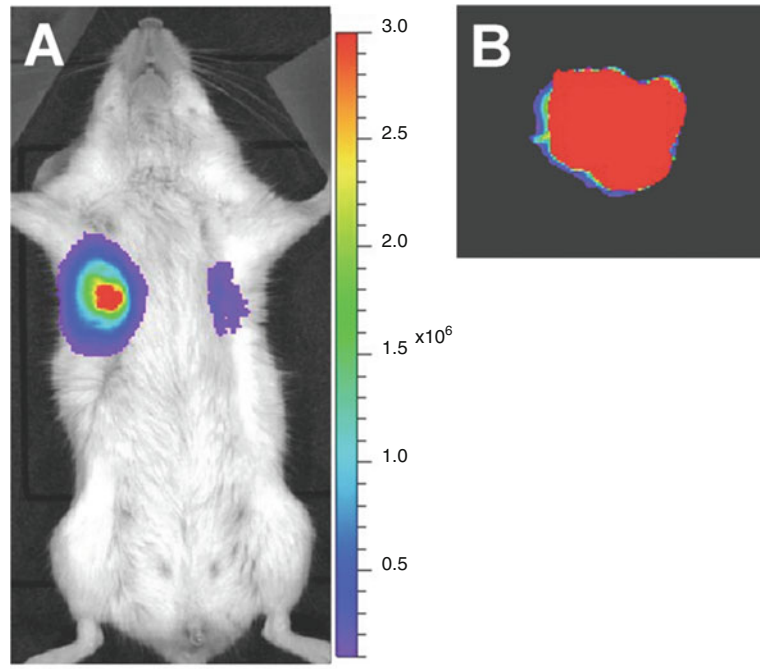

Fig. 3 Tumor in vivo imaging. Imaging of mouse (**a**) and lung (**b**) at day 14 after i.v. injection of 0.1×10^6 4T1-luc2 cells. The color bar shows the relationship between color and light intensity in arbitrary units (counts) for both images, acquired with the same setting (*see* **Note 11**)

5. In accordance to available fluorochrome-conjugated antibodies and cytometer characteristics, a single tube or two different tubes should be prepared for each sample (1) myeloid cells: CD45, CD11b, Ly6C, Ly6G, F4/80; (2) lymphoid cells: CD45, CD11b, CD3, CD19.
6. In order to determine the absolute number of leukocyte subpopulations in the sample, a fluorescent beads method should be used (e.g., BD Trucount tubes).
7. Instillation of the lung is strongly recommended in order to avoid alveolar collapse that could interfere with evaluation of lung metastases. Lung instillation is obtained by pinching closed the upper part of the trachea, inserting a syringe filled with 0.5 ml of fixative into the trachea, and injecting slowly the fixative until the lung will expand.
8. In case it is not possible to process samples for paraffin embedding after 48 h of fixation, move cassettes into 70 % ethanol to avoid overfixation of tissues that could hinder later immunohistochemical analysis and process them as soon as possible (within few days).
9. Isoflurane gas manifold is commonly housed in the Imaging System, but it could be substituted by an injectable anesthesia as ketamine-xylazine, which maintains mice anesthetized for 1 h.

10. Luciferin subcutaneous injections (s.c.) can be made into the scruff of the neck. The distribution kinetics of s.c. injected luciferin are similar to i.p. injections due to the high vascularization of the neck area. Sometimes, for bioluminescent imaging studies on bioluminescent-tumor bearing mice, the s.c. injection of luciferin is recommended, because i.p. injections can lead to an overestimation of signals [14].
11. The field of view (FOV) parameter affects the distance of the CCD camera from the imaging chamber platform, and therefore the extent of the sample that will be acquired. This parameter can be changed accordingly with the number of animals to be acquired simultaneously, the required details on specific body areas, as well as the strength of the bioluminescence signal. Depending on tumor size, and consequently on signal brightness, the imaging time, f-stop, and pixel binning can be optimized to prevent saturated images. These parameters can be changed during an experiment, resulting in no impact on the quantitative result.
12. At the beginning of the acquisition, the instrument acquires a photographic image of the animal. At the end of the acquisition, bioluminescent signal is displayed as an intensity map, expressed in photons per second, and overlaid on this image. Contrast and resolution can be adjusted to provide best appearance, without affecting quantitation. To ensure consistent photon flux, image mouse immediately after 10 min after luciferin injection. For acquisition, it is recommended to use the "counts" display mode. In this way it is possible to adjust camera settings to optimize the signal levels, which should be above the background noise (>100 counts) and below the saturation value (65535 counts).
13. Luminescence output is expressed as total flux; therefore it is possible to compare data obtained with different instrument settings (exposure times, binning, FOV, etc.). The bioluminescent signal released from the 4T1-luc2 cells can be measured in the different body areas/ex vivo organs using the region of interest (ROI) tool. It is possible to draw ROI of different shapes and sizes, by using the Circle, Square, or Grid buttons. To quantify signals using ROI or to compare several images, it is recommended to use the "photons" display mode. This modality outputs the measurements adjusted and normalized in function of the different exposure times, binning, f/stop, and FOV. The photon flux emitted by the tumor directly correlates with tumor size, being proportional to the number of cells that express bioluminescence. When all the ROIs are drawn and placed, by clicking the measure button data are displayed in a table containing also the other experimental parameters related to the image. The table can be exported for further analyses. BLI unique features of in vivo imaging can be exploited to perform

longitudinal studies covering seconds to months, in which multiple images can be acquired from the same animal and compared, in order to study, e.g., the tumor progression.

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Chapter 9

T Cells as Antigen Carriers for Anti-tumor Vaccination

Catia Traversari and Vincenzo Russo

Abstract

The exploitation of the physiologic processing and presenting machinery of dendritic cells (DCs) by in vivo loading of tumor-associated antigens may improve the immunogenic potential and clinical efficacy of DC-based cancer vaccines. The approach developed by our group was based on the clinical observation that some patients treated with the infusion of donor lymphocytes transduced to express the HSV-TK suicide gene for relapse of hematologic malignancies, after allogeneic hematopoietic stem cell transplantation, developed a T cell-mediated immune response specifically directed against the HSV-TK gene product.

We demonstrated that lymphocytes genetically modified to express HSV-TK as well as self/tumor antigens, acting as antigen carriers, efficiently target DCs in vivo in tumor-bearing mice. The infusion of TRP-2-transduced lymphocytes induced the establishment of protective immunity and long-term memory in tumor-bearing mice by cross-presentation of the antigen mediated by the $CD11c^{+}CD8a^{+}$ DCs subset. A similar approach was applied in a clinical setting. Ten patients affected by MAGE-3^{+} metastatic melanoma were treated with autologous lymphocytes retrovirally transduced to express the MAGE-3 tumor antigen. In three patients, the treatment led to the increase of MAGE-3 specific CD8+ and CD4+ effectors and the development of long-term memory, which ultimately correlated with a favorable clinical outcome. Transduced lymphocytes represent an efficient way for in vivo loading of tumor-associated antigens of DCs.

Key words Active immunotherapy, Cross-presentation, Tumor antigens

1 Introduction

The active role of the immune system in controlling tumor growth has been widely investigated in preclinical models. In humans, since the first melanoma-associated antigen has been identified [1], several phase I/II clinical studies were performed in order to evaluate the immunologic and clinical efficacy of cancer vaccines [2, 3]. Recently, the therapeutic effect of cancer vaccines has been reported in patients affected by high-grade vulvar intraepithelial neoplasia vaccinated with a mix of long peptides from the HPV-16 viral oncoproteins E6 and E7 [4], and in a phase III study combining vaccination with gp100:209–217 (210 M) and

Attilio Bondanza and Monica Casucci (eds.), *Tumor Immunology: Methods and Protocols*, Methods in Molecular Biology, vol. 1393, DOI 10.1007/978-1-4939-3338-9_9,

high-dose interleukin-2 in metastatic melanoma patients [5]. Indeed, a cancer-specific vaccine has recently received the FDA approval for the treatment of patients with prostate cancer [6].

The vast majority of cancer vaccines rely on HLA-I-specific peptides. However, this therapy suffers from some weaknesses [7], such as the presence of suitable HLA class I molecules able to bind the peptide used as immunogen, the need for the peptide to succeed in binding the antigen presenting cells at the injection site or, in case of ex vivo pulsing, the low stability of the HLA-peptide complex [8], all limiting the effectiveness and the large-scale applicability of the majority of the studies performed [7].

Our group explored an alternative approach, based on the results of clinical trials carried out in patients affected by hematological malignancies and relapsing after allogeneic bone marrow transplantation. These patients were treated with donor lymphocytes in order to control the disease relapse [9, 10]. Donor lymphocytes may mediate a therapeutic immune response against the hematological malignancy. However, in a high percentage of cases, donor lymphocytes also induce the Graft versus Host Disease (GvHD), an often-lethal reaction of the donor lymphocytes against the host. In our studies, the donor lymphocytes were genetically engineered with a retroviral vector encoding the suicide gene HSV-TK and a marker gene, the low-affinity receptor of Nerve Growth Factor (ΔLNGFR), deleted of its intracellular portion and therefore biologically inactive [11]. The presence of ΔLNGFR allows for in vitro selection of pure populations of transduced lymphocytes, whereas the suicide gene HSV-TK allows for the GvHD control by the administration of the prodrug ganciclovir [9]. Two different vectors were used, encoding either the entire HSV-TK (i.e., SFCMM3 vector) or the HSV-TK/neo fusion protein (TN), a bifunctional protein carrying both the HSV-TK activity and the neomycin resistance (i.e., SFCMM2 vector). Both the suicide genes allowed the killing of the transduced cells following the infusion of ganciclovir, when GvHD occurred [10].

Genetically modified lymphocytes (GML) were detected in the circulation of treated patients by both polymerase chain reaction (PCR) and FACS analysis for the expression of the cell surface marker ΔLNGFR. During the follow-up, in some patients the genetically modified cells decreased below the level of PCR detection, thus suggesting the development of an immune response responsible for the clearance of the transduced cells. Indeed, the patients treated when their immune system was entirely reconstituted developed a strong immune response against the transduced cells, which led to the elimination of the transduced cells from the circulation [12]. Effectors of this immune response were cytotoxic T lymphocytes recognizing the exogenous HSV-TK and neo transgenic products [12]. The transgene-specific immune response could be enhanced by the administration of ganciclovir,

which selectively kills the GML [12]; thus, suggesting that the induction of the anti-TK immune response would require cross-presentation of the cell-associated antigens mediated by host antigen presenting cells (APCs). By using appropriate mouse models, such as mice deficient for MHC-I molecules (i.e., β2 microglobulin KO mice), we confirmed this hypothesis [13]. Indeed, GML behaved as antigen carriers delivering transgene products to a specific subset of lymphoid organ-resident DC, i.e., $CD11c^+CD8\alpha^+$ DCs, which in turn elicited T cell priming [13]. Notably, we showed that GML elicit T cell priming not only against non-self antigens (HSV-TK) but also against self-tumor antigens, such as the melanoma differentiation antigen TRP-2. Indeed, mice injected with TRP-2-GML developed anti-TRP-2 T cells that protected them from a subsequent challenge of the mouse melanoma B16 [13]. Based on these results, we performed a phase I clinical trial to evaluate the immunologic and clinical efficacy of the infusion of autologous lymphocytes genetically engineered to express the MAGE-A3 tumor antigen in patients with advanced melanomas [14]. We observed the induction of anti-MAGE-A3 T cells in three out of ten melanoma patients treated with autologous MAGE-A3-GML [14]. The elicited MAGE-A3-specific T cells, both $CD4^+$ and $CD8^+$, the latter recognizing multiple epitopes of MAGE-A3, were able to traffic through inflamed and neoplastic tissues [14]. In these three patients, we observed a correlation between the development of anti-MAGE-A3 T cells in periphery as well as in inflamed (DTH) or tumor tissues and a favorable clinical outcome [14]. These results were confirmed in a phase I/II clinical trial performed in 23 $MAGE\text{-}A3^+$ melanoma patients [15]. The increase of anti-MAGE-A3 T cells after vaccination was observed in 27 % of patients. Among 19 patients with measurable disease, we observed a disease control rate of 26 %, with one objective clinical response, and four durable, stable diseases. Interestingly, we reported a clinical benefit only in patients experiencing MAGE-A3-specific immune responses [15]. Altogether, these results provide evidence that GML in some circumstances may behave as cancer vaccines by targeting in vivo a subset of DC residing in secondary lymphoid organs, which in turn elicit a tumor-specific immune response that may induce clinical benefit in some patients.

2 Materials (*see* Note 1)

2.1 Isolation of PBMCs from Buffy Coat of Peripheral Blood or from Lymphocyte Apheresis

1. Buffy coat of 1 unit of human blood, or lymphocyte apheresis.
2. Phosphate buffered saline (PBS) without calcium and magnesium.
3. Lymphoprep (density 1.077 g/mL; Fresenius Kabi).
4. 50-ml polypropylene conical tubes.

5. 4-ml freezing tubes (Nalgene).
6. Trypan blue.
7. A hemocytometer (counting chamber).
8. A light microscope with phase contrast.
9. Freezing medium:
   - Cell Growth medium.
   - 2 mM l-glutamine.
   - 20 % Human plasma (Kendrion SpA).
   - 10 % DMSO.

2.2 Transduction and Selection of T Lymphocytes

1. T25 tissue culture flasks.
2. Complete Culture medium:
   - Cellgro medium (CellGenix).
   - 3 % Human plasma (Kendrion SpA).
   - 2 mM l-glutamine.
   - 600 IU/ml human IL-2.
3. CD3 Pure (Miltenyi).
4. Protamine hydrochloride (Hoffman-La Roche).
5. G418 disulfate salt solution (SIGMA).
6. Retroviral vector supernatant (*see* **Note 1**).
7. Saline solution (NaCl 0.9 %).
8. HSA (Human Serum Albumin) 25 % (Kendrion S.p.A).
9. DMSO (SIGMA D2650).
10. PBS.
11. Trypan Blue.

3 Methods

3.1 Isolation of PBMCs from Buffy Coat of Peripheral Blood or from Lymphocyte Apheresis

1. Distribute the buffy coat or the lymphocyte apheresis uniformly (*see* **Note 2**) in four 50-ml conical tubes (about 12 ml per tube).
2. Dilute the cell solution by adding 23 ml of PBS, mix buffy coat and PBS by pipetting up and down.
3. Layer 13 ml of Lymphoprep under the mixture, being careful to minimize mixing of blood with Lymphoprep.
4. Centrifuge tubes for 25 min at room temperature, 580 × *g*, without brake.
5. Recover the layer of PBMCs that accumulate at the interface with Lymphoprep (*see* **Note 3**).

6. Transfer PBMCs to two new 50-mL conical tubes.
7. Wash PBMCs with 50 mL of PBS and centrifuge for 10 min, $450 \times g$.
8. Collect all the PBMCs in one 50-mL conical tube and wash twice with 50 mL of PBS. Centrifuge for 10 min at $240 \times g$ to remove platelets. Before the last centrifugation harvest a small aliquot of cell suspension to count the cells.
9. Count PBMCs with trypan blue in hemocytometer under microscope.
10. Resuspend the cells freezing medium (12×10^6/ml).
11. Fill the Nalgene freezing vials (4 ml) with the desired volume (2–4 ml).
12. Freeze the cells at –80 °C, under conditions of decreasing temperature using a cryo-freezing container (Nalgene).
13. Store the frozen cells in liquid nitrogen.

3.2 Transduction and Selection of T Lymphocytes

3.2.1 Cells Thawing (Day 0)

1. Thaw the lymphocytes in order to obtain about 1×10^8 viable cells at the end of the process.
2. Centrifuge at $450 \times g$ for 10 min.
3. Resuspend the cells in complete medium.
4. Count lymphocytes with trypan blue in hemocytometer under microscope.
5. Plate the cells in flasks at the concentration of 10^6/ml/cm^2.
6. Stimulate the lymphocytes by adding CD3 Pure at 30 ng/ml.
7. Incubate at 37 °C for 48 h.

3.2.2 First round of Transduction (Day 2)

1. Count lymphocytes with trypan blue in hemocytometer under microscope.
2. Harvest 50×10^6 of lymphocytes.
3. Centrifuge at $450 \times g$ for 10 min.
4. Resuspend the cell in the retroviral-containing supernatant (1×10^6/ml) in a T25 flask (*see* **Note 4**).
5. Add to the cells suspension the protamine at 4 μg/ml.
6. Plate the cells in T25 flasks (25 ml/flask).
7. Centrifuge the flasks for 2 h at $1160 \times g$ at 32 °C, without brake.
8. Collect all the cells putting attention to detach them from the flask walls by pipetting.
9. Centrifuge the cells at $450 \times g$ for 10 min.
10. Resuspend the cells in 10–20 ml of complete medium.

11. Count lymphocytes with trypan blue in hemocytometer under microscope.
12. Resuspend the cell in complete culture medium (1×10^6/ml) in flask (maximum volumes are: 20 ml/T25; 50 ml/T75; 125 ml/T162).
13. Incubate at 37 °C for 24 h.

3.2.3 Second round of Transduction (Day 3)

1. Harvest and count with trypan blue in hemocytometer the lymphocytes transduced at day 2.
2. Centrifuge the cells at 450 × *g* for 10 min.
3. Resuspend the cell in the retroviral-containing supernatant (1×10^6/ml) in a T25 flask.
4. Add to the cells suspension the protamine at 4 μg/ml.
5. Plate the cells in T25 flasks (25 ml/flask).
6. Centrifuge the flasks for 2 h at 1160 × *g* at 32 °C, without brake.
7. Collect all the cells putting attention to detach them from the flask walls by pipetting.
8. Centrifuge the cells at 450 × *g* for 10 min.
9. Resuspend the cells in 10–20 ml of complete medium.
10. Count lymphocytes with trypan blue in hemocytometer under microscope.
11. Resuspend the cell in complete culture medium (1×10^6/ml) in flask (maximum volumes are: 20 ml/T25; 50 ml/T75; 125 ml/T162).
12. Incubate at 37 °C for 24 h.

3.2.4 First Step of Selection (Day 4)

1. Harvest and count with trypan blue in hemocytometer the transduced lymphocytes.
2. Centrifuge the cells at 450 × *g* for 10 min.
3. Resuspend the cells in complete culture medium (1×10^6/ml) containing G418 (0.8 mg/ml).
4. Plate the cells in flasks (maximum volumes are: 20 ml/T25; 50 ml/T75; 125 ml/T162).
5. Incubate at 37 °C for 3 days.

3.2.5 Second Step of Selection (Day 7)

1. Harvest and count with trypan blue in hemocytometer the transduced lymphocytes.
2. Centrifuge the cells at 450 × *g* for 10 min.
3. Resuspend the cells in complete culture medium (1×10^6/ml) containing G418 (0.8 mg/ml).

4. Plate the cells in flasks (maximum volumes are: 20 ml/T25; 50 ml/T75; 125 ml/T162).
5. Incubate at 37 °C for 3 days.

3.2.6 Expansion of the Transduced Cells (Day 10)

1. Harvest and count with trypan blue in hemocytometer the transduced lymphocytes.
2. Centrifuge the cells at $450 \times g$ for 10 min.
3. Resuspend the cells in complete culture medium (0.5×10^6/ml).
4. Plate the cells in flasks (maximum volumes are: 20 ml/T25; 50 ml/T75; 125 ml/T162).
5. Incubate at 37 °C for 4 days.

3.2.7 Freezing of the Transduced Cells (Day 14)

1. Harvest and count with trypan blue in hemocytometer the transduced lymphocytes.
2. Centrifuge the cells at $450 \times g$ for 10 min.
3. Resuspend the cells in freezing medium (12×10^6/ml).
4. Fill the Nalgene freezing vials (4 ml) with the desired volume (2–4 ml).
5. Freeze the cells at –80 °C, under conditions of decreasing temperature using a cryo-freezing container (Nalgene).
6. Store the frozen cells in liquid nitrogen.

4 Notes

1. The materials and methods described below refer to a transduction-selection procedure performed in standard research laboratories approved for the use of MOGM of group II (genetically modified organism) but not for the generation of clinical grade material. The production of material for use in human required the application of fully GMP rules to each single step of the procedure, starting from production environment, personnel training, materials, methods, and manufacturing procedures.
2. Take necessary precautions when working with human blood products. Assume that all blood-derived products are to be considered potential hazards.
3. To facilitate the recovery of the PBMC layer, aspirate platelets containing supernatant to 5 ml above the dense layer of cells at the interface, then pipet PBMC out.
4. The protocol described can be applied to a retroviral vector encoding the Antigen of interest and for the neomycin-resistance gene as selection marker.

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Chapter 10

Exploiting Secreted Luciferases to Monitor Tumor Progression In Vivo

Laura Falcone and Monica Casucci

Abstract

Secreted luciferases provide a simple, accurate, and cost-effective tool to monitor tumor response after treatment in small animal models. This protocol describes all the steps required to generate human tumor cell lines expressing this reporter gene and to monitor in vivo tumor progression after injection in immunocompromised mice by means of serial peripheral blood sampling and analysis.

Key words Secreted luciferases, Animal models, Cancer therapy, Monitoring tumor growth in vivo, In vivo imaging

1 Introduction

The development of novel compounds or cell products for cancer therapy unquestionably requires accurate in vivo testing. Being able to longitudinally monitor the different phases of tumor response to treatment (regression, clearance, relapse) is mandatory, but sometimes challenging. Indeed, several tumors cannot be followed in mice using standard techniques, like flow cytometry or volumetric analysis through a caliber. For this kind of tumors, genetic modification with luciferases enabling in vivo imaging can be extremely beneficial. Luciferases encompass a wide range of enzymes used for bioluminescence, defined as the emission of light produced by a living organism. Luciferases have become precious bio-indicators for life science research and drug discovery, owing to their remarkable sensitivity, lack of toxicity, and wide dynamic range of quantitation [1–3]. The best-studied luciferases, derived from the firefly and the sea pansy *Renilla*, are intracellular reporters and are suitable for monitoring tumor growth by in vivo imaging [4]. Another class of luciferases, derived from marine bioluminescent organisms, exhibits the great advantage of being naturally secreted from cells in an active form, allowing simple

Attilio Bondanza and Monica Casucci (eds.), *Tumor Immunology: Methods and Protocols*, Methods in Molecular Biology, vol. 1393, DOI 10.1007/978-1-4939-3338-9_10,

detection directly from the cell culture medium or from the blood, serum, or urine of mice engrafted with luciferase-expressing tumors, avoiding frequent systemic anesthesia and substrate injection required for the in vivo imaging [5, 6].

This protocol describes the use of a commercially available secreted luciferase, engineered to have superior properties in mammalian cells compared to natural secreted luciferases, to develop a xenograft mouse model for monitoring tumor progression and response to therapy.

2 Materials

2.1 Generation and Production of the Lentiviral Vector Carrying the LUCIA-luciferase *Gene*

1. Lentiviral vector backbone carrying a marker gene for selection.
2. Plasmid carrying the *LUCIA-luciferase* gene (InvivoGen).
3. Restriction enzymes and corresponding buffers (New England Bio Labs).
4. Thermomixer.
5. Agarose (Sigma-Aldrich).
6. TE buffer (100 ml: 1 ml 1 M Tris–HCl pH = 8, 0.2 ml 0.5 M EDTA, and double distilled water).
7. Wizard Gel and PCR Clean Up System (Promega).
8. Quick Ligase or T4 Ligase and corresponding buffers (New England Bio Labs).
9. LB broth (Sigma-Aldrich).
10. One Shot TOP10 chemically Competent *E. coli* (Life Technologies).
11. Wizard Plus Minipreps Purification System (Promega).
12. Petri dish (MONOLAB).
13. 293 T cells.
14. 15 cm tissue culture-treated dish (BD Falcon).
15. Packaging plasmids.
16. $CaCl_2$.

2.2 Tumor Cell Transduction, FACS Analysis and Sorting

1. 24-well plates (Costar).
2. T25 flasks (Corning).
3. Viral supernatant.
4. 5-ml polystyrene tubes (Falcon).
5. FACS washing buffer (PBS + 2 % FBS).
6. Fluorochrome-conjugated mAbs specific for the selection marker (Biolegend).
7. FACS Calibur or FACS Canto system (Becton Dickinson).

8. FlowJo software for data analysis (Tree Star, Inc.).
9. Paramagnetic MicroBeads for cell sorting (Miltenyi).
10. MACS Columns and MACS support for cell sorting with MicroBeads (Miltenyi).

2.3 In Vitro and In Vivo LUCIA-luciferase Assay

1. Polyethylene tubing PE50 (Becton Dickinson).
2. Heparin (Hospira Italia).
3. Heparin-coated 5-ml tubes for blood sampling (BD Bioscience).
4. White 96-well plates for bioluminescence (Costar).
5. QUANTI-Luc, coelenterazine-based luminescence assay reagent (InvivoGen).
6. Luminometer.

2.4 In Vivo Imaging

1. 1-ml syringes for tumor cell inoculation (Terumo).
2. Coelenterazine native (Nanolight technology).
3. Methanol (Sigma-Aldrich).
4. HCl (Sigma-Aldrich).
5. Gaseous anesthesia.
6. A cooled CCD camera for in vivo bioluminescence imaging.

3 Methods

3.1 Generation and Production of a Lentiviral Construct Carrying the LUCIA-luciferase Gene

1. If not already present, order the *LUCIA-luciferase* gene flanked by restriction sites for cloning into a lentiviral vector carrying a selection marker.
2. Cut the insert and the vector with the corresponding restriction enzymes and load the reaction products on an agarose gel.
3. Cut the bands corresponding to the *cut insert* and the *cut backbone* and purify them with standard kits.
4. Ligate the insert and the vector with a quick (15 min) or a T4 (2 h) ligase (*see* **Note 1**).
5. Transform competent bacteria with the ligation product by heat shock (30 min in ice, 45 s at 42 °C, and 2 min in ice).
6. Seed bacteria in Petri dish prepared with LB agar supplemented with the appropriate antibiotic (according to vector resistance) and let them grow overnight at 37 °C.
7. Pick some colonies and let them grow in 4 ml of LB medium supplemented with antibiotic for at least 6 h.
8. Process the minipreps.
9. Identify the correct clone by specific cut or direct sequencing.
10. Let this clone grow in 250 ml of LB medium supplemented with antibiotic overnight at 37 °C.

11. Process the maxiprep and store the transfer vector at −20 °C.
12. For vector production, seed 18×10^6 293 T cells in 22 ml of complete medium on a 15 cm dish and incubate overnight at 37 °C.
13. Prepare the plasmid DNA mix (9 μg of the ENV plasmid, 12.5 μg of the PACKAGING plasmid, 6.25 μg of the REV plasmid, and 37.5 μg of the TRANSFER vector) and perform standard $CaCl_2$-mediated transfection of 293 T cells.
14. After 12–14 h replace the media with 16 ml of fresh media.
15. Collect the viral supernatant 30 h after changing the media.
16. Titrate the viral supernatant by FACS (taking advantage of the selection marker) or by molecular analysis.

3.2 Tumor Cell Transduction, FACS Analysis and Sorting (see Fig. 1)

1. Plate tumor cells in 24w plate with 500 μl/well of complete media and incubate for 6 h at 37 °C (*see* **Note 2**).
2. Wash the cells with 2 ml of PBS.
3. Add 500 μl of fresh complete medium.
4. Add the required amount of the lentiviral supernatant accordingly to the viral titer (*see* **Note 3**) and incubate for 24 h at 37 °C.
5. Wash the cells with PBS and transfer them in T25 flask with fresh medium.
6. 10 days after, determine the transduction efficiency by FACS by assessing the percentage of cells expressing the selection marker.
7. If transduction efficiency is under 100 %, sort transduced cells with appropriate paramagnetic MicroBeads.

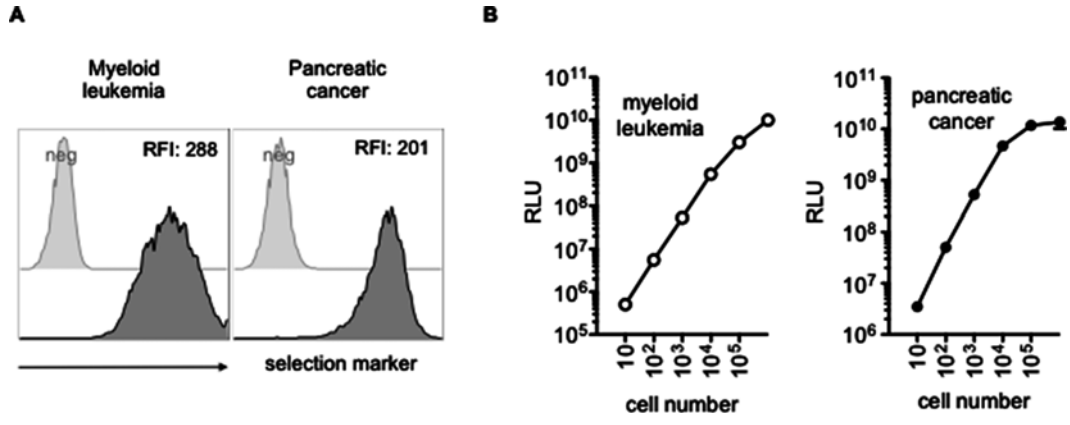

Fig. 1 A myeloid leukemia cell line and a pancreatic cancer cell line were transduced with the **LUCIA-luciferase** lentiviral vector, purified thanks to the expression of the selection marker (**a**) and their supernatant tested with the bioluminescence assay (**b**)

3.3 *In Vitro* LUCIA-luciferase *Assay* (see *Fig. 1*)

1. Plate increasing amounts of transduced tumor cells (1, 10, 10^2, 10^3, 10^4, 10^5, 10^6) in 96-well plate with 200 µl/well of complete media and incubate for 24 h at 37 °C.
2. Measure the LUCIA-luciferase activity using a plate luminometer:
   (a) Transfer 5 µl aliquot of the cell supernatant to a well of a white 96-well plate (*see* **Note 4** and **5**).
   (b) Dispense 50 µl of the QUANTI-Luc reagent including coelenterazine.
   (c) Select the *kinetic mode*.
   (d) Set the total counting time at 300 s.
   (e) Set the counting time for each read at 10 s.
   (f) Read.
3. Obtain the emission curve, identify the pick, and subtract the blank.
4. Analyze the data by plotting the relative light units (*y* axes) with respect to the cell number (*x* axes). Verify the presence of a linear correlation.

3.4 *In Vivo* LUCIA-luciferase *Blood Assay for Monitoring Tumor Growth* (see *Fig. 2*)

1. Inject *LUCIA-luciferase+* tumor cells into 6/8-week-old NSG mice (*see* **Note 6**).
2. Weekly collect 5 µl of blood by placing a small heparin-coated catheter into the ocular vein.

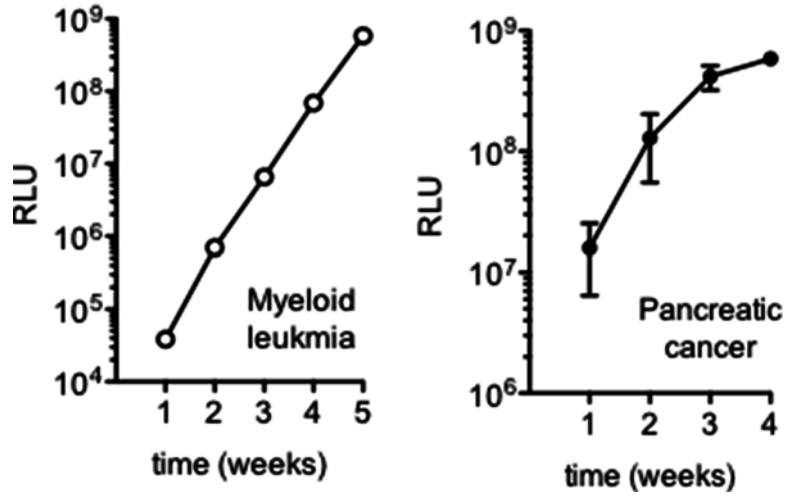

Fig. 2 LUCIA-luciferase+ leukemia cells and **LUCIA-luciferase+** pancreatic cancer cells were infused in NSG mice (intravenously and intrapancreas, respectively) and weekly bleedings were performed to monitor tumor progression with the bioluminescence assay

3. Measure the *LUCIA-luciferase* activity using a plate luminometer as in point 3.
4. Analyze tumor growth kinetic by plotting the RLUs (*y* axes) with respect to time (*x* axes).

3.5 In Vivo Imaging with LUCIA-luciferase (see Note 7)

1. Anesthetize the mice with a gaseous mixture of isoflurane (1–4 %) and oxygen (0.5–0.7 %).
2. Dissolve coelenterazine in acidified methanol (add one drop of concentrated HCl to 10 ml of methanol) to the concentration of 5 mg/ml. Aliquots can be stored at −80 °C for 2 years. The working concentration will be 4 mg/Kg (*see* **Note 8**).
3. Inject the substrate into the tail vein of mice, 200 μl/mouse (*see* **Note 9**).
4. Acquire photon counts over a 1–5 min period (about 10 s for each read) using a cooled CCD camera with no illumination (*see* **Note 10**).
5. Take the images.
6. Define the regions of interest around the tumor (ROIs).
7. Calculate the sum of the photon counts in the ROIs and identify the pick.
8. If required, overlay the bioluminescence images with the white light surface images to allow the unambiguous identification of tumor localization.

4 Notes

1. To increase the chance of positive ligation, try different vector-to-insert ratios, like 1:3, 1:5, and 1:10.
2. Plate non-adherent and adherent cells at 0.5×10^6 and 0.1×10^6 cells/well, respectively.
3. Use MOI 5–10 to have high vector copy number.
4. It is important to use white instead of clear plates to avoid the transfer of the bioluminescence signal from one well to the other.
5. Aliquots of the cell supernatant may be stored at −80 °C for more than 1 month.
6. Inject hematopoietic tumors intravenously (250 μl/mouse) and solid tumors subcutaneously or intra-organ (30 μl /mouse, 50 % of ice-cold Matrigel-50 % of cells).
7. Before performing the experiments with the drug of interest, verify the possibility to monitor the specific tumor cell line by in vivo imaging. Indeed, *LUCIA-luciferase* emits in the blue light, which can be adsorbed by tissues preventing its emergence

and measurement. This may especially occur when the tumor localizes in big and deep organs, like the liver, or when the tumor spreads rather than accumulating in a specific location.

8. In order to stabilize this substrate that is prone to auto-oxidation, dilute it to the working concentration of 4 mg/ml in DMEM and incubate 40 min in the dark at 20–25 °C before use.
9. To avoid high background a-specific signal, do not inject the substrate intraperitoneally.
10. Due to the flash kinetic of the *LUCIA-luciferase*, image the animals immediately after coelenterazine injection (1 min).

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Chapter 11

Efficient RNA Interference of Primary Leukemic Cells for Loss-of-Function Studies in Xenograft Mouse Models

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Abstract

RNA interference (RNAi) is a powerful tool for efficient and highly specific gene silencing. Transduction with lentiviral vectors provides stable and long-term gene expression in slowly and non-dividing cells. This chapter describes how to couple these two technologies to efficiently silence specific genes in primary acute myeloid leukemia (AML) cells for subsequent xenotransplantation in immunocompromised mice. This approach could be used for loss-of-function studies aimed at identifying oncogenic targets in AML.

Key words shRNA, RNAi, AML, Xenograft models

1 Introduction

1.1 RNA Interference: An Overview

RNA interference (RNAi) is a specific gene silencing mechanism that mammalian cells use to regulate their RNA expression. Briefly, endogenous microRNAs (miRNAs) are transcribed by Pol II and processed to form a short double-strand RNA, known as small interfering RNA (siRNA) recognized in the cytoplasm by the RNA-Induced Silencing Complex (RISC) that unwinds it. In particular, one strand of the siRNA, named "guide," is loaded into RISC and functions as a template for mRNA screening. When a complementary mRNA is encountered, RISC inhibits its translation or induces its degradation [1, 2].

Since RNAi discovery in 1998, there have been many improvements in the design and delivery of siRNA. One strategy relies on the direct transfection of chemically synthetized siRNAs, RNA duplexes of 19–22 nucleotides [3], which are straightforwardly recognized by RISC. This approach, however, induces only a transient downregulation of the gene of interest and implies the

The original version of this chapter was revised. An erratum to this chapter can be found at DOI 10.1007/978-1-4939-3338-9_16

Attilio Bondanza and Monica Casucci (eds.), *Tumor Immunology: Methods and Protocols*, Methods in Molecular Biology, vol. 1393, DOI 10.1007/978-1-4939-3338-9_11, © Springer Science+Business Media New York 2016

dilution of the siRNA upon cell proliferation. The high amount of siRNA molecules required to overcome these problems could cause off-target activity and RISC saturation, unbalancing the endogenous miRNA regulation. In addition, this strategy is quite expensive and many cell types are difficult to be transfected [4, 5].

The first relevant improvement was the design of plasmids containing sequences that can be encapsulated into an adeno-, retro- or lentivirus to achieve stable siRNA expression, allowing a stable knockdown of the gene of interest.

The development of short-hairpin RNAs (shRNAs) represented the second crucial improvement. Short-hairpin RNAs consist of 50–70 bp ssRNAs (single-strand RNAs), are directly transcribed by the target cell, and are designed to form stem and loop structures recognized by the endogenous enzyme DICER, which in turn cleaves it to form siRNAs for subsequent complexing with RISC.

Finally, shRNAs deployed in a miRNA structure (shRNA-mir) were proposed. Short-hairpin RNA-mir are constructed starting from the backbone of an endogenous miRNA in which only the guide and the passenger strands have been replaced with the targeting sequences. This strategy ensures stable and potent gene silencing, thanks to a more efficient engagement of the endogenous miRNA processing machinery. As a consequence, the choice of an optimal endogenous miRNA scaffold and its validation criteria are crucial and challenging issues in RNAi-based approaches [6].

Despite the undoubted potential for gene silencing, the different RNAi techniques bring with them some toxicity concerns. In particular, three are the main factors to take into account:

1. High transduction efficiencies are required. Considering that RNAi is a dynamic process, in which there is a competition between the siRNA-RISC complex and the ribosome for the same mRNAs, the highest are the siRNA levels, and the greatest are the chances that the mRNA of interest will be intercepted and degraded.
2. Off-target toxicities are a constant threat. Even though the design of the passenger and guide strand should uniquely recognize the target sequence, there is always the possibility of silencing other mRNAs. To overcome this problem, it is suggested to use in parallel two siRNA sequences, as it is very unlikely that both would lead to the same off-target toxicity.
3. Saturation of the endogenous miRNA machinery may occur. The most recent RNAi strategies provide the cells with precursors that have to be processed by endogenous enzymes to become functional, potentially causing overload of the miRNA machinery. Under this scenario, endogenous miRNA processing will be impaired causing a general deregulation of mRNA expression. The use of a scrambled shRNA within the same backbone or the transduction of similar cell types that do not express the target gene could reveal whether this saturation is occurring.

1.2 Efficient Lentiviral Transduction with shRNAs

The xenotransplantation model has become a fundamental tool for leukemia research, since it is the best setting in which primary leukemic cells can propagate and expand. In this context, the exploitation of the RNAi approach may be extremely informative to study the contribution of particular genes in leukemic-cell survival and proliferation.

Gene transduction has been traditionally achieved with Moloney-derived retroviral vectors, which guarantee stable transgene expression. Unfortunately, retroviruses do not integrate in non-dividing cells, rendering this approach inadequate when working with primary leukemic cells. Consequently, a lentiviral vector platform is required [7]. Moreover, due to the poor survival of primary blasts in vitro, a cocktail of cytokines is required to preserve cells viability.

To achieve high transduction efficiency of primary blasts some precautions in designing the plasmid are necessary. Firstly, the choice of the promoter is critical. It has been shown that the *Spleen Focus Forming Virus* (SFFV) promoter induces a more robust and stable expression in both multipotent and lineage-committed myeloid cell lines, compared with the *cytomegalovirus* (CMV) promoter [8]. In addition, the use of the shRNA-mir approach could increase the expression and activity of the siRNA. In particular, Naldini group developed an shRNA-mir based on the backbone of the miR-223, a hematopoietic-specific miRNA that is highly expressed in myelomonocytic cells (>10^3 copies/pg small RNA). Advocating that a strong expression correlates with an efficient posttranscriptional processing, this backbone could be a good choice for increasing AML transduction efficiency [6].

This chapter will describe how to isolate primary blasts, transduce them with a lentiviral vector encoding the shRNA-mir, and verify gene silencing and/or toxicity. Moreover, loss-of-function studies in a xenotransplant model will be discussed to investigate the contribution of a specific gene in a setting allowing AML expansion and proliferation.

2 Materials

1. AML samples from peripheral blood or bone marrow biopsy.
2. AML cell lines.
3. Six-to-eight weeks-old immunodeficient mice, NSG (Jackson).
4. RPMI-1640 (Gibco-Brl) and IMDM (Gibco-Brl) containing 10 % Fetal Bovine Serum (FBS; BioWhittaker) and supplemented with penicillin (100 UI/ml; Pharmacia), streptomycin (100 UI/ml; Bistrol-Meyers Squibb), and glutamine (2 mM; Gibco-Brl).
5. X-Vivo15 (BioWhittaker) containing 2 % Human Serum (HS; BioWhittaker) supplemented with penicillin, streptomycin, and glutamine.

6. Cytokines: IL-3, Stem Cell Factor (SCF), Thrombopoietin (TPO), Granulocyte Macrophages-Colony stimulating factor (GM-CSF) (Peprotech).
7. Phosphate-buffered Saline (PBS), Calcium- and Magnesium-free (EuroClone Life Science Division).
8. Washing Buffer: PBS supplemented with 5 % FBS and 0.2 % $NaNO_3$.
9. 293T cell line.
10. Agarose.
11. Wizard SV Gel and PCR Clean-Up System and SV Miniprep (Promega).
12. Digestion Enzymes, Alkaline Phosphatase, and Quick Ligation Kit (New England BioLab).
13. Competent Bacteria: (Top10, Invitrogen; GC5, Sigma-Aldrich; home made bacteria).
14. LB-Agar and Ampicillin.
15. Pure link HiPure Purification Filter MaxiPrep (Invitrogen).
16. ENV plasmid (VSV-G; AddGene), packaging plasmid (pMDLg/pRRE; AddGene), REV plasmid (AddGene).
17. TE/H_2O, 2.5 M $CaCl_2$, HBS or GeneJuice or Lipofectamine.
18. Flow cytometer: FACS canto III, LRS II, and/or similar (BD Bioscience).
19. Fluorescent-Activated cell sorter: FACSaria (BD Bioscience) and/or MoFlow (Dako).
20. Labeled Antibodies for FACS analysis: target gene, CD45, CD33, CD117, CD14 and/or the Ig referred to the LAIP of the specific leukemia (BD Bioscience and Biolegend).
21. CD3 MicroBeads, human and separation columns (MACS, Miltenyi Biotec).

3 Methods

3.1 ShRNA Lentiviral Vector Design and Construction

3.1.1 Identification of the siRNA Target Sequence and Oligo Design

There are some guidelines to design a functional and efficient siRNA.

1. The length of the target sequence should be 19–22 nucleotides and start with AA dinucleotide.
2. The GC content should be 30–50 %.
3. Stretches of ≥ 4 T shouldn't be included.
4. Avoid 5′-UGUGU-3′ and 5′-GUCCUUCA-3′.
5. If designing an shRNA, the loop should be between 4 and 11 nucleotides to allow a good annealing of the complementary sequences [9].

6. It is suggested to choose as target 2–4 sequences in different regions of the mRNA.
7. Analyze the mRNA II structure conformation to choose those sequences that are more accessible for siRNA annealing (*see* **Note 1**).
8. Check the complementarity with other mRNA; a minimum of three mismatches is required. Check also the complementarity of the antisense, since it could act as a siRNA as well (*see* **Notes 2** and **3**).

3.1.2 Cloning of the shRNA into the Backbone

Once the siRNA sequence has been identified, it has to be cloned into a lentiviral vector.

1. Get the single-strand oligonucleotides synthetized with the desired sticky ends and anneal them into a double-strand structure at 95 °C for 10 min and allow the heat to cool down to 37 °C.
2. Linearize the backbone vector with the enzymes that will be compatible with the overhangs of the annealed oligonucleotides (*see* **Note 4**):
   (a) 5 μg of the vector.
   (b) 10–20 kU of the single enzymes.
   (c) 3 μl of the specific Buffer.
   (d) Add H_2O up to 20 μl.
   (e) Incubate for the required time at 37 °C.
3. Run the linearized backbone into a 1.5 % agarose gel and purify it with Wizard SV gel and PCR Clean-Up System (*see* **Note 5**).
4. Ligate the annealed oligos and the linearized backbone for 15 min at RT with Quick Ligation Kit.
5. Transfect competent bacteria and plate 100 μl on LB plates containing the correct antibiotic.
6. Check the colonies after overnight incubation at 37 °C, pick single colonies, extract the plasmid with SV Minipreps, and digest to verify the ligation has worked.
7. Check whether the inserted shRNA sequence is correct by sequencing.

3.1.3 Lentiviral Vector Production

The production of the lentiviral particles can be obtained by transient transfection of 293 T through $CaCl_2$ precipitation:

1. Plate 9×10^6 293 T cells in 15 mm dish the day before the transfection in 20 ml of IMDM medium.
2. Change the medium 2 h before transfection.

3. Prepare the plasmid DNA mix, by adding:
   (a) 9 μg of ENV plasmid.
   (b) 12.5 μg of packaging plasmid.
   (c) 6.25 μg of REV plasmid.
   (d) 37.5 μg of the transfer vector.
   (e) Add 0.1× TE/H_2O to yield 1125 μl total volume.
4. Add 125 μl of 2.5 M $CaCl_2$ and rotate 15 min at RT.
5. Add dropwise 1250 μl of 2× HBS while vortexing to form the precipitates.
6. Add immediately the precipitate to the 293 T.
7. Leave for 14 h at 37 °C, 5 % CO_2, then change the medium.
8. After 30 h, collect and filter the supernatant (*see* **Note 6**).
9. Aliquot and store at −80 °C.

3.2 Stable Transduction of Cell Lines: Validation and Toxicities

When performing RNA interference, it is necessary to verify the downregulation of the targeted gene, the specificity, and the possible toxicities of the siRNA before proceeding any further. To set these parameters it is suggested the use of cell lines, since they proliferate in vitro and are easily transduced.

3.2.1 Transduction of AML and MM Cell Lines

1. Use a cell line that expresses the gene of interest (*see* **Note 7**).
2. Plate $0.1–0.2 \times 10^6$ cells into a 24-well plate in 1 ml.
3. Transduce them at a MOI of 10.
4. After 24 h change the medium.
5. Depending on the reporter gene contained in the shRNA-encoding plasmid:
   (a) Perform a FACS analysis at day 3, 5, and 7 to verify the transduction efficiency. If less than 98 % of transduction is obtained, a FACS sorting is required. To obtain cells with an optimal downregulation profile, sort the cells expressing high levels of the reporter gene and low levels of the target molecule.
   (b) If the reporter gene is a drug resistance gene, a drug selection will be necessary (*see* **Note 8**).

3.2.2 shRNA-Mediated Knockdown Validation: mRNA Analysis

Depending on the targeted protein, the verification of its down-regulation could be achieved through different approaches, but an RT-qPCR is always required.

1. Design custom or commercial primers specific for your mRNA:
   (a) If you are not targeting a specific exon-containing splice isoform, design the primers on two different exons.

(b) The length of the amplified fragment should be around 100–150 bp.

(c) Check OligoAnalyzer provided by Integrated DNA Technology for assistance in the choice of the primers (www.idtdna.com/analyzer/Applications/OligoAnalyzer).

2. Isolate total RNA from 2×10^6 cells at different time points by Trizol or RNAeasy MiniKit (*see* **Note 9**).
3. Prepare the cDNA:
   (a) Prepare 1 μg of RNA into a final volume of 15 μl of nuclease-free H_2O.
   (b) Prepare the reaction mix:
      – 8 μl of 5× First Strand Buffer.
      – 8 μl of 25 mM dNTPs.
      – 2 μl of Oligo(dT) (500 μg/ml).
      – 4 μl of 0.1 M DDT.
      – 1 μl of RNAse Inhibitor.
   (c) Mix the content of the tubes and add 1 μl of M-MLV Reverse Transcriptase.
   (d) Incubate at 42 °C for 1 h.
4. Prepare the PCR mix for the gene of interest and the housekeeping gene (like GAPDH, ß-actin, or ß$_2$-microglobulin) for a triplicate:
   (a) 12.5 μl of SYBR Green.
   (b) 1 μl of forward primer (7.5 μM stock solution).
   (c) 1 μl reverse primer (7.5 μM stock solution).
5. Dilute the cDNA 1:1000 (*see* **Note 10**), dispense 10.5 μl in a 96-well plate in triplicate, and add 14.5 μl of the PCR mix.
6. Run the plate on an AB Prism 7500 or a similar thermocycler:
   (a) 50 °C for 2 min.
   (b) 95 °C for 10 min.
   (c) 95 °C for 15 s.
   (d) 60 °C for 1 min.
   (e) Repeat **step** c and d for 40 cycles.
   (f) Run the melting curve when using Syber Green (*see* **Note 11**).
7. Standardize the values on the housekeeping gene and calculate the relative amount using the comparative Ct method (*see* **Note 12**).

3.2.3 shRNA-Mediated Knockdown Validation: Protein Analysis

It is important to verify the correspondence between mRNA degradation and protein downregulation. There are two methods that can be used depending on the targeted gene. Briefly:

1. If the targeted protein is (trans)membrane, perform a FACS analysis using the corresponding fluorochrome-labeled mAb.
2. If the targeted protein is not expressed on the surface, a western blot or an intracellular staining analysis is required.
3. Once it has been verified that one or two of the shRNA designed are efficiently downregulating the targeted protein, it is possible to move towards primary AML cells.

3.2.4 ShRNA Knockdown Toxicities

As anticipated in the introduction, it is very important to address possible toxicities. To correctly control the specificity and safety of the procedure:

1. Two different shRNAs specific for the same gene have to be used.
2. Transduce with scrambled shRNA or mock lentiviral vector.
3. Transduce with the shRNA a cell line that does not express the target gene.

3.3 Stable Transduction of AML Primary Blasts with shRNA-Encoding Lentiviral Vector

3.3.1 Prepare AML Cells

1. Isolate mononuclear cells from fresh peripheral blood or bone marrow samples by Ficoll-Hypaque gradient separation and cryopreserve until use.
2. Culture the cells in complete X-vivo15 (*see* **Note 13**).
3. Perform a FACS analysis according to the leukemia-associated immunophenotype (LAIP).
4. Deplete the CD3 population using anti-human CD3 Microbeads (*see* **Note 14**).
5. Since not all the AML samples are able to engraft, perform a prescreening.
   (a) Sublethally irradiate the mice 6 h–1 day before leukemia inoculation (*see* **Note 15**).
   (b) Resuspend $5–10 \times 10^6$ cells in 200–250 μl of PBS or complete X-vivo15 medium and inject intravenously in NSG mice (three mice per sample).
   (c) Follow the engraftment as explained in Subheading 4, **steps 1** and **2**.

3.3.2 Transduce AML Primary Blasts

AML samples differentially grow in vivo and survive in vitro (*see* **Note 16**).

1. Thaw the cells, considering that this usually results in a 20–50 % of cell death.

2. Incubate the cells at a concentration of $1.5–2 \times 10^6$ cells/ml in complete X-vivo15 medium and DNAse for 2–3 h.
3. Count viable cells and plate 1×10^6 cells in a 24-well plate in X-vivo15 complete medium, supplemented with SCF (50 ng/ml), TPO (10 ng/ml), IL-3 (25 ng/ml), and G-CSF (10 ng/ml).
4. Add the virus at the MOI of 20–40 to a final volume of 1 ml and incubate for 24 h.
5. Change the medium and culture them for another 2 days.
6. Check the transduction efficiency and the downregulation of the protein (by FACS or WB).
7. Depending on the results obtained with the experiment on the cell lines, perform another analysis at day 5 and 7 (*see* **Note 17**) (Fig. 1).
8. Analyze cell viability, by staining for the apoptotic marker Annexin V and the death marker 7AAD (or DAPI) to verify that the transduction is not inducing cell death unrelated to the shRNA.
9. With primary blasts it is unlikely to obtain a 100 % of transduction. Indeed it is necessary to perform a flow cytometry sorting of the transduced cells based on the gene reporter and, if possible, the targeted protein (*see* **Note 18** and Fig. 2).

3.4 Xenograft Model

3.4.1 Engraftment of Silenced-AML Primary Blasts in Immunodeficient Mice

1. According to the prescreening experiment, sublethally irradiate the mice prior to the injection of the prepared cells.
2. Inject $5–10 \times 10^6$ cells intravenously into 200–250 µl of complete medium or PBS.
   (a) Mock or scrambled shRNA.
   (b) ShRNA-specific #1.
   (c) ShRNA-specific #2 (*see* **Note 19**).

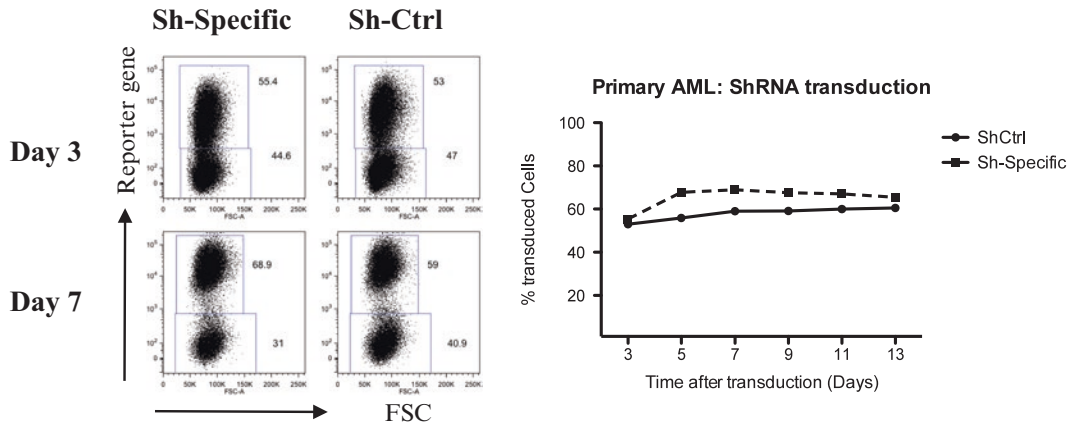

Fig. 1 Example of how to follow the proportion of primary AML cells transduced with the control vector or the vector encoding the specific shRNA

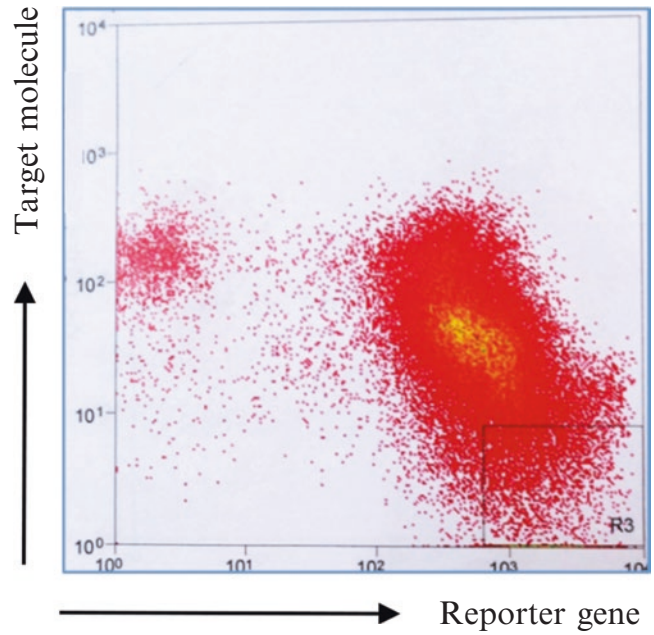

Fig. 2 How to choose the gate for FACS sorting (high expression of the reporter gene/low expression of the target molecule)

3.4.2 Follow the Engraftment

Collect weekly blood samples (ca. 100 μl) and perform a FACS analysis (*see* **Note 20**).

1. Take 50 μl and perform a white cell count using a Hemochrome Reader.
2. Take 50 μl in a FACS tube and stain with the specific mAb.
3. After 15 min, add 2 ml of warm ACK for 7 min vortexing every 2–3 min.
4. Centrifuge at 453 × *g* for 5 min and wash once with Washing Buffer.
5. Perform FACS analysis according to the Leukemia-associated Immunophenotype (LAIP) and the reporter surface marker.

3.4.3 At Sacrifice: Bone Marrow and Spleen Analysis

Sacrifice the mice between 4 and 12 weeks or when the blood chimerism is higher than 70 % using either cervical dislocation or terminal anesthesia.

1. Collect the spleen and dissect the femurs from the mice removing all the connective tissue and store in a 1.5 ml eppendorf with PBS at RT.
2. Cut the edges of the bone and place it into a 0.5 eppendorf tube previously punctured at the bottom with a 25-G needle. Place it into a 1.5 ml eppendorf and centrifuge at 18,121 × *g* for 4 min.
3. Remove the eppendorf and discard the bones; the bone marrow has been flushed at the bottom of the 1.5 ml eppendorf and the bone appears white.

4. Add 1 ml of washing buffer and pass the content 2–3 times through a 25-G needle until it is well resuspended.
5. Pass it through a 70 μm Nylon Cell Strainer, count the viable cells using a hemocytometer, and store in ice until antibody labeling.
6. Take the spleen and smash it through a 70 μm Nylon Cell Strainer with a plunger of a 2.5 ml syringe and resuspend in washing buffer.
7. Count the viable cells and store in ice until antibody labeling.
8. Dispense BM and spleen cells into FACS tubes, centrifuge at 450 × *g* for 5 min, and resuspend in 50 μl.
9. Add 50 μl of the antibody mix and incubate for 15 min.
10. If the pellet appears red, add 2 ml of warm ACK for 1–2 min, centrifuge at 450 × *g* for 5 min.
11. Wash with washing buffer and store in ice until analysis.

3.4.4 FACS Analysis

1. Label the cells to exclude death cells and mouse cells (mCD45) and to identify the AML.
2. Set the compensation according to the specific spectra overlap.
3. Set the threshold at 30,000, to eliminate the record and display of debris.
4. Figure 3 will demonstrate how to perform a FACS analysis on chimerism.

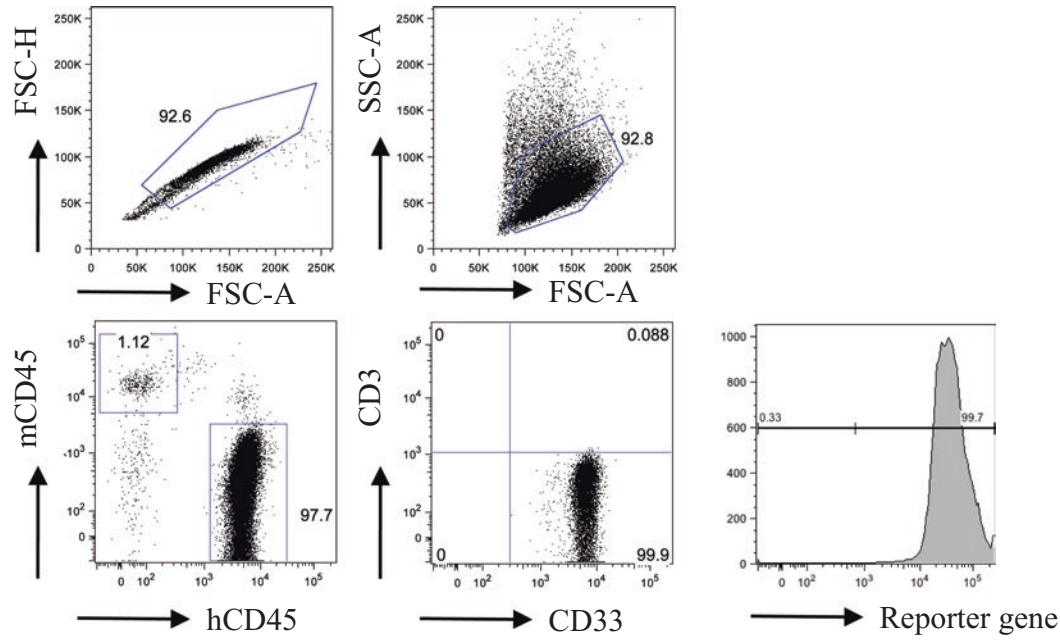

Fig. 3 Example of FACS analysis of blood samples from the xenograft model. (**a**) Exclude doublets and triplets gating on the cell population placed on the bisector (FSC-A/FSC-H). (**b**) Exclude debris (FSC-A/SSC-A). (**c**) Gate on human CD45+ cells (hCD45/mCD45). (**d**) Gate on AML CD33+CD3− cells (hCD33/hCD3). (**e**) Check the expression of the reporter gene

5. Record about 30,000 events (*see* **Note 21**) on the FCS and SSC.
6. AML chimerism was calculated as follows: human $CD45^+CD3^-$cells/(human $CD45^+CD3^-$ cell + mouse $CD45^+$ cells) × 100.

4 Notes

1. We indicate GeneBee Service to have a prediction of the mRNA II structure (www.genebee.msu.su).
2. We suggest using BLAST (www.ncbi.nlm.nih.gov/BLAST) or Ensemble (www.ensembl.org/Multi/blastview).
3. We suggest checking the Ambion's siRNA Target Finder that describes the guidelines in detail and can search for you possible siRNA sequences based on a specific mRNA sequence (www.ambion.com/techlib/misc/siRNA_finder.htlm). Remember that the tool does not analyze the mRNA II structure nor the complementarities with other mRNAs.
4. For the specificities of the reaction's time and buffer to use consult the NEB website (www.neb.com).
5. A dephosphorylating step is suggested to avoid self-ligation (especially if blunting ends are generated) with Antarctic Phosphatase (BioLab) for 1 h at 37 °C.
6. It is possible to ultracentrifuge it, to obtain a higher titer or use directly to infect the cells.
7. When possible, test another cell line that is negative for the marker of interest. This would be a great control for miRNA machinery saturation and specificity of the shRNA.
8. In general, the fluorescent reporter gene is preferable, since it is possible to verify the strength of the transduction and it is possible to sort only those cells that have been transduced the most, which will probably be the one with higher capability of downregulating the target protein.
9. For primary AML samples, we suggest isolating the total RNA with Trizol.
10. The dilution depends on the quantity of the specific mRNA and the performance of the designed primers, so the first time a serial dilutions step is required to obtain the correct dilution.
11. If using a commercial fluorescent probe (TaqMan) instead of Syber Green, the melting curve is not needed.
12. Do not expect a complete elimination of the targeted mRNA but a three- to tenfold of silencing.
13. If the cells were frozen, thaw them and incubate for at least 3 h before proceeding.

14. For an engraftment experiment in NSG mice, it is important to have less than 3–5 % of CD3 to minimize the risk of a xeno-GvHD. After the separation, stain the cells and check the purity again by FACS. If a threshold of 95 % is not reached, repeat the depletion a second time.
15. The dose of irradiation depends on many factors: mouse strain, age, weight, sex, and the source of the irradiator used. We suggest verifying the correct dose and time of irradiation before performing the experiment. When animals lose more than 20 % of their weight or have signs of illness (e.g., hump, rough fur, reduced motility), sacrifice them.
16. Since the transduction of the cells and the downregulation of the targeted protein require several days of culture in vitro, remember that their potential ability to engraft could be reduced.
17. Usually, in 2 days, the promoter will start to express the shRNA and the reporter gene. Consider other 3–5 days to observe the downregulation of the target molecule, depending on the turnover of the protein, the stability of the mRNA etc. All these issues should be investigated on the cell lines.
18. In general, choose the blasts that are transduced the most, since they will express the less amount of the protein of interest.
19. If two of the designed shRNA efficiently downregulate the target, it is suggested to use both of them, as it is very unlikely that they will have the same off-target toxicities.
20. Blood samples could not be informative for all AML samples, since sometimes the chimerism in the blood does not match the level of engraftment in the bone marrow. The prescreening analysis previously performed will help in the evaluation of the time of sacrifice.
21. The number of event to record and display depends on the type of experiment (e.g., to assess AML homing a minimum of 1,000,000 events have to be recorded).

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Chapter 12

Modeling Human Graft-Versus-Host Disease in Immunocompromised Mice

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Abstract

Hematopoietic stem cell transplantation (HSCT) from an allogeneic donor is an effective form of cancer immunotherapy, especially for acute leukemias. HSCT is however frequently complicated by the occurrence of graft-versus-host disease (GVHD). Immunocompromised mice infused with human T cells often develop a clinical syndrome resembling human GVHD (xenogeneic or X-GVHD). Herein, we describe a method for inducing X-GVHD in a highly reproducible manner. Given the human nature of immune effectors, this xenogeneic model can be routinely adopted for screening the efficacy of new treatments for GVHD.

Key words Hematopoietic stem cell transplantation (HSCT), Xenogeneic graft-versus-host disease (X-GVHD), Immunocompromised mice

1 Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is increasingly used as a form of adoptive immunotherapy for acute leukemias. Despite its efficacy, HSCT is however often complicated by graft-versus-host disease (GVHD), an immune attack of the few alloreactive T cells present in the graft against the recipient's tissues. Xenograft mouse models based on the infusion of human peripheral blood mononuclear cells (PBMCs) in severely combined immunodeficient (scid) mice, i.e., mice lacking T and B cells, have been introduced more than 25 years ago to study immune function in vivo [1]. Crossing scid mice with the NOD background first (NOD/scid) [2] and then with the common-gamma chain double-knockout offspring (NSG) [3, 4] has led to a more profound immunodeficiency, including macrophage defects and absence of NK cells, respectively.

In the absence of any barrier to human engraftment, infusing PBMCs in sublethally irradiated NSG mice therefore results in xenogeneic GVHD (X-GVHD), which is caused by the recognition

Attilio Bondanza and Monica Casucci (eds.), *Tumor Immunology: Methods and Protocols*, Methods in Molecular Biology, vol. 1393, DOI 10.1007/978-1-4939-3338-9_12,

of mouse major histocompatibility complex (MHC) class I and class II by human T cells [5]. Given the human nature of effectors, this model has been used for preclinically evaluating the efficacy of novel therapeutic strategies for GVHD, including the co-infusion of regulatory T cells (Tregs) [6] and tolerogenic dendritic cells [7], or the activation of a suicide gene expressed in T cells [5, 8–14]. Herein, we show an optimized protocol for reproducibly inducing X-GVHD in NSG mice. Using specific criteria detailed in the next sections, it is possible to estimate in a quantitative manner the efficacy of pharmacological compounds or cell therapy approaches in preventing and/or treating xGVHD. The protocol is divided into three main sections:

1. Isolation of human PBMCs
2. Infusion of human PBMCs in sublethally irradiated NSG mice
3. Monitoring and sacrifice of NSG mice

2 Materials

Before starting with the experiments, it is mandatory to obtain the specific approval by the Institutional Animal Care and Use Committee (IACUC). These experiments require the presence of an animal facility able of hosting severely immunocompromised mice under specific pathogen-free conditions (SPF) in individually ventilated cages. All the procedure before infusing the mice must be performed in sterile conditions, using laminar flow hoods for isolating human PBMCs.

2.1 Isolation of Human PBMCs

1. A photocopier to scan and track the donor PBMC's barcode on the buffy coat.
2. Laminar flow hood.
3. Scissors to open the buffy coat unit.
4. Falcon tubes 50 mL (BD Falcon).
5. Saline (S.A.L.F.).
6. Lymphoprep (Sentinel Diagnostic).
7. 50 ml syringe (PIC).
8. Spinal needle (Terumo).
9. Centrifuge without brake.
10. ACK (ammonium-chloride-potassium) lysing buffer.
11. FBS (fetal bovine serum, Euroclone).
12. Burker chamber or analogue.

2.2 Infusion of Human PBMCs in Sublethally Irradiated NSG Mice

1. Irradiator and appropriate mouse restrainer.
2. 1 mL syringe with needle (Terumo).

2.3 Monitoring and Sacrifice of NSG Mice

1. Weight scale.
2. Disposable sterile scalpel.
3. CO_2 chamber.
4. Falcon 50 ml (BD Falcon).
5. Eppendorf 1.5 ml (Eppendorf).
6. Cell strainer 70 μm (BD Falcon).
7. Syringe 2.5 ml without needle (PIC).
8. Scissors, tweezers.
9. PBS (Euroclone).
10. Formalin (Bio-Optica).
11. FACS tubes (BD Falcon).
12. ACK solution (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2 EDTA in H_2O).
13. Washing buffer: PBS-FBS 1 % (For 500 mL: 495 mL PBS + 5 ml FBS).
14. Fluorochrome-conjugated monoclonal antibodies (mAb) to mouse CD45, human CD3, human CD4, human CD8, and human CD45 to be used for FACS staining. The dye 4′,6-diamidino-2-phenylindole (DAPI) to exclude dying cells (Biolegend, BD Bioscience).
15. Flow cytometer (BD FACS Canto II).
16. Software to analyze FACS data (FlowJo).

3 Methods

The experimental procedure is subdivided into three main sections:

3.1 Isolation of Human PBMCs

A single buffy coat unit is made of 40–50 ml of concentrated blood, whose PBMC content is sufficient even in the case of complex designs requiring numerous experimental conditions (*see* **Note 1**). Usually, cohorts of $n = 5$ NSG mice per experimental condition are used.

1. Pre-warm the Lymphoprep at 37 °C.
2. Transfer the content of the buffy coat unit in a 50 mL tube.
3. Divide the content in other tubes in order to have between 15 and 18 mL per tube.

4. Add saline to the buffy coat in order to reach 35 mL (e.g., if the tube contains 15 mL of buffy coat, add 20 mL of saline), and mix well for at least five times.
5. Pour the Lymphoprep needed in a 50 mL tube.
6. Mount the spinal needle on a 50 mL syringe and use it to stratify the Lymphoprep on the bottom of the tube up to reaching 45 mL (10 mL approximately of Lymphoprep).
7. Centrifuge at 580×*g* without brake for 25 min.
8. Discard the upper phase with a 10 mL pipette, and then collect the ring of PBMCs with a 5 mL pipette. Discard the rest.
9. Wash the PBMCs by adding saline and centrifuging at 580×*g* with brake for 10 min (repeat two to three times).
10. Discard the supernatant, and add ACK solution 5 mL to the cell pellet prior to 5-min incubation.
11. Add FBS 5 mL to block the ACK solution and centrifuge at 800–1000 rpm for 10 min in order to eliminate platelet excess (repeat two times).
12. Count the PBMCs in a Burker chamber.

3.2 Infusion of Human PBMCs in Sublethally Irradiated NSG Mice (See Notes 2 *and* 3)

You can start either from freshly isolated PBMCs (according to Subheading 3.1) or from previously vitally frozen cells. At the time of PBMC infusion or at later time points, NSG mice can be treated with pharmacological compounds and/or cell therapy approaches according to the experimental design.

1. At day 0, weight and irradiate NSG mice at 150–200 rad (*see* **Notes 4** and **5**).
2. Resuspend human PBMCs 5–10×10E6 in 250 μL saline (*see* **Note 6**). This is the cell number/volume that will be infused in each NSG mouse.
3. Infuse NSG mice intraperitoneally in the lower left flank.

3.3 Monitoring and Sacrifice of NSG Mice (See Note 7)

All clinical X-GVHD signs in NSG mice should be recorded daily, on a Monday-to-Friday schedule, by the same person in a blinded fashion to the experimental conditions in order to avoid subjective biases. X-GVHD signs must be accompanied by weekly measuring T cell engraftment in the peripheral blood of mice by FACS.

1. Weight the mice, and score them as follows: 0 = no weight loss; 1 = more than 5 % loss from initial weight; 2 = more than 10 % loss from initial weight; 3 = more than 15 % loss from initial weight. When the weight score reaches score 3, euthanize the mice for humane reasons.
2. Give a score from 0 to 2 at each of the following X-GVHD signs based on the severity: ulcers at the paws (from 0 to 2), loss or ruffled fur (from 0 to 2), presence of hunched back

(from 0 to 2), loss of motility (from 0 to 2). When more than three parameters reach score 2, euthanize the mice for humane reasons.

3. Once a week, bleed the mice from the tail vein and obtain 50 μl of peripheral blood to measure the levels of human T cell engraftment by FACS after adding the ACK solution for lysing red blood cells. Human T cell engraftment is calculated as follows: % of human $CD3^+$ cells/(% of mouse $CD45^+$ cells + % of human $CD45^+$ cells). If needed, also human $CD4^+$ and/or human $CD8^+$ engraftment can be measured. When human T cell engraftment reaches 10 % in concomitance with body weight loss of more than 10 % (score 2) record the event as "X-GVHD." This event, along with spontaneous death (*see* **Note 4**) and sacrifice for any of the humane reasons as stated above, can be used for drawing Kaplan-Meyer curves.
4. After 6–8 weeks from PBMC infusion in the absence of X-GVHD, sacrifice the animals and prepare single-cell suspensions from the bone marrow and/or the spleen for subsequent FACS analysis. Perform necropsy and sample the following organs for histopathology: ear skin, lung, liver, and gut. These organs are in fact particularly involved during an X-GVHD reaction.

4 Notes

1. In our experience, there is a high inter-donor variability in xenoreactivity between human donors and, for reasons that are at present unknown, in approximately 1 out of 7 cases X-GVHD does not ensue, even at higher PBMC doses.
2. Move NSG mice from individually ventilated cages to a zone of the SPF animal facility where conventional mice are hosted at least one week before PBMC infusion. This will allow the gut microflora to be populated with commensals causing bacterial translocation upon sub-lethal irradiation and the ensuing inflammation needed for X-GVHD priming.
3. Do not use NSG mice older than 8 weeks, as leakage in the genetic defects leading to immunodeficiency may lead to a reduced human T-cell engraftment.
4. Be very careful with the irradiation dose. Choose this dose according to previous, but recent, experience with the irradiator at the same animal facility, with NSG mice from the same provider and at the same age and sex, in order to avoid premature death due to irradiation toxicity (usually within the first week).
5. If the X-GVHD induced from the PBMCs from an individual human donor is too aggressive (death within the first two

weeks), and for experimental reasons you want to use that donor, you may decide to lower the PBMC dose (in any case, never below $5x10^6$ cells/mouse) or to apply a milder irradiation (reduce the dose of 25%).

6. Do not resuspend the cells to infuse in complete medium because high concentration of FBS may be toxic.
7. Use the same balance to weight mice.

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Chapter 13

ICOS Expression as Immunologic Marker in Immune Activating Monoclonal Antibodies

Riccardo Danielli, Ornella Cutaia, Carolina Fazio, Erika Bertocci, Ester Fonsatti, Michele Maio, and Luana Calabrò

Abstract

Compelling experimental evidences point to monitoring of the absolute number of circulating ICOS-positive T cells as an early predictive marker of clinical activity of anti-CTLA-4 antibodies in cancer patients. Here, we report available data focusing on this issue and operative procedures to detect ICOS expression on circulating peripheral blood mononuclear cells during CTLA-4 blockade therapy.

Key words ICOS, CTLA-4, Flow cytometry, Immunomodulating antibodies, Solid tumors

1 Introduction

In the last few years several monoclonal antibodies with immunomodulating properties have been developed in clinic in several tumor histotypes. Among these, ipilimumab (YERVOY) is a fully human CTLA-4 blocking IgG1 that improved overall survival (OS) in patients with previously untreated or treated metastatic melanoma, utilized alone [1] or in combination with dacarbazine [2]. Along this line, atypical patterns of clinical response [3] and immune related side effects have been observed in ipilimumab patients [4], and several pharmacodynamic markers have been reported to have impact on the treatment [5]. Among these, an increase in absolute lymphocyte count after two or three doses (ALC) [6] or in eosinophil count [7] after the first dose has been correlated with improved survival in metastatic melanoma patient. Moreover, earlier studies reported on increased frequency of circulating CD4+HLA-DR+ and or CD4+CD45RO+ T cells after ipilimumab therapy although no correlation with OS was found at early time-points [5]. Nevertheless, to date, molecular basis and mechanisms of action of ipilimumab have not been systematically documented in vivo, and there is a great need to identify biomarkers

Attilio Bondanza and Monica Casucci (eds.), *Tumor Immunology: Methods and Protocols*, Methods in Molecular Biology, vol. 1393, DOI 10.1007/978-1-4939-3338-9_13,

predictive of clinical effect and response as well as onset of immune related side effects of ipilimumab [5]. Along this line, inducible costimulator (ICOS) is a member of immunoglobulin gene family, structurally related to CD28 and CTLA-4, that is expressed on CD4+ and CD8+ T cells after their activation and that can be detected by flow cytometry analysis. ICOS function on activated T cells is associated with increased effector T cell survival, required for Th 17 compartment expansion, and likely responsible in part for the antitumor effect of ipilimumab [8, 9]. The first correlation between CTLA-4 blockade activity and ICOS expression was described in 12 bladder cancer patients that received ipilimumab at 3 mg/kg or 10 mg/kg in a preoperative setting [10]. The analysis of both peripheral blood cells (collected after 3 and 7 weeks post-treatment) and tumor tissues revealed a positive correlation between CD4+ICOS+ T cells frequencies and efficacy of ipilimumab treatment [10]. In addition, increased circulating CD4+ICOS+ T cells were reported to be more pronounced after treatment with ipilimumab at 10 mg/kg, with a concomitant increases in CD8+ICOS$^+$ T cells that were not noted with the 3 mg/kg of antibody [10].

Moreover, after ipilimumab therapy, circulating CD4+ICOS$^+$ cells were found to produce higher levels of interferon gamma compared to those collected at baseline, suggesting enhancement of CD28 costimulatory signals in the absence of the inhibitory effects mediated by CTLA-4. In addition, a retrospective analysis conducted in 14 metastatic melanoma patients treated with ipilimumab at 10 mg/kg identified an increased frequency of CD4+ICOS$^+$ T cells, sustained over a period of 12 weeks, as a correlate of OS [10]. Consistently, a retrospective analysis in 55 stage IIIC or IV resected metastatic melanoma patients treated with ipilimumab at different dosages, confirmed a significant increase of level of ICOS expression on both CD4+ and CD8+ peripheral T cells [5]. However, these changes in ICOS expression on CD4+ or CD8+ T cells were not associated with clinical outcome [5]. Our analysis conducted in 17 metastatic melanoma patients treated with ipilimumab at 10 mg/kg within an expanded access program, also revealed a significant increase in the number of peripheral CD4+ICOS+ and CD8+ICOS+ T cells at week 7, 12, 24 and at 7, 12 respectively [11]. Among patients who achieved disease control, the increase in both CD4+ICOS+ and CD8+ICOS+ T cells at week 7 and 12 was fivefold higher than that observed at baseline. In particular, patients with a fold increase higher than four or five in CD4+ICOS+ or CD8+ICOS+ T cell, respectively, at week 7 had a significant better rates of survival [11]. Finally, a significant increase of the number of circulating CD4+ICOS+ cells was reported in 6 patient affected by metastatic breast cancer treated with exemestane and the anti-CTLA4 monoclonal antibody tremelimumab [12], an IgG2 fully human antibody in clinical development in different tumor histotypes. Altogether, available data indicate the

absolute number of circulating ICOS+ T cells as an early prognostic marker of clinical efficacy of CTLA-4 blockade treatment, suggesting its routinely analysis on patients treated with anti-CTLA4 mAb. Continuous monitoring of this T cell subpopulation and standardization of its analysis will provide further clue on its value as marker of clinical efficacy. Here, we report our proposed procedure. The number of circulating ICOS+ T cells are investigated by direct multiparameter immunofluorescence followed by flow cytometry on cryopreserved peripheral blood mononuclear cells (PBMCs) separated from EDTA whole blood by density gradient centrifugation.

2 Materials

2.1 Isolation and Cryopreservation of Lymphocytes

1. Hank's balanced salt solution (HBSS) (Life Technologies Monza, MI, Italy).
2. Biocoll separating solution (Biochrom AG—Berlin, Germany).
3. 15 or 50 mL conical centrifugation tubes (Greiner Bio-One—Frickenhausen, Germany).
4. Leucosep 50 mL (Greiner Bio-One—Frickenhausen, Germany).
5. RPMI 1640 medium ((Biochrom AG—Berlin, Germany).
6. NH_4Cl 0.87 %.
7. Human serum AB (EuroClone—Milan, Italy).
8. Dimethyl sulfoxide minimum 99.5 % (Sigma-Aldrich—Milan, Italy).
9. 1.5 mL cryogenic vials for storing biological materials (Nalgene—Rochester, NY, USA).

2.2 Immunofluorescence Analysis of ICOS Expression

1. For wash solution: PBS 1×, EDTA 2 mM, and 0.5 % BSA added immediately before use.
2. 5 mL polystyrene round-bottom tubes (BD ®Falcon).
3. Fluorochrome-conjugated mAb:
   (a) FITC Mouse Anti-Human IgG1 (BD Pharmigen™ clone MOPC-21).
   (b) PE Mouse Anti-Human IgG1 (BD Pharmingen™ clone MOPC-21).
   (c) Pecy5 Mouse Anti-Human IgG1 (BD Pharmingen™ clone MOPC-21).
   (d) APC Mouse Anti-Human IgG1 (BD Pharmingen™ clone MOPC-21).
   (e) FITC-CD3 (BD Pharmingen™ ref 555332, clone UCHT1);

(f) CD278 PE-inducible costimulatory (ICOS): (BD Pharmigen™ clone DX29).

(g) Pecy5-CD4 (BD Pharmingen ™ clone RPA-T4).

(h) APC-CD8 (BD Pharmingen™ clone RPA-T8).

3 Methods

3.1 Lymphocytes Isolation (see Note 1)

All steps, other than centrifugations, must be performed under sterile conditions in a Biological Safety cabinet, using sterile solutions and materials.

1. Warm up separation medium (Biocoll separating solution density 1077 g/mL n°L6115) to room temperature (RT) protected from light.
2. Fill the LeucoSep™ tube (Greiner Bio-One material n° 227290) with 3 mL when using 15 mL tubes or 15 mL when using 50 mL tubes of separation medium.
3. Close the tubes containing the separation medium with the screw-cap and centrifuge for 30 s at 1000 × *g* and RT. (The separation medium is now located below the porous barrier).
4. Dilute the blood 1:2 with Hank's Balanced Salt Solution (HBSS) w/o Ca^{++}, Mg^{++} in a conical tube.
5. Gently pipette the diluted blood (maximum volume 30 mL when using 50 mL tube or 11 mL when using 15 mL tube) on top of LeucoSep™ tube.
6. Centrifuge for 30 min at 620 × *g* (20 °C). No BRAKE!
7. Remove the upper plasma fraction and transfer the white blood cell ring fraction (PBMC) to a new 50 mL or 15 mL sterile tube.
8. Add HBSS to each tube containing collected cells to bring the volume up to 45 mL when using 50 mL tubes or 13 mL when using 15 mL tubes.
9. Wash cells with HBSS by centrifugation at 620 × *g* for 10 min at 20 °C. Brake ON!
10. Discard the supernatant.
11. Resuspend the cells with HBSS.
12. Pool cells from the same donor bleed.
13. Wash cells with HBSS by centrifugation at 620 × *g* for 10 min at 20 °C. Brake ON!
14. Discard the supernatant.

N.B. All the following steps have to be performed at 4 °C (on ice).

N.B. **Steps 15–18** have to be performed only if red blood cells are still present in the pellet, otherwise go ahead with **step 19**.

15. Resuspend the pellet in 2 mL of complete medium (RPMI 1640 supplemented with 10 % heat-inactivated FCS and 2 mM L-glutamine).
16. Add 8 mL of lysing solution (ammonium chloride 0.87 %).
17. Incubate for 10 min on ice.
18. Add HBSS to each tube containing collected cells to bring the volume up to 45 mL when using 50 mL tubes or 13 mL when using 15 mL tubes.
19. Wash cells with HBSS by centrifugation at 250 × *g* for 10 min at 4 °C to eliminate platelets. Brake ON!
20. Discard the supernatant.
21. Resuspend cells in HBSS for counting.
22. After cell counting, centrifuge cell suspensions at 620 × *g* for 10 min at 4 °C.
23. Proceed with the analysis for ICOS or freeze PBMC for further analysis.
24. Remove the supernatant, and then resuspend cells in the appropriate volume of cold sterile freezing medium (RPMI 1640 + 10 % DMSO + 30 % Human AB serum; from 1×10^6 to 1×10^7 PBMC per mL of freezing medium), in sterile polypropylene cryovials.
25. Transfer cryovials in −80 °C freezer for at least 24 h, but no longer than 1 week.
26. Transfer and store PBMC aliquots in liquid nitrogen for long term conservation.

3.2 Analysis of ICOS Expression on Lymphocytes

1. Thaw out PBMC using 10 mL of wash solution and centrifuge RT for 5 min at 620 × *g*. Discard the supernatant (*see* **Note 2**).
2. Resuspend PBMC in 200 μL (4,00,000–7,00,000 cells/100 μL) of wash solution and incubate for 10 min on ice.
3. Utilize two different 5 mL polystyrene round-bottom tubes.
4. In the first tube distribute 10 μL of each BD isotype controls (IgG PE, FITC, PECy5, APC).
5. In the second tube distribute 10 μL of each BD labeled antibodies, CD3 FITC, CD278 PE (ICOS), CD4 Pecy5, CD8 APC (*see* **Note 3**).
6. Add 100 μL of cell for each tube and incubate at 4 °C for 30 min with minimal exposure to light.
7. Wash with 2 mL of wash solution at 4 °C at 500 × *g* for 10 min. Discard the supernatant.
8. Resuspend the cells in 200 μL of PBS 1× and proceed with the acquisition within 2 h.

9. Optional: add 300 μL of 1 % formaldehyde in PBS and store max 24 h at 4 °C before the acquisition.
10. We recommend optimizing forward scatter and side scatter voltages to visualize lymphocytes as separate from debris, red cell ghosts, and/or platelets before acquisition. Acquire at least 40,000 lymphocytes.
11. The results are expressed as absolute lymphocyte counts calculated on the ALC (routinely obtained for each sample).
12. ICOS CD4+ Absolute value = (CD4 % × ALC)/100 × ICOS % /100.
13. ICOS CD8+ Absolute value = (CD8 % × ALC)/100 × ICOS % /100.
14. ALC is obtained from a complete blood count and measured by an accredited clinical laboratory.
15. Data are analyzed using descriptive statistics, such as mean and standard deviation, Student's *t* test for paired data, and *P*-value <0.05 was considered statistically significant.

4 Notes

1. The flow cytometer analysis can be done on fresh or frozen isolated lymphocytes. Make sure that all the analyses are performed in the same conditions.
2. After thawing of PBMC proceed to count perform a cell count and viability analysis.
3. For optimal performance of fluorochrome conjugated antibodies, store vials at 4 °C in the dark. Do not freeze. We recommend to not vortex the antibody vials. Use for all experiment always the same amount of antibody.

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Chapter 14

Identifying NK Alloreactive Donors for Haploidentical Hematopoietic Stem Cell Transplantation

Loredana Ruggeri, Antonella Mancusi, Elena Urbani, and Andrea Velardi

Abstract

NK cell alloreactivity mediated by donor NK cells is a fundamental therapeutic tool in HLA haplotype mismatched hematopoietic transplantation in adult acute myeloid leukemia and pediatric acute lymphoblastic leukemias. NK cell is mediated by donor NK cells recovering very early after transplant. The self donor KIR ligands educates the donor NK repertoire and arms functional NK cells which express inhibitory killer cell immunoglobulin-like receptor(s) (KIRs) for self-class I ligand(s), They sense missing expression of donor KIR ligand(s) in the recipient and mediate alloreactivity. Donor-versus-recipient NK cell alloreactivity is evaluated by KIR genotyping and phenotyping and functional assay.

Key words HLA haplotype mismatched hematopoietic transplantation, NK cells, KIR genotyping, KIR phenotyping, NK cell alloreactivity

1 Introduction

Full HLA haplotype-mismatched (haploidentical) transplants are the setting for natural killer (NK) cell alloreactivity which, when directed from the donor to the recipient, strongly impacts beneficially on transplant outcomes. They reduced the risk of leukemia relapse, did not cause GvHD, and markedly improved event-free survival in haploidentical transplantation for adult acute myeloid leukemia (AML) and pediatric acute lymphoblastic leukemia (ALL) and in cord blood transplantations [1–4]. Mouse models of haploidentical bone marrow transplantation provided proof that alloreactive NK cells ablated (1) leukemic cells, (2) recipient T cells which reject the graft, and (3) recipient dendritic cells (DCs) which trigger Graft-versus-Host Disease (GvHD), thus protecting from GvHD and allowing for mismatched T cell replete transplantation without GvHD [1].

Human NK cells possess clonally distributed inhibitory and activating receptors but only NK cells which express inhibitory KIRs for self HLA ligands become "licensed/educated" upon

Attilio Bondanza and Monica Casucci (eds.), *Tumor Immunology: Methods and Protocols*, Methods in Molecular Biology, vol. 1393,
DOI 10.1007/978-1-4939-3338-9_14,

interaction with donor HLA molecules and thus exert alloreactivity against mismatched allogeneic targets which do not express donor HLA inhibitory KIR ligands ("missing self" recognition). KIR2DL2/3, receptors for HLA-C group 1, are present in all individuals, KIR2DL1, receptor for HLA-C group 2 is found in 97 % and KIR3DL1, receptor for HLA-Bw4 alleles, is found in ~95 %. All KIR genes are randomly expressed; KIR distribution varies on NK cells. When, as their only inhibitory receptor for self, NK cells express a KIR whose ligand is a HLA class I group which is absent on allogeneic targets, they sense the missing expression of the self class I KIR ligand and mediate alloreactions [5–9]. Activating KIR are not found in all individuals. In fact, 25 % of Caucasians are homozygous for KIR gene A haplotypes which contain inhibitory KIR genes and only the KIR2DS4 activating KIR which is non-functional in 2/3 of individuals. The other 75 % are either heterozygous or homozygous for B haplotypes which carry not only inhibitory KIRs but also various combinations of activating KIRs, e.g., KIR2DS1-2-5 and KIR3DS1. Knowledge of the ligand specificity of activating KIR homologues is limited. Studies reported a weak interaction between KIR2DS1 and Lys^{80} HLA-C molecules, despite its homology to KIR2DL1 and an even weaker interaction between KIR2DS2 and Asn^{80} HLA-C [10, 11]. The beneficial effects of donor-vs.-recipient NK alloreactivity in haploidentical transplants have been confirmed in an updated analysis of 135 high-risk AML patients. Transplantation from NK alloreactive donor was associated with significantly lower relapse rates and better event-free survival. Evaluation of donor NK cell alloreactivity is become one of the criteria for donor selection. Donor-versus-recipient NK cell alloreactivity is evaluated by KIR genotyping and phenotyping and functional assay.

2 Materials and Methods

2.1 Evaluation of Donor and Recipient by Four-Digit HLA Typing

A family donor is considered potentially able to exert donor-vs.-recipient NK cell alloreactivity when HLA-C and HLA-B typing shows KIR ligands incompatibility in the GvH direction, i.e., when the recipient does not express one KIR ligand of HLA-C allele group (C1 or C2) and/or the HLA-Bw4 group which are present in the donor.

First, four-digit HLA typing of the recipient is necessary to evaluate the potential susceptibility to donor alloreactive NK cell lysis. Those individuals who express all three principal KIR ligands (HLA-C group 1, HLA-C group 2, and HLA-Bw4 alleles) recognized by 2DL1/2/3 and 3DL1 KIRs will block all NK cells from every donor and will be resistant to alloreactive NK killing (approximately 1/3 of the population).

Patients who express only one or two of these inhibitory KIR ligands are susceptible to alloreactive NK cell lysis and may find in the family members an NK alloreactive donor.

Four-digit HLA typing of the familiars will identify those haploidentical family member(s) who expresses the class I group(s) missing in the recipient and who have, therefore, the potential to be a donor whose NK cells exert alloreactivity against the recipient.

2.2 KIR Genotyping and Phenotyping

Donors must possess the relevant KIR gene(s) for missing self-recognition on recipient targets.

Donor KIR genotyping is performed to ensure the donor possesses the relevant inhibitory KIR gene to exert alloreactivity. In particular, as already anticipated in the introduction, 3 % of individuals do not possess KIR2DL1 gene and 5–7 % of individuals do not possess KIR3DL1.

Moreover, KIR genotyping identifies donors who also possess activating KIR genes (approximately 2/3 of Caucasian population). We and other groups suggest a relevant role of activating KIRs in NK cell alloreactivity and haploidentical transplantation outcome. In our laboratory, highly pure DNA is extracted from peripheral blood mononuclear cells (NucleoSpin Tissue, MACHEREY-NAGEL Gmbh & Co. KG, Düren, German) and KIR genotyping is performed using a PCR-SSP assay (Olerup SSP KIR Genotyping, Olerup SSP AB, Stockholm, Sweden) following the manufacturer's instructions. This assay identifies all the alleles available in the IPD KIR Sequence Database (www.ebi.ac.uk/ipd/kir) for 16 KIR genes (2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 2DS1, 2DS2, 2DS3, 2DS4, 2DS5, 3DL1, 3DL2, 3DL3, 3DS1, 2DP1, 3DP1).

KIR phenotyping can be used as an alternative method. It checks surface expression of KIR2DL1 and KIR3DL1. Moreover, four-color immunofluorescence analysis identify and quantify alloreactive donor NK cell repertoire. Alloreactive donor NK cells are those cells that express, as their only inhibitory receptor for self, the KIR for which there is no ligand in the recipient. Gating into CD56+/CD3- cells (APC-conjugated anti-CD56, IgG1; Miltenyi, Bergisch Gladbach, Germany/PE-Cy7-conjugated anti-CD3, IgG1; BD Bioscience, San Diego, CA), KIR2DL2/3/S2 single-positive NK cells are identified with anti-KIR2DL2/3/S2 antibody FITC-conjugated (clone CH-L, IgG2b, BD Bioscience, Franklin Lakes, NJ) in combination with a cocktail of PE-conjugated anti-KIR2DL1 (clone 143211, IgG1, R&D, Minneapolis, MI), anti-KIR3DL1 (clone DX9, IgG1, Miltenyi), and anti-NKG2A (clone Z199, IgG2b, Beckman Coulter, Brea, CA) mouse antibodies.

KIR2DL1 single-positive NK cells were identified with PE-conjugated anti-KIR2DL1 (clone 143211, IgG1, R&D, Minneapolis, MI) in combination with a cocktail of FITC-conjugated anti-KIR3DL1 (clone DX9, IgG1, Miltenyi) and anti-KIR2DL2/3S2 (clone CH-L, IgG2b) and anti-NKG2A

(clone Z199, IgG2b, Beckman Coulter, Brea, CA) mouse antibodies developed with FITC-conjugated goat anti-mouse IgG2b antibodies (Southern-Biotech).

KIR3DL1 single-positive NK cells were identified with PE-conjugated anti-KIR3DL1 (clone DX9, IgG1, Miltenyi) in combination with a cocktail of FITC-conjugated anti-KIR2DL1 (clone 143211, IgG1, R&D, Minneapolis, MI) and anti-KIR2DL2/3S2 (clone CH-L, IgG2b) and anti-NKG2A (clone Z199, IgG2b, Beckman Coulter, Brea, CA) mouse antibodies developed with FITC-conjugated goat anti-mouse IgG2b antibodies (Southern-Biotech).

2.3 Functional Assessment of Alloreactive NK Cell Repertoires

This analysis aims to confirm donor NK alloreactivity against the recipient by formally identifying donor NK cells which kill allogeneic recipient targets, such as PHA lymphoblasts and/or leukemia cells. This analysis is also used to study the post transplant donor NK cell repertoire, reconstituting into the patients.

Large numbers of donor NK clones are generated by limiting dilution and cytotoxicity assays against recipient target cells are used to detect the frequency of alloreactive NK clones. Peripheral blood mononuclear cells are enriched of purified NK cells by human NK cell isolation KIT (Miltenyi).

NK cells are plated under limiting-dilution conditions, activated with phytohemagglutinin (PHA; Biochrom KG, Berlin, Germany), and cultured with interleukin-2 (Chiron BV, Amsterdam, Netherlands) and irradiated feeder cells. Feeder cells are obtained by pooling buffy coats from five to nine healthy donors. Cloning efficiencies ranges from one in five to one in ten plated NK cells. Cloned NK cells are screened for alloreactivity by standard 51-Cromium release cytotoxicity at an effector-to-target ratio of 10:1 against recipient PHA lymphoblasts and leukemic cells. Approximately 100 NK clones from each person are screened. Clones exhibiting greater than 30 % lysis are scored as alloreactive. The assay is considered positive when the frequency of lytic clones was more than 1 in 50. When tested in large donor cohorts, functional analyses detected high-frequency alloreactive NK clones against either HLA-C1 or HLA-C2 group-mismatched allogeneic targets. On the contrary, only 2/3 of HLA-Bw4-positive individuals with the KIR3DL1 gene display alloreactive NK clones against allogeneic HLA-Bw4-negative targets. Failure to detect alloreactive NK clones may be due to their highly variable frequencies, or because certain allelic KIR3DL1 variants do not allow receptor expression at the cell membrane. In donor-recipient pairs that are not KIR ligand-mismatched in the graft-vs.-host direction, no donor alloreactive NK clones are found, indicating that KIR ligand mismatching is a prerequisite for NK cell alloreactivity.

3 Conclusion

Haploidentical transplantation from NK alloreactive donors is associated with better outcome because of protection from leukemia relapse.

For this reason identification of an NK alloreactive donor has become a major criterion of donor selection in the setting of haploidentical transplantation.

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Chapter 15

Human Microtumors Generated in 3D: Novel Tools for Integrated In Situ Studies of Cancer Immunotherapies

Lothar Hambach, Andreas Buser, Marcel Vermeij, Nadine Pouw, Theo van der Kwast, and Els Goulmy

Abstract

Cellular immunotherapy targeting human tumor antigens is a promising strategy to treat solid tumors. Yet clinical results of cellular immunotherapy are disappointing. Moreover, the currently available in vitro human tumor models are not designed to study the optimization of T-cell therapies of solid tumors. Here, we describe a novel assay for multiparametric in situ analysis of therapeutic effects on individual human three-dimensional (3D) tumors. In this assay, tumors of several millimeter diameter are generated from human cancer cell lines of different tumor entities in a collagen type I microenvironment. A newly developed approach for efficient morphological analysis reveals that these in vitro tumors resemble many characteristics of the corresponding clinical cancers such as histological features, immunohistochemical staining patterns, distinct tumor growth compartments and heterogeneous protein expression. To assess the response to therapy with tumor antigen specific T-cells, standardized protocols are described to determine T-cell infiltration and tumor destruction by monitoring soluble factors and tumor growth. Human tumors engineered in 3D collagen scaffolds are excellent in vitro surrogates for avascular tumor stages allowing integrated analyses of the antitumor efficacy of cancer specific immunotherapy in situ.

Key words In vitro tumor model, Multiparametric assay, Targeted therapies, Cell adhesion and extracellular matrix, Novel assay technology

1 Introduction

The overall success of cancer immunotherapy with tumor antigen specific T-cells is still low [1]. Particularly, the in situ interaction of human T-cells with the tumor cells is poorly understood. Most preclinical studies use suspension cell assays determining the antitumor efficacy of T-cells by single parameters such as cytotoxicity and cytokine release. Notably, three-dimensional (3D) growth, i.e., the central hallmark of clinical cancers, strongly affects the tumor response to cellular immunotherapy for the following reasons. First, 3D tumor growth represents a hurdle for tumor specific T-cells, since they need to infiltrate into

Attilio Bondanza and Monica Casucci (eds.), *Tumor Immunology: Methods and Protocols*, Methods in Molecular Biology, vol. 1393, DOI 10.1007/978-1-4939-3338-9_15,

the tumors in order to be effective [2–4]. Thus, appropriate assays modeling the T-cell–tumor interaction require tumors large enough to allow tumor infiltration by T-cells. Second, the gene expression profile of 3D tumor cell cultures is strongly altered compared to tumor cells growing in 2D [5, 6]. This alteration typically results in downregulation [7] and heterogeneity [8] of tumor-associated antigens (TAA), known as an important problem in clinical cancer immunotherapy [8–10]. Third, spatially distinct tumor niches in 3D show heterogeneous dynamics in cell growth and death (e.g., outer proliferative versus inner necrotic tumor regions) [5]. Previous studies already indicated a heterogeneous chemosensitivity of these different niches [11, 12]. Fourth, the polarity of tumor cells organized in 3D and the tumor cell interactions with extracellular matrix (ECM) lead to differences in the intracellular signaling compared to tumor cells growing in 2D [13–16]. Thereby, 3D growth can increase apoptosis resistance of tumor cells [5, 17, 18] and impair their response to treatment.

The tumor spheroid model, i.e., non-adherently cultured tumor cell agglomerates, was a first 3D in vitro approach [5, 19]. However, many tumor cell lines fail to create spheroids [20]. The addition of ECM as key regulator of the tissue phenotype facilitated the generation of microtumors. Thereby, 3D tumor foci in matrigel [21] or synthetic polylactide-co-glycolide scaffolds [22] can be reliably engineered in vitro. However, these models neither combine growth of individual tumors nor do they create the possibility to assess multiple parameters of their response to T-cell treatment in one assay. Moreover, an efficient histological analysis of individual tumors is cumbersome due the small tumor size in these assays. Ideal in vitro assays to study cancer immunotherapy require single 3D human tumors that (1) are large enough to facilitate studies on T-cell infiltration, (2) have defined properties (e.g., known presence or absence of the target antigens), (3) reflect the heterogeneity of the target antigen expression present in many clinical tumors, (4) resemble relevant morphological aspects of 3D clinical tumors, including their tumor growth patters and their heterogeneous growth niches, and (5) can be easily and individually analyzed for multiple parameters of their response to treatment. In order to fulfill all 5 latter requirements, we adapted a previously published method by Wei et al. in the murine setting [23]. Herewith, we generated single human solid tumors of several millimeter diameter in vitro in collagen type I scaffolds and monitored their response to cellular immunotherapy over a period of 7 days. Using a newly developed approach for the efficient histological analysis of these tumors, we found that 3D tumors generated from various well-defined human tumor cell lines resemble a series of histological features of the corresponding clinical cancers [24]. These 3D tumors enabled us to study, as proof of principle, the antitumor efficacy of

human CTLs directed against minor histocompatibility antigens (mHags). MHags are HLA-restricted polymorphic antigens capable of mediating strong immune responses after allogeneic stem cell transplantation [24, 25].

In the current report, we describe a standardized approach to generate individual human solid tumors in vitro and to analyze their response to antigen-specific T-cells. We show the details of (1) our new approach to study the histology of tiny tumors of several millimeter diameter, (2) the morphological and immunohistochemical features of these tumors and (3) the multiparametric assessment of the tumor response to cellular immunotherapy.

2 Materials

1. Adherently growing human cancer cell lines (*see* **Note 1**).
2. Antigen specific CTL clones (*see* **Note 2**).
3. Rat tail collagen (BD Biosciences Europe, Erembodegem, Belgium; 1.75 mg/ml in PBS prepared according to the instructions of the manufacturer).
4. Micropipette (Eppendorf Reference for 0.5–10 µl, Hamburg, Germany).
5. Round tips (Sorenson Bioscience, Miniflex Round Tips 0.1–10 µl, Salt Lake City, Utah, USA).
6. Inverted microscope (Axiovert 25, Zeiss, Jena, Germany).
7. Software: AxioVision 40 LE Version 4.5, Sliedrecht, The Netherlands.
8. Watchmaker's forceps.
9. Metal base molds (Klinipath BV, Duiven, The Netherlands).
10. Slide warmer (SW 85, Adamas instrumenten BV, Rhenen, The Netherlands).
11. 2 % liquefied agar (Agar select, Sigma, Zwijndrecht, Netherlands).
12. Stereo dissecting microscope (Leica MZ 7.5, Leica, Rijswijk, The Netherlands).
13. Tissue cassettes (Klinipath BV, Duiven, The Netherlands).
14. Tissue processor Excelsior (Thermo Fisher, Landsmeer, The Netherlands).
15. Variwax paraffin (7079-1, Klinipath, BV, Duiven, The Netherlands).
16. Paraffin embedding station (Leica EG1150H, Leica, Rijswijk, The Netherlands).
17. Microtome (Microm HM 335 E, Wallorf, Germany).
18. EDTA buffer 1 mM, pH 8.0 (for CD8).
19. Tris–EDTA buffer, pH 9.0 (for all other antibodies).

20. 0.5 % Protifar (Protifar Nutricia Cuji, The Netherlands) blocking solution.
21. Antibodies against CD8 (clone 4B11, Novocastra, UK), pankeratin (clone AE1/AE3, Neomarkers, CA), EMA (clone E29, DAKO, Haverlee, Belgium), Vimentin (clone V9, DAKO), CD10 (clone 5bc6, Monosan, Uden, The Netherlands), gp100 (clone HMB45, DAKO), Melan A (clone A103, Neomarkers), Estrogen receptor (clone 1D5, DAKO), S100 (DAKO), Ki-67 (clone MIB-1, DAKO).
22. DAKO Envision Peroxidase/DAB kit according to the instructions of the manufacturer (DAKO, Heverlee, The Netherlands).
23. Pelikline ELISA kit (CLB, Amsterdam, The Netherlands).

3 Methods

3.1 Generation of Microtumors

Similar to the previous murine work of Wei et al. [23], individual 3D microtumors are generated from adherent tumor cell lines by embedding tumor cell agglomerates between two layers of collagen type I on day 0. Collagen type I is used as scaffold for tumor growth because of its capability to facilitate the generation of complex morphologies in vitro [26, 27] and its high optical transparency allowing photographic documentation of microtumors in culture.

1. A base layer of 50 µl rat tail collagen (BD Biosciences Europe, Erembodegem, Belgium; 1.75 mg/ml in PBS according to the instructions of the manufacturer) is added to a 96-well flat bottom culture plate well and incubated at 37 °C for 2 h with closed lid.
2. After 2 h, a cancer cell agglomerate of 6×10^4 tumor cells in 0.6 µl collagen (3.65 mg/ml) is placed on the base layer using a micropipette (Eppendorf Reference for 0.5–10 µl, Hamburg, Germany) with round tips (Sorenson Bioscience, Miniflex Round Tips 0.1–10 µl, Salt Lake City, Utah, USA).
3. After incubation at 37 °C for 45 min with slightly opened lid and additional 15 min without lid in the flow cabinet, a top layer of 50 µl collagen (1.31 mg/ml) is gently added.
4. After incubation at 37 °C for 30 min with closed lid, 100 µl of 20 % pooled human serum (HS) in IMDM is added. This procedure results in 1 microtumor per well. The generation of microtumors is shown in Fig. 1a, b. Note, that the microtumor generation using very small volumes of tumor cell suspensions requires serious training.

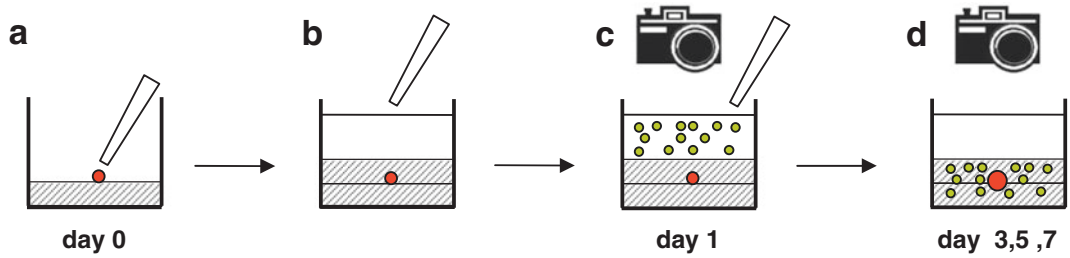

Fig. 1 Microtumor generation. (**a**, **b**) Microtumors were generated on day 0 by embedding of tumor cells between two layers of collagen type I in a 96-well plate well and subsequently covered with medium. (**c**) In order to study the in situ response of human tumor-epitope specific CTLs against human microtumors, CTLs were added to the microtumor cultures on day 1. (**d**) On day 1, 3, 5, and 7 tumor size was quantified and supernatant was stored for cytokine analysis

3.2 Treatment of Microtumors

After 1 day, microtumors of comparable size and shape are selected for further analysis and equally distributed to the various treatment groups. Microtumors are treated on day 1 with CTLs in 100 µl 20 % HS in IMDM supplemented with 40 Cetus U/ml interleukin-2 (Chiron, Amsterdam, The Netherlands) or medium only (*see* Fig. 1c).

3.3 Monitoring of Microtumor Growth and Cytokine Release

3.3.1 Monitoring of Microtumor Growth

1. Similar to the previous work of Wei et al. [23], microtumor growth is photographically documented on day 3, 5, and 7 with an inverted microscope (Axiovert 25, Zeiss, Jena, Germany) (*see* Fig. 1d).
2. Two-dimensional microtumor area π*r1*r2 is calculated after determination of the two maximal orthogonal microtumor radii r1 and r2 (AxioVision 40 LE Version 4.5, Sliedrecht, The Netherlands) in these photographs. This allows a comparison of antitumor efficacy between different treatments.

In our experiments, microtumors were generated from the cancer cell lines MDA-MB-231, BB65-RCC, 518A2, and MCF-7, which have differential expression of the mHags HA-1 and H-Y (*see* **Note 1**). These microtumors were treated with mHag HA-1 or H-Y specific CTLs (*see* **Note 2**) or medium only. HA-1 CTLs only inhibited the growth of the HA-1$^+$ microtumors MDA-MB 231 (*see* Fig. 2a, c) and BB65-RCC (data not shown), while H-Y CTLs only inhibited the growth of the male derived H-Y$^+$ microtumors 518A2 (*see* Fig. 2b, d) and BB65-RCC. Growth of microtumors not expressing the relevant mHag targeted by the CTLs was not affected even at high effector to target (E/T) ratios of 15:1. HA-1 CTLs induced dose-dependent antitumor effects (*see* Fig. 2c) with reduced MDA-MB 231 microtumor progression at a 5:1 E/T ratio and total growth inhibition at a 15:1 E/T ratio. These data show that the suppression of tumor growth by human CTLs is antigen-specific in this model.

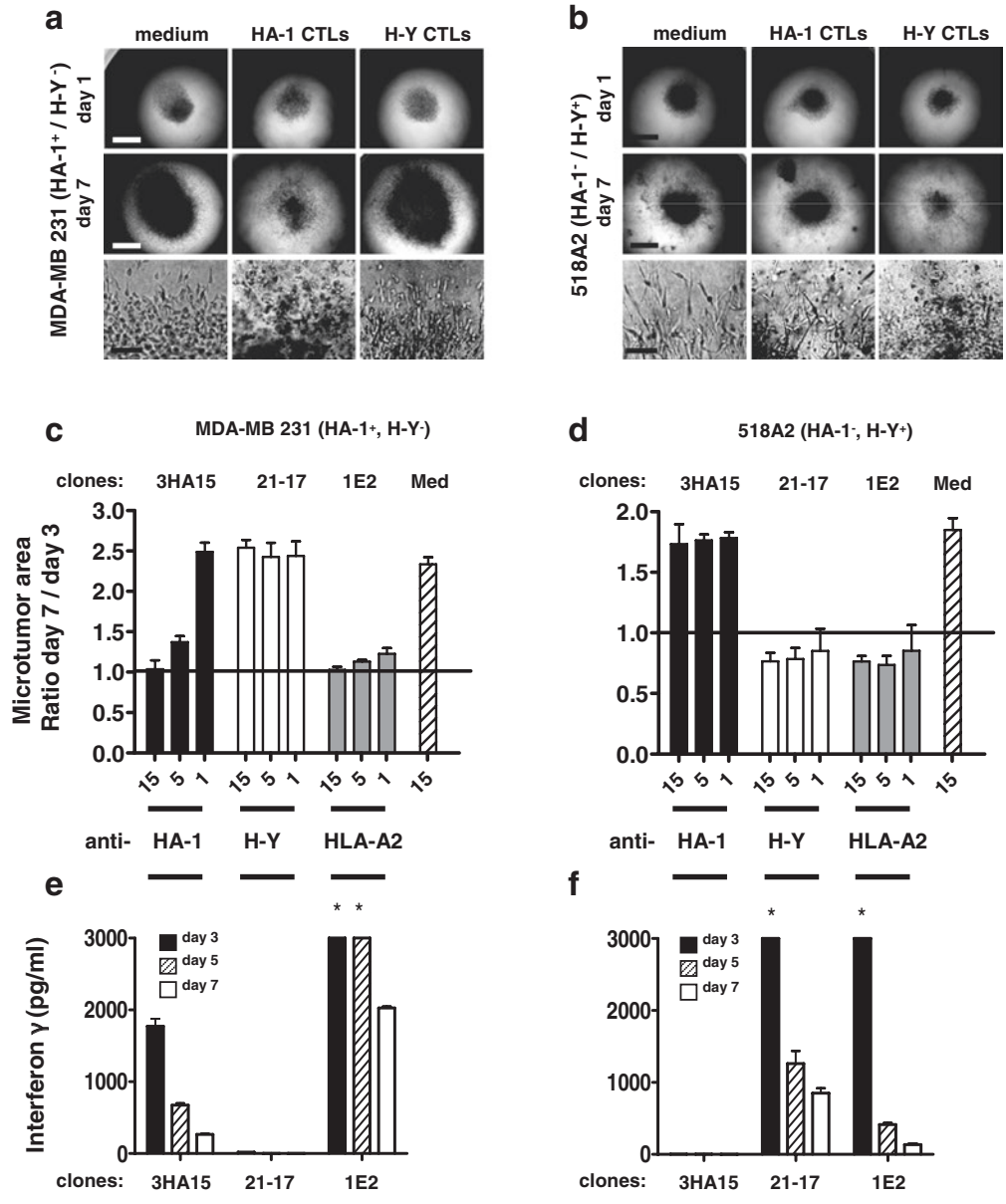

Fig. 2 Monitoring of antitumor responses mediated by tumor antigen specific CTLs. (**a**–**b**) MDA-MB 231 and 518A2 microtumors were used. Microtumors on day 1 (**top row**) and on day 7 (**middle** and **bottom row**) after medium or mHag HA-1 or H-Y CTL treatment (effector to target ratio: 15:1); **top** and **middle row**: bar, 1 mm; **bottom row**: bar, 100 μm. Intact tumors on day 7 (**bottom row**) contain spindle shaped tumor cells; destroyed tumors contain mainly cell debris. (**c**–**d**) Microtumor growth inhibition after addition of the anti-HA-1, H-Y, or HLA-A2 CTLs. **x**-axis, CTL clones each in 3 different effector–target ratios (15:1, 5:1, 1:1) or medium; **y**-axis, ratio between the microtumor areas in photographs of day 7 and day 3. Bars correspond to mean +/– S.E.M. (data are pooled from three independent experiments resulting in median 3 (range 2–8) microtumors per condition). (**e**–**f**) IFN-γ in the supernatant of microtumors after addition of anti-HA-1, H-Y CTL, and HLA-A2 CTLs in an effector to target ratio of 15:1. **x**-axis, day 3, 5, and 7 after addition of different CTL clones; **y**-axis, IFN-γ in the supernatant. Bars correspond to mean +/– S.E.M. (four measurements per sample). **Stars**: off-scale measurement. (**c**–**f**) This research was originally published in **Blood**. Hambach, L., M. Vermeij, A. Buser, Z. Aghai, K. T. van der, and E. Goulmy. Targeting a single mismatched minor histocompatibility antigen with tumor-restricted expression eradicates human solid tumors. **Blood.** 2008; 112:1844–1852. © the American Society of Hematology

3.3.2 Monitoring of Cytokine Release by mHag CTLs

IFN-γ concentration in the supernatant was determined with the Pelikline ELISA kit (CLB, Amsterdam, The Netherlands). Detection limit was 2 pg/ml. Interferon-γ (IFN-γ) was quantified in the supernatant of CTL-treated MDA-MB-231 and 518A2 microtumors on day 3, 5, and 7.

IFN-γ was only detectable in response to CTLs specific for mHags expressed by the microtumors. IFN-γ levels were maximal on day 3 and subsequently declined (*see* Fig. 2e, f). These data show that the collagen is sufficiently permeable for soluble factors for quantification in subsequent assays. Moreover, this demonstrates that cytokine release by human CTLs is antigen-specific in this model.

3.4 Embedding of the Microtumors in Agar and Paraffin

Histological analysis of microtumors is performed after processing and paraffin embedding according to a newly developed technique. This methodology ensures that serial sectioning of paraffin-embedded tumors starts directly at the microtumor borders (*see* Fig. 3) without the need to search the tumors during serial sectioning in the paraffin block. This approach was crucial for the rapid histological analysis of microtumors.

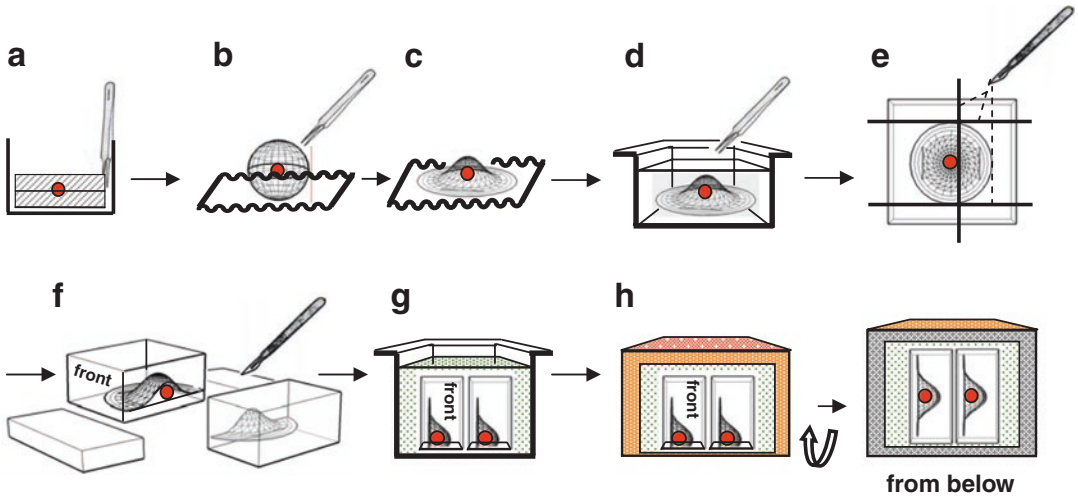

Fig. 3 Processsing of microtumors for histological analysis. For histological analysis, microtumors in collagen were formalin fixed. The microtumor containing collagen clot was separated from the well wall with a watchmaker's forceps (**a**) and transferred onto a tissue paper to absorb excessive formalin (**b**). The shape of the cylindrical collagen clot (**a**) changed upon transfer to a round (**b**) and after fluid drainage to a bell shape (**c**). The collagen clot was stabilized by embedding into liquid agar at 65 °C in a tissue mold (**d**). After solidification at room temperature, the agar block was dissected along the microtumor border and then laterally trimmed (**e**, **f**). Several microtumor containing segments were placed with the microtumor cutting edges facing downwards and in perpendicular position into new liquid agar (**g**). After solidification, the agar block was paraffin-embedded with the tumor cutting edges at the bottom (**h**) and serial sections were made

3.4.1 Embedding of the Microtumors in Agar

1. Microtumors in collagen type I are fixed with 4 % formalin for 24 h at 4 °C.
2. Subsequently, microtumor containing collagen clots are detached from the well wall with a watchmaker's forceps (*see* Fig. 3a) and transferred to a tissue paper to drain off excess formalin (*see* Fig. 3b, c). The shape of the collagen clot changes upon transfer from a cylindrical to a round shape (*see* Fig. 3b) and upon fluid removal from a round to a bell shape (*see* Fig. 3c).
3. When there is no further shrinkage anymore, the collagen clot is transferred into a metal tissue mold heated on a hot plate filled with 2 % liquefied agar (agar select, Sigma, Zwijndrecht, Netherlands) at 65 °C (*see* Fig. 3d).
4. After solidification at room temperature (RT), the microtumor containing agar block is removed from the tissue mold with a small spatula. The agar block is placed on a dark background so that the microtumor is visible as white nodule under a stereo dissecting microscope (Leica MZ 7.5, Rijswijk, The Netherlands).
5. The agar block is dissected along the microtumor border and laterally trimmed with a scalpel (*see* Fig. 3e, f).
6. The microtumor containing segment is flipped into upright position and up to three agar segments with microtumors treated under the same condition are embedded into new liquid 2 % agar at 65 °C in a perpendicular way and with the microtumor cutting edge to the bottom (*see* Fig. 3g).

3.4.2 Embedding of the Microtumors in Paraffin

After solidification at RT, the agar block is removed from the tissue mold, placed into a tissue cassette, processed in a tissue processor and embedded in paraffin with the microtumor cutting edge on the bottom (*see* Fig. 3h). This ensures that serial sectioning starts directly at the microtumor border.

3.5 Histological and Immunohistochemical Analysis of Microtumors

3.5.1 Histological Analysis of Microtumors

Paraffin embedded material is sectioned at 4 µm with 100 µm interval. Sections are stained with hematoxylin and eosin (H and E). Microtumors were generated from the tumor cell lines BB65-RCC, MDA-MB 231, 518A2, and MCF-7. Tumor cells of all cell lines in monolayer cultures had a spindle shaped morphology (except MCF7, which had an epitheloid morphology) (data not shown). Microtumors in culture were clearly visible by light microscopy (*see* Fig. 4a). Histology revealed compact 3D microtumors with distinct morphologies already on day 3 of culture (*see* Fig. 4b,c).

3.5.2 Immunohistochemical Analysis of Microtumors

1. Deparaffinized and rehydrated slides are not pretreated (for S100 stain) or pretreated with microwave for 20 min in EDTA 1 mM, pH 8.0 (CD8) or with Tris–EDTA buffer, pH 9.0 (all other antibodies).

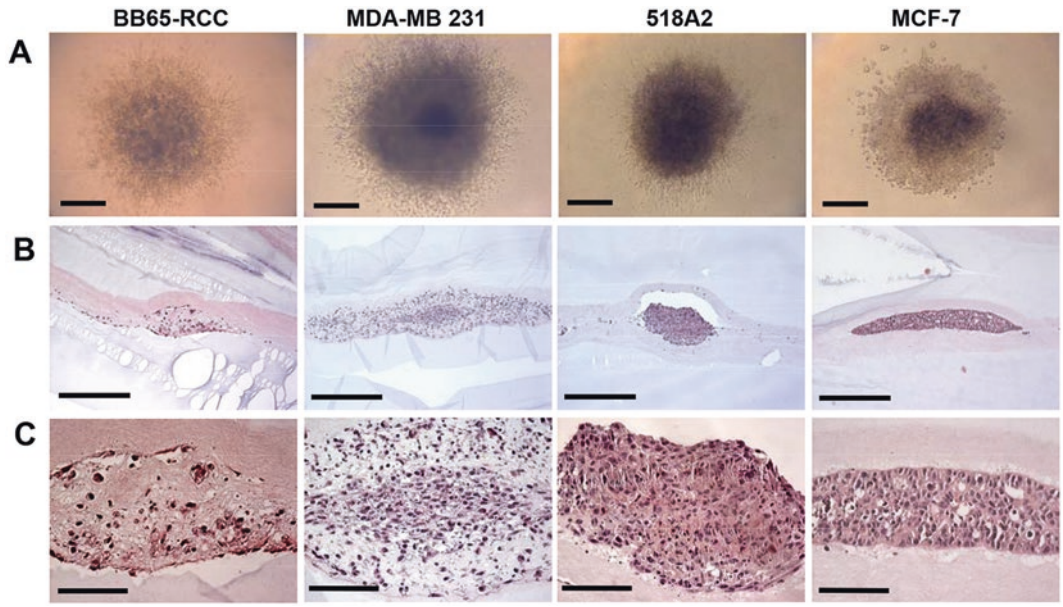

Fig. 4 Microtumors are already 3D on day 3 of in vitro culture. Depicted are microtumors derived from the cell lines BB65-RCC, MDA-MB 231, 518A2, and MCF-7 after 3 days of in vitro culture. (**a**) microtumor in culture; (**b, c**) H and E stainings of central longitudinal cross sections of microtumors; bar 500 μm (**a, b**) or 100 μm (**c**)

2. After washing 3× with 1 % Tween in PBS and incubation with 0.5 % Protifar (Protifar Nutricia Cuji, The Netherlands) blocking solution in 1 % Tween in PBS for 30 min, slides are stained overnight at 4 °C: with antibodies against CD8 (clone 4B11, dilution 1/20, Novocastra, UK), pankeratin (clone AE1/AE3, dilution 1/200, Neomarkers, CA), EMA (clone E29, dilution 1/500, DAKO, Haverlee, Belgium), Vimentin (clone V9, 1/50, DAKO), CD10 (clone 5bc6, dilution 1/20, Monosan, Uden, The Netherlands), gp100 (clone HMB45, dilution 1/100, DAKO), Melan A (clone A103, dilution 1/200, Neomarkers), Estrogen receptor (clone 1D5, dilution 1/10, DAKO), S100 (1/400, DAKO), Ki-67 (clone MIB-1, dilution 1:100, DAKO).
3. After washing 3× with 1 % Tween in PBS, primary antibodies are detected with DAKO Envision Peroxidase/DAB kit according to the instructions of the manufacturer (DAKO, Heverlee, The Netherlands). Sections are counterstained with Mayer hematoxylin.

3.5.3 *Application*

1. Comparison of microtumors on day 7 of in vitro culture with primary clinical tumors.

   The validity of the generated microtumors as surrogates for clinical cancers was determined by histological and immunohistochemical analysis on day 7. Microtumors strongly resembled histopathological (*see* Fig. 5b) and immunohistochemical features

(*see* Fig. 5c) of the analogous clinical cancers from which the tumor cell lines were derived (*see* Fig. 5a). The tumor cell line BB65-RCC was derived from a primary human kidney tumor defined as a clear cell carcinoma, grade II–III [24]. BB65-RCC microtumors (*see* Fig. 5b) showed the typical morphology of clear cells similar to a primary intermediately differentiated renal clear cell carcinoma (*see* Fig. 5a). BB65-RCC microtumors stained characteristically for CD10, vimentin and reticulin fibers (*see* Fig. 5c), which was compatible with a renal clear cell carcinoma [28]. Gomorri silver staining of reticulin was performed as described [29]. The tumor cell line MDA-MB 231 was derived from the pleural effusion of a patient with a low differentiated adenocarcinoma of the breast [30]. MDA-MB 231 microtumors (*see* Fig. 5b) contained spindle formed cells without orientation similar to a primary breast spindle cell carcinoma (*see* Fig. 5a). Immunohistochemistry revealed - like in primary spindle cell carcinomas [31–33] the simultaneous presence of the epithelial markers keratin and EMA and the mesenchymal marker Vimentin (*see* Fig. 5c). Accordingly, MDA-MB 231 microtumors were also estrogen receptor negative (data not shown). Thus, MDA-MB 231 might be derived from a spindle cell component of the primary tumor. The tumor cell line 518A2 was derived from a metastasized melanoma [34]. 518A2 microtumors (*see* Fig. 5b) showed sheets of cells similar to a primary melanoma (*see* Fig. 5a). The positivity for only one (Melan A) of the three tested melanocytic antigens (Melan A, gp100, S100; *see* Fig. 5c) is in accordance with the strong heterogeneity of staining mostly observed in individual primary melanomas [8]. The tumor cell line MCF-7 was derived from the pleural effusion of a mammary adenocarcinoma with ductal structures [35]. MCF-7 microtumors showed acinus like structures similar to a primary ductal carcinoma of the breast (*see* Fig. 5a). Positivity for keratin and EMA (*see* Fig. 5c) and negativity for vimentin (data not shown) supported the epithelial origin of this tumor. Moreover, MCF-microtumors were positive for estrogen receptor (*see* Fig. 5c), which was compatible with ductal breast carcinoma [36]. Overall, microtumors originating from different cancer cell lines show tumor entity-specific features.

2. Microtumors contain distinct niches of growth.

   Ki-67 staining revealed in all microtumors a similarly heterogeneous growth pattern as in primary tumors with a most pronounced proliferative activity at the borders of all investigated tumors (*see* Fig. 6a). In order to find indications for central necrosis as in primary tumors in vivo, we prolonged the culture period of MDA-MB 231 and MCF-7 microtumors from 7 days to 14 days. Only MCF-7 microtumors showed a central necrosis (*see* Fig. 6b), while MDA-MB 231 microtumors did not contain necrotic areas (data not shown).

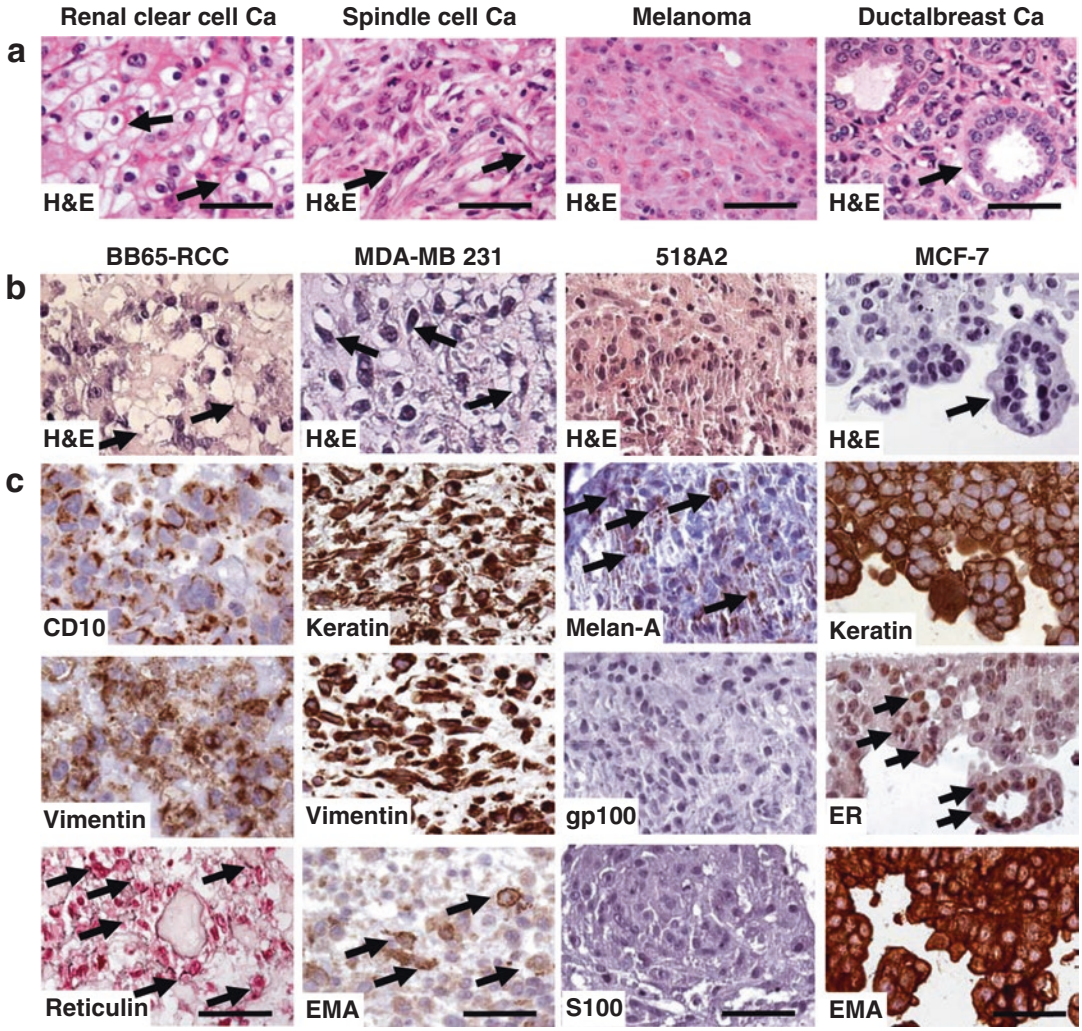

Fig. 5 Microtumors have morphological features of primary tumors. Histopathological features (H and E stainings) of primary tumors (**a**) are resembled by microtumors generated from various cancer cell lines after 7 days of in vitro growth (**b**). Important characteristics are: clear cells in renal cell carcinoma (**arrowheads**), spindle formed cells without orientation in spindle cell carcinoma (**arrowheads**), sheets of atypical melanocytes in melanoma (dense growth pattern) and acinus structures in ductal breast cancer (**arrowheads**). (**c**) The in vitro generated microtumors resembled characteristic immunohistochemical staining patterns of the corresponding clinical cancers. **Arrowheads** indicate: extracellular reticulin, cytoplasmatic staining for Melan-A, and nuclear staining for estrogen receptor (ER). Bar, 100 μm. (**a**, **b**) This research was originally published in part in **Blood**. Hambach, L., M. Vermeij, A. Buser, Z. Aghai, K. T. van der, and E. Goulmy. Targeting a single mismatched minor histocompatibility antigen with tumor-restricted expression eradicates human solid tumors. **Blood.** 2008; 112:1844–1852. © The American Society of Hematology

3. Analysis of tumor infiltration and tumor destruction by mHag CTLs.

   Microtumors were generated from BB65-RCC, MDA-MB-231, 518A2, and MCF-7 tumor cells on day 0 and HA-1 CTLs, H-Y CTLs or medium only were added on day 1. On day 3,

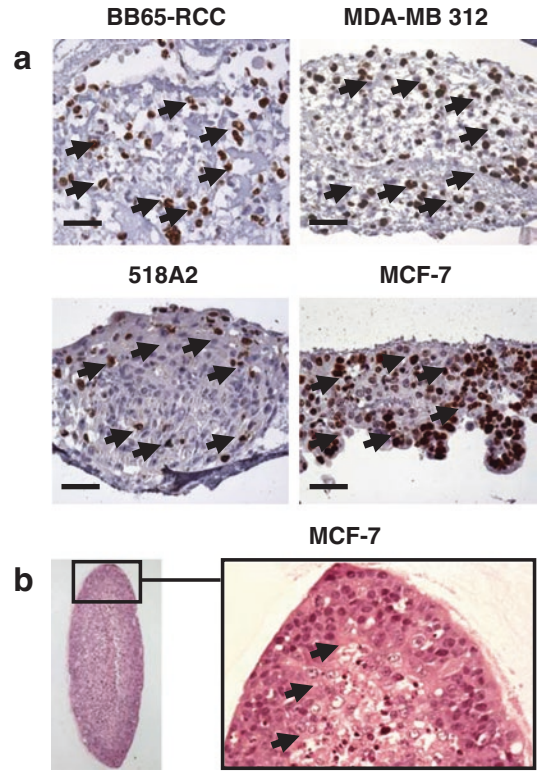

Fig. 6 Growth activity and central necrosis in vitro and tumor morphology in vivo. (**a**) Ki67 stainings on BB65-RCC, MDA-MB 231, 518A2, and MCF-7 microtumors on day 7 show heterogeneous growth activities throughout the tumors, most pronounced in the tumor periphery. **Arrowheads** indicate Ki67 positive cells; bar, 50 μm. (**b**) MCF-7 microtumors on day 14 are centrally necrotic. bar, 250 μm; insert: bar, 50 μm. **Arrowheads** indicate the transition from viable to necrotic areas

3D microtumors were fixed with paraformaldehyde and subjected to serial sections. CD8 immunohistochemistry on the serial sections revealed microtumor infiltration by HA-1 and H-Y CTLs both in microtumors positive and negative for the relevant antigen (*see* Fig. 7). Microtumor destruction on day 3 by HA-1 and H-Y CTLs was specific for the presence of the relevant antigen on the microtumors (*see* Fig. 7).

3.6 Conclusion

The presented in vitro culture system provides an unlimited source of experimental solid tumors of different entities and with defined properties, such as the presence or absence of the tumor target molecules. Since the 3D tumors in our culture system are not vascularized, the described 3D tumors can serve as models for avascular tumor stages or micrometastases. The human tumors engineered in 3D scaffolds allow monitoring of multiple parameters in response to treatment. Thereby, these 3D tumors can provide an integrated view on the mechanisms and the antitumor efficacy of cellular immunotherapy in situ.

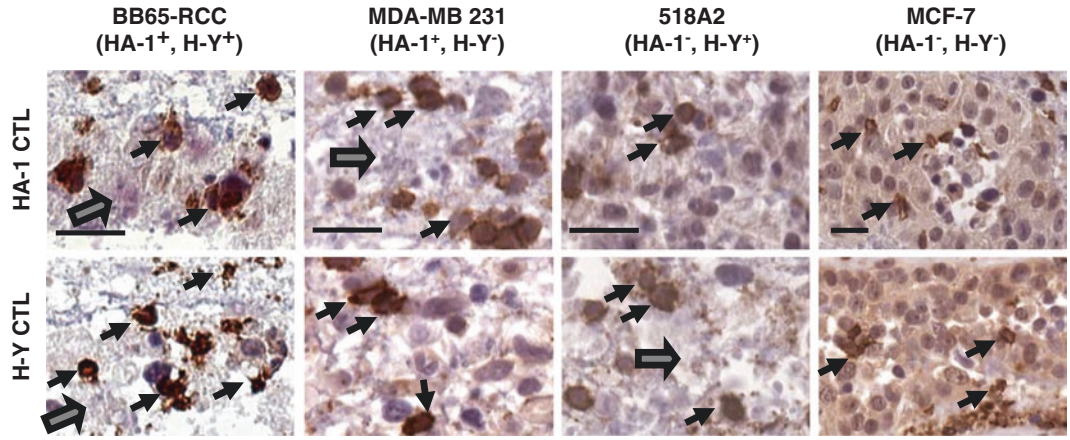

Fig. 7 Tumor antigen specific CTLs infiltrate and eradicate microtumors. The different tumor cell lines show a differential expression of the mHags HA-1 and H-Y. Microtumors generated from these tumor cell lines were coincubated with HA-1 and H-Y CTLs. After 3 days, CD8 staining on serial sections of these microtumors revealed that HA-1 CTLs (**upper row**) and H-Y CTLs (**lower row**) had infiltrated into the microtumors (***closed* arrowheads**: CD8+ CTLs). Interaction of HA-1 CTLs and H-Y CTLs with microtumors expressing the specific mHags induced tumor cell killing and cell debris (**open arrowheads**); bar, 50 μm

4 Notes

1. *Cancer cell lines.* The breast cancer cell lines MCF-7 [35] and MDA-MB-231 [30] (American Type Culture Collection, Rockville, USA), the melanoma cell line 518A2 [34] (kindly provided by Dr. P. Schrier, Leiden University Medical Center, The Netherlands) and the RCC cell line BB65-RCC (provided by Dr. B. Van den Eynde, Ludwig Institute for Cancer Research, Brussels, Belgium) were cultured in 5 % fetal calf serum (FCS) in Iscove's modified Dulbecco's medium (IMDM, Invitrogen Life Technologies, Breda, The Netherlands). All cell lines were genomically typed for the minor histocompatibility antigens (mHags) HA-1 and H-Y by allele specific PCR [37] and analyzed for HA-1 mRNA expression as described [38]. Tumor cell lines were designated "HA-1^{+}", if positive for the immunogenic HA-1^{H} allele and for HA-1 mRNA. MHag expression was associated with antigen-specific tumor cell killing by HA-1 or H-Y specific CTLs (HA-1 or H-Y CTLs) in a standard 4 h $^{51Cr\text{-release}}$ assay [24, 38, 39].
2. *mHag HA-1 and H-Y specific CTLs.* MHag specific CTL lines were generated from peripheral blood mononuclear cells (PBMCs) of HLA-A2^{+}/HA-1^{-} healthy donors as described earlier [40] or were isolated from patients after allogeneic stem cell transplantation [41]. mHag CTL clones were recovered by

limiting dilution of the mHag CTL lines. Approval was obtained from the Leiden University Medical Centre review board and informed consent was provided according to the Declaration of Helsinki.

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ERRATUM TO

Efficient RNA Interference of Primary Leukemic Cells for Loss-of-Function Studies in Xenograft Mouse Models

B. Nicolis di Robilant and Maddalena Noviello

Attilio Bondanza and Monica Casucci (eds.), *Tumor Immunology: Methods and Protocols*, Methods in Molecular Biology, vol. 1393,
DOI 10.1007/978-1-4939-3338-9_11, © Springer Science+Business Media New York 2016

DOI 10.1007/978-1-4939-3338-9_16

The spelling of the [Benedetta Nicolis de Robilant] name was incorrect in Chapter, Table of Contents and in the list of Contributors.

The name should read: B. Nicolis di Robilant

The updated original online version for this chapter can be found at
DOI 10.1007/978-1-4939-3338-9_11

INDEX

Attilio Bondanza and Monica Casucci (eds.), *Tumor Immunology: Methods and Protocols*, Methods in Molecular Biology, vol. 1393, DOI 10.1007/978-1-4939-3338-9, © Springer Science+Business Media New York 2016

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