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Dual-promoter lentiviral system allows inducible expression of noxious proteins in macrophages

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Abstract

In-depth studies of innate immunity require efficient genetic manipulation of macrophages, which is especially difficult in primary macrophages. We have developed a lentiviral system for inducible gene expression both in macrophage cell lines and in primary macrophages. A transgenic mouse strain C3H.TgN(SRA-rtTA) that expresses reverse tetracycline transactivator (rtTA) under the control of macrophage-specific promoter, a modified human scavenger receptor A (SR-A) promoter was generated. For gene delivery, we constructed a dual-promoter lentiviral vector, in which expression of a "gene-of-interest" is driven by a doxycycline-inducible promoter and the expression of a selectable surface marker is driven by an independent constitutive promoter UBC. This vector is used for transduction of bone marrow-derived macrophage precursors. The transduced cells can be enriched to 95–99% purity using marker-specific monoclonal antibodies, expanded and differentiated into mature macrophages or myeloid dendritic cells. We also successfully used this approach for inducible protein expression in hard to transfect macrophage cell lines.

Because many proteins, which are expressed by activated or infected macrophages, possess cytotoxic, anti-proliferative or pro-apoptotic activities, generation of stable macrophage cell lines that constitutively express those proteins is impossible. Our method will be especially useful to study immunity-related macrophage proteins in their physiological context during macrophage activation or infection.

Keywords

Lentiviral vectors; dual-promoter; macrophages; tetracycline-inducible expression; magnetic cell sorting; *Ipr1* gene; interferon-activation; protein-protein interaction

1. Introduction

Macrophages are versatile cells that play diverse roles in host defenses, tissue homeostasis and remodeling under normal and pathologic conditions such as the onset, progression and

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resolution of various inflammatory and infectious diseases, as well as in the control of tumor progression (Ross and Auger, 2002; Sasmono and Hume, 2004; Henson and Hume, 2006). Macrophages perform both regulatory and effector functions by producing secreted proinflammatory and proangiogenic mediators, growth factors, antibacterial peptides, reactive oxygen and nitrogen species as well as via direct contact with target cells. Macrophages also ingest particles and live pathogens. They can either destroy the ingested pathogens or serve as reservoirs for their propagation promoting survival and dissemination of infectious agents, such as HIV and *Mycobacterium tuberculosis* among others. The ability to understand genetic control of macrophage function will certainly allow development of rational interventions to enhance beneficial and prevent disease-promoting macrophage activities.

Modern approaches to dissecting cellular functions broadly utilize expression of modified genes and reporter constructs in target cells. However, the delivery of recombinant genetic constructs into macrophages is difficult, which limits utilization of powerful molecular approaches to the studies of the macrophage biology. It is well known that the widely used transfection methods using synthetic carriers such as liposomes, lipoplexes or diethylaminoethyl (DEAE)-dextran, to deliver naked plasmid DNA, are inefficient in primary macrophages (Heider et al., 2000). Only a few monocyte or macrophage cell lines, such as THP-1 or RAW264.7, can be efficiently transfected by plasmid DNA. Electroporation has been used to transfer plasmid DNA into monocytic cell lines or macrophages with high efficiency (Weir and Meltzer, 1993; Hume et al., 2001). Usually, electroporation causes significant cell death and release of intracellular contents, which is known to induce macrophage responses (Krysko et al., 2006). Moreover, both transfection and electroporation with plasmid DNA containing unmethylated CpG (cytosine followed by guanine) dinucleotides may activate macrophages through Toll-like receptor (TLR)-9 to produce proinflammatory cytokines (Stacey et al., 1996; Sester et al., 1999; Jiang et al., 2006).Thus, the existing methods of plasmid delivery perturb the normal physiological status of the macrophage population and complicate interpretation of the experimental data. Recombinant vectors based on adenovirus (De et al., 1998; Foxwell et al., 1998; Heider et al., 2000) and lentivirus (Naldini et al., 1996; Corbeau et al., 1998; Schroers et al., 2000) have been used to deliver genetic constructs into macrophages much more efficiently. This includes attempts to express therapeutic genes in macrophages, and use of the transduced macrophages as a vehicle for adoptive immunotherapy (Burke et al., 2002).

The efficiency of transduction of primary macrophages is much lower as compared to standard cell lines, which requires selection of the transduced cells. A popular strategy for identification and enrichment of the virally transduced cells is based on bicistronic constructs, in which two genes, a gene-of-interest and a selectable marker, are encoded by the same transcript, and therefore, are expressed simultaneously. Thus, the cells expressing the gene-of-interest may be identified and positively selected. However, many genes related to innate immunity are expressed in activated or infected macrophages only in an inducible manner. These genes often possess cytotoxic, anti-proliferative or pro-apoptotic activities, which are directly related to their role in immunity to pathogens. This precludes stable long-term expression of those immune-related macrophage genes in dividing cells, such as hematopoietic stem cells, and makes genetic complementation tests in a context of experimental infection *in vivo* impossible.

Macrophages are the primary host cells of *M. tuberculosis* (MTB) as well as many other intracellular bacteria. Previously, we have mapped the *sst1* locus on mouse chromosome 1 (*s*upers*s*usceptibility to *t*uberculosis, 49–52 cM) that mediates host resistance to tuberculosis (Kramnik et al., 2000). A strong candidate gene *Ipr1* (*i*ntracellular *p*athogen *r*esistance 1) has been identified within the *sst1* locus using a positional cloning approach. This is an inducible protein which is expressed in interferon-activated and/or MTB-infected macrophages of the *sst1*-resistant (C57BL/6J), but not *sst1*-susceptible (C3HeB/FeJ) mice (Pan et al., 2005). To

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perform complementation tests and to study the molecular basis of Ipr1-mediated macrophage function(s), we attempted expression of the *Ipr1* gene in hematopoietic stem cells and bone marrow-derived macrophages of the *Ipr1*-negative C3HeB/FeJ mice using a standard bicistronic lentiviral vector, and failed, because overexpression of Ipr1 blocked cell division and increased apoptosis. Therefore, we have developed a system for efficient genetic manipulation of both macrophage cell lines and primary mature macrophages based on a set of dual-promoter lentiviral vectors. These enabled us to study the Ipr1 protein function in a physiologically-relevant context of activated and MTB-infected macrophages.

2. Materials and methods

2.1. Mice

C3HeB/FeJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). To make a transgene construct we used a pBlueScript KS (Stratagene, Cedar Creek, TX, USA) derived plasmid containing the reverse tetracycline transactivator (rtTA) and the SV40 polyadenylation signal (SV40pA). The *Xho*I-*Eco*RI DNA fragment of a modified human Scavenger Receptor A (SRA) promoter was derived from plasmid pAL1 (a generous gift of Dr Christopher Glass (Horvai et al., 1995)). This promoter fragment was inserted into the pBlueScript KS upstream of the rtTA using the same restriction sites. To generate the transgenic mouse strain C3H.TgN(SRA-rtTA), the above plasmid was digested with *Xho*I and *Not*I to isolate the 6777-bp SRA-rtTA-SV40pA transgene. The fragment was purified from agarose gel and introduced into fertilized C3HeB/FeJ oocytes by pronuclear injection. Offspring was genotyped by PCR with primers rtTA-4F (5'- CGC TAG ACG ATT TCG ATC TGG AC -3') and rtTA-4R (5'- TTC CAA GGG CAT CGG TAA ACA -3'). Transgene-bearing founder mice were backcrossed to the C3HeB/FeJ mice. The transcription of the transgene was confirmed by RT-PCR. Homozygote transgenic mice were generated by intercrossing transgene-positive animals and selection for the transgene homozygotes using quantitative PCR. The sequence of the SRA-rtTA-SV40pA transgene is available upon request. Mice were bred and maintained under specific-pathogen-free conditions in animal facility at the Harvard Medical School and given autoclaved chow and water *ad libitum*. All experiments were performed with the full knowledge and approval of the Standing Committee on Animals at Harvard Medical School.

2.2. Cell lines and BMDMs culture

Human renal epithelial cell line 293T, mouse fibroblast cell line NIH/3T3, mouse macrophage cell lines RAW264.7 and J774A.1 were cultured in DMEM/F12 (Mediatech, Inc., Herndon, VA, USA) containing 7.5% Tet-system approved fetal bovine serum (FBS, Clontech, Mountain View, CA, USA) and 10 mM Hepes buffer (Mediatech, Inc). Isolation of mouse bone marrow and culture of BMDMs were described previously (Pan et al., 2005). Briefly, mouse femurs and tibias were homogenized in DMEM (Mediatech, Inc) containing 2% FBS. Bone marrow cells were filtered through a 70-μm strainer (BD Biosciences, San Jose, CA, USA) and further purified on a gradient of NycoPrep A-1.077 (Axis-Shield Plc, Dundee, UK). Purified bone marrow cells were cultured in DMEM/F12 containing 10% FBS, 1 ng/mL recombinant mouse interleukin-3 (rmIL-3, R&D Systems, Minneapolis, MN, USA), and 10% L-929 cell conditioned medium as a source of colony stimulation factor-1 (CSF-1) for 3 days. Non-adherent cells were collected and expanded in the same medium in the ultra-low cluster plates (Corning, Acton, MA, USA) for additional 6–20 days with medium changed every 2 days. Appropriate number of cells were plated in tissue culture plastic ware in DMEM/F12 containing 10% FBS and 20% L-929 conditioned medium (w/o IL-3) to form a monolayer of macrophages.

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2.3. Construction of dual-promoter lentiviral vectors

The pHAGE backbone lentiviral vector used in the experiments was an optimized self-inactivating nonreplicative vector derived from the pHR'CMV-lacZ vector (Naldini et al., 1996). The original pHAGE vector will be described in detail elsewhere (Balazs et al., in preparation). Briefly, the original pHR'CMV-lacZ from Naldini *et al.* was modified to create the pHRST vector by adding a polypurine tract between the 3' end of the *env* sequence and the 5' end of the CMV promoter. In addition, the LacZ gene was changed to eGFP and a Woodchuck Hepatitis virus post transcriptional regulatory element (WPRE) was cloned downstream of eGFP at the *Kpn*I site. In order to remove extra sequences and alleviate cloning strategies the pHRST vector was further modified to create the pHAGE vector, by first moving the viral backbone from 5' LTR to 3' flanking region into a minimal pUC backbone containing an SV40 origin of replication. Subsequent cloning re-created the central polypurine tract and added a unique *Spe*I cloning site to the 5'end and *Not*I site at the 3' end of the CMV promoter region to simplify swapping of the internal promoter, and removed a large amount of the exogenous 3' flanking sequence that remained from the original viral integrant. This pHAGE vector served as the basis for the creation of all other pHAGE derivatives. For inducible gene expression, the CMV promoter was replaced by the TRE promoter that contains seven copies of the 42-bp Tet operator sequence and the minimal CMV promoter and was obtained from pLP-RevTRE vector (Clontech) by digestion with XhoI and EcoRI. The truncated human cell surface selection markers LNGFR and CD4 were cloned from plasmid pMACS-LNGFR and pMACS4.1 (Miltenyi Biotec, Auburn, CA, USA) by PCR using primers LNGFR-NdeI 5'-TTT CAT ATG GGG GCA GGT GCC ACC GGC CGC GCC AT-3', LNGFR-ClaI 5'-AAA ATC GAT CTA TCA CCT CTT GAA GGC TAT GTA GGC CAC AAG ACC CAC AAC CAC AGC A-3', and CD4-NdeI 5'-TTT CAT ATG AAC CGG GGA GTC CCT TTT AGG CAC TTG CTT C-3', CD4-ClaI 5'-AAA ATC GAT CTA TCA GTG CCG GCA CCT GAC ACA GAA GAA GAT G-3' respectively, followed by digestion with *Nde*I and *Cla*I, and inserted in the same restriction sites downstream of the UBC promoter. The rtTA was cloned from plasmid pTet-ON (Clontech) by PCR using primers rtTA-NotI 5'-TTT GCG GCC GCC ATG TCT AGA TTA GAT AAA AGT AAA GTG ATT-3' and rtTA-BamHI 5'-AAG GAT CCT TAC TAC CCA CCG TAC TCG TCA ATT CCA AGG GCA TCG GTA AAC-3'. The Ipr1 cDNA was cloned by PCR from lung of C3H.B6-sst1 mice as described previously (Pan et al., 2005). Sequences of the pHAGE constructs are available upon request.

2.4. Lentivirus production

Recombinant lentiviruses were produced by a five-plasmid transfection procedure (Mostoslavsky et al., 2005). Briefly, 293T cells were co-transfected using Trans IT-293 liposome reagent (Mirus, Madison, WI, USA) and the pHAGE or pHRST backbone lentiviral vector together with four expression vectors encoding the packaging proteins Gag-Pol, Rev, Tat and the G-protein of the vesicular stomatitis virus (VSVG). The virus supernatants were collected 24, 36, 48 and 60 hours after transfection, pooled and filtered through 0.45-μm filters. If necessary, the viral supernatants were concentrated to about 1/100 volume by ultracentrifugation at 15,000 g for 3 hours. Viral titers were determined by the percentage of LNGFR+ or CD4+ 293T cells transduced with serial dilutions of lentivirus supernatants.

2.5. Lentiviral transduction

Transduction of cell lines was performed in 6-well plate in 4 mL volume per well. Lentivirus supernatants and 10 μg/mL Polybrene were added to 1×10^6 target cells at m.o.i.of 10 (or 5 for each virus in a co-transduction) in DMEM/F12 medium containing 2% FBS, 10 mM Hepes buffer. Transduction of BMDMs was performed in the ultra-low cluster 6-well plate (Corning) at m.o.i of 10 and in DMEM/F12 medium containing 10% FBS, 10% L-929 conditioned medium, and 1 ng/mL rmIL-3. To achieve higher efficiency of transduction, plates were spun

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at 1,000 g for 1 hour at room temperature (Kotani et al., 1994). Cells were then immediately washed twice with medium and cultured for at least 24 hours before FACS analysis or magnetic selection.

2.6. Magnetic selection and FACS analysis

Transduced cells were enriched using the MACSelect systems (Miltenyi Biotec) according to manufacturer's instruction. Briefly, adherent cells were collected using cell scrapers after being incubated in PBS buffer (Mediatech, Inc) containing 1% FBS and 5 mM EDTA for 20 min. 4×10^7 cells were resuspended in 4 mL PBS buffer containing 0.5% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) and 5 mM EDTA. Cells were incubated with 4 μg low-endotoxin rat anti-mouse CD16/CD32 (FcγRIII/II) (AbD Serotec, Raleigh, USA) on ice for 15 min, and then with 80 μL anti-LNGFR or anti-CD4 Microbeads (Miltenyi Biotec) for additional 15 min on ice. Cells were passed through a pre-separation filter to remove clumps and loaded on a pre-equilibrated MASC LS column in a magnetic holder, followed by washing 4 times with 3 mL PBS buffer containing 0.5% BSA and 5 mM EDTA. The columns were removed from the magnetic holder and the antibody-bound cells were flashed out with 5 mL cell medium. Either immediately or 2 days culturing after magnetic selection, cells were pre-blocked with rat anti-mouse CD16/CD32 and then labeled with Allophycocyanin (APC)-conjugated anti-LNGFR or PE-conjugated anti-CD4 (Miltenyi Biotec) and analyzed using a FACScan flow cytometry (Becton-Dickinson, San Jose, CA, USA).

2.7. RNA isolation and RT-PCR

Total RNA was isolated by using TRIzol (Invitrogen, Carlsbad, CA, USA) and further cleaned up by using RNeasy Mini kit (Qiagen, Valencia, CA, USA) with DNase I digestion. 2 μg total RNA was reverse transcripted by using oligo-dT primers and SuperScript II (Invitrogen). One twenty-fifth of each product was amplified by 3-primer PCR with primers Ifi75-9F 5'- AGA CAT TAA GAC ATC TGG AGC AGA AAG-3', Ifi75-9R 5'-GCA CAT ATC AGG TCA GGA GTT CAT C-3', and UBC-1R 5'-CGG GCG GAA GGA TCA GGA-3'.

2.8. Cell cloning by limiting dilution

Transduced J774A.1 cells were seeded in 96-well plates at a density of approximately 0.5 cell per well, so that 20–30% of the wells had clones grown up. About 400 clones were picked and transferred into duplicate plates. One plate was left untreated, while doxycycline was added to another plate. Cells were examined under the fluorescent microscope for the nuclear eGFP expression 24 and 48 hours later. Clones with undetectable basal level of the eGFP-Ipr1 fusion protein expression and doxycycline-induced eGFP-Ipr1 expression were selected and further tested by flow cytometry.

2.9. Cell lysis, nuclear extraction, antibodies and immunoblotting

2×10^7 cells were washed twice with PBS, and scraped in 1.2 mL RIPA buffer (50 mM TrisCl pH7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% Sodium deoxycholate, 0.1% SDS) to prepare whole cell lysate. Alternatively, cells were scraped in 1.2 mL hypotonic buffer (10 mM HEPES pH7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.2% Nonidet P-40). Cytosolic lysate and nuclei were separated by centrifugation at 15,000 ×g for 3 min. Nuclei were washed with 0.6 mL hypotonic buffer and then extracted with 200 μL nuclear extraction buffer (20 mM HEPES pH7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 25% Glycerol) on a rotary mixer at 4°C for 2 hours. All buffers were supplemented with 1% protease inhibitor cocktail and phosphatase inhibitor cocktails I and II (Sigma-Aldrich). Protein concentration was measured by using BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). 20 μg protein was purified by using PAGEprep kit (Pierce Biotechnology) and separated by 10% SDS-PAGE. Proteins were transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA) and immunoblotted. All membranes were

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developed with SuperSignal chemiluminescent substrate (Pierce Biotechnology). The mouse anti-FLAG M2 monoclonal antibody was purchased from Sigma-Aldrich, and the rat anti-Hsc70 monoclonal antibody was from Assay Designs, Inc (Ann Arbor, MI, USA). The Ipr1-specific rabbit anti-serium was generated by using the service of Covance Research Products, Inc (Denver, CO, USA).

2.10. Immunoprecipitation and Nano-LC tandem mass spectrometry

Immunoprecipitation was performed by using μMACS GFP-tagged protein isolation kit (Miltenyi Biotec). Briefly, 200 μg nuclear extract was diluted with 9 volumes of IP dilution buffer (50 mM TrisCl pH7.4, 150 mM NaCl, 1% Triton X-100) and immunoprecipitated with 50 μL anti-GFP Microbeads according to manufacturer's manual. The eluate were separated by 10% SDS-PAGE and analyzed by either immunoblotting or Coomassie Blue R-250 staining. The Coomassie stained bands were excised from the gels and digested with trypsin. Protein digests were desalted on a C18 solid phase extraction ("trapping") column, eluted and separated on a Nano-LC revered-phase self packed fused silica column (75 μm i.d. × 15 mm; HCPF) with linear gradient of eluent. The eluent was introduced into the LCQ Deca XP Plus mass spectrometer (MS) by nanoelectrospray. The MS operation and MS data processing was performed by investigators at the Harvard School of Public Health Core Proteomics Facility (HCPF).

3. Results

3.1. Failure to constitutively express the Ipr1 gene in mouse bone marrow-derived macrophages using a standard bicistronic lentiviral vector

Initialy, to express the *Ipr1* gene in bone marrow-derived macrophages (BMDMs) of the Ipr1-negative C3HeB/FeJ mice, we used the bicistronic lentiviral vector, in which the co-expression of the *Ipr1* gene and selectable marker eGFP was driven by a constitutive CMV promoter (pHRST-CMV.Ipr1-IRES.eGFP.W.MAR, see Fig. 1) (Mostoslavsky et al., 2005). We found that less than 10% of the cells could be transduced. Attempts to expand the Ipr1 expressing BMDMs after enrichment using FACS sorting were unsuccessful because GFP-positive (Ipr1-expressing) cells rapidly declined after sorting (data not shown). Taken together, our failure to constitutively express the functional Ipr1 protein in macrophages suggested that it might have deleterious effects on proliferation or survival of these cells both *in vivo* and *in vitro*. This is consistent with the previous report that overexpression of the human homologue of *Ipr1*, *SP110b/IFI41*, was "toxic" for a cell line (Kadereit et al., 1993). Therefore, we wanted to develop a gene delivery system, which would overcome difficulties associated with low transducibility of macrophages, avoid effects of foreign DNA and viral particles on macrophage activation status during experiments and allow inducible expression of potentially "toxic" products. Ideally, this system should be applicable to studies of macrophage function *in vitro* and *in vivo*.

3.2. Dual promoter lentiviral vectors do allow separate control of the expression of both the gene-of-interest and a selectable marker in macrophages using constitutive and inducible promoters

The backbone lentiviral vectors pHRST and pHAGE used in our experiments are derivatives of the self-inactivating nonreplicative vector pHR'CMV-lacZ. They both have an intact HIV-1-derived 5'-long terminal repeat (5'-LTR) and a 3'-LTR deleted of U3 region (Naldini et al., 1996). Both backbone vectors also contain several *cis*-acting elements, which enhance the transduction efficiency or gene expression (Fig. 1). The Rev-responsive element (RRE) is the binding site of the HIV-1 Rev protein, which helps exporting the viral RNA genomes to the cytoplasm of virus-producing cells and thus increases the production of large-size recombinant lentiviruses (Pollard and Malim, 1998). The central polypurine tract (cPPT) is a *cis*-acting

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determinant for the nuclear import of HIV pre-integration complex, which is necessary for transduction of non-dividing cells (Follenzi et al., 2000; Zennou et al., 2000). The woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) enhances gene expression by facilitating the nuclear export of RNA transcripts (Zufferey et al., 1999). In addition, the pHRST vector contains 7 copies of the human interferon-β gene matrix attachment region (MAR) which has been shown to increase gene expression (Murray et al., 2000).

3.2.1. Constitutive expression—To achieve separate control of expression of a selectable marker and a gene-of-interest, we developed dual promoter lentiviral vectors (Fig. 1). The upstream promoters, either a constitutive CMV promoter or an inducible promoter composed of tetracycline response element (TRE) and a minimal promoter, were used to control the expression of a gene-of-interest. A constitutive ubiquitin C (UBC) promoter was placed downstream and used to control expression of one of two selectable surface markers, either the human low-affinity nerve growth factor receptor (LNGFR) or human CD4. The cytoplasmic domains of both selectable markers were deleted to eliminate signaling through these molecules. Expression of the selectable markers on the cell surface allowed identification of the transduced cells by FACS using specific antibodies as well as their enrichment using magnetic cell sorting (MACS) (Gaines and Wojchowski, 1999) (Fig. 2D and 4B).

To determine whether the dual promoter lentiviral vectors can simultaneously co-express the gene-of-interest and a selectable marker, we transduced the mouse fibroblast NIH/3T3 cell line with a lentiviral vector encoding the *Ipr1* or FLAG-tagged *Ipr1* (FLAG-Ipr1) under the control of the CMV promoter and the cell surface marker LNGFR under the control of the UBC promoter (pHAGE-CMV.FLAG-Ipr1.UBC.dLNGFR.W or pHAGE-CMV.FLAG-Ipr1.UBC.dLNGFR.W, see Fig. 1). Seven days post transduction, about 90% of the cells expressed the cell surface marker LNGFR (Fig. 2B). Meanwhile, strong expression of the Ipr1 or FLAG-Ipr1 was detected in the nuclear extracts using immunoblot with either FLAG- or Ipr1-specific antibodies (Fig. 2A). No expression of the endogenous Ipr1 was detectable in the control sample (mock transduction). Next, we used the same lentiviral construct to transduce a mouse macrophage cell line J774A.1. However, in this macrophage cell line, the expression level of the FLAG-Ipr1 protein rapidly declined as compared to that in NIH/3T3 fibroblasts. Meanwhile, the transcript level of the vector-encoded FLAG-Ipr1 (Fig. 2C), as well as the proportion of the LNGFR-expressing (transduced) cells, which represented about 97% of the population after magnetic sorting (Fig. 2D) remained stable. Similar results were observed using another mouse macrophage cell line RAW264.7 (data not shown).

When the eGFP was substituted for the FLAG-Ipr1 in these constructs, we could achieve high-level expression of both the eGFP and LNGFR proteins in J774A.1 macrophage cell line (data not shown), thus, indicating that there was no interference between the promoters of the dual promoter lentiviral vectors neither in fibroblast nor in macrophage cell lines. Perhaps, post-transcriptional regulation was responsible for the dramatic decrease of the Ipr1 protein expression over time specifically in macrophage, but not fibroblast cell lines. Thus, to study the Ipr1 protein in macrophages, we developed an inducible system.

3.2.2. Inducible Expression of eGFP—Two lentiviral vectors were constructed for inducible gene expression in cell lines. The first vector contained the reverse tetracycline transactivator (rtTA) driven by the constitutive CMV promoter and the CD4 under the control of the UBC promoter (pHAGE-CMV.rtTA.UBC.dCD4.W, Fig. 2). The second vector was used to deliver gene-of-interest (eGFP) under the control of tetracycline inducible promoter (TRE) and a selectable surface marker LNGFR driven by the UBC promoter (pHAGE-TRE.eGFP.UBC.dLNGFR.W, Fig. 2). The cell lines were co-transduced with the two lentiviral constructs at m.o.i. of 5. The efficiency of the transduction was evaluated by double staining using the LNGFR- and CD4-specific antibodies 24 hours post transduction (Fig. 3A, upper

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panels). A significant proportion of double-positive cells, i.e. transduced with both lentiviral constructs, were observed after either simultaneous or sequential co-transduction. High efficiency of co-transduction with the vectors was observed in 293T, RAW264.7 and J774A.1 cell lines.

Next, we assessed the doxycycline-inducible expression of the TRE-driven eGFP in this system using FACS analysis. The levels of GFP were determined 24 hours after adding doxycycline to the cell cultures. As shown in Fig. 3A (lower panels), a proportion of CD4 and LNGFR double-positive cells did express GFP. However, low basal level expression of GFP was also detected in some of the CD4-negative, LNGFR-positive cells, i.e. in the absence of rtTA. In addition, the inducibility in 293T cell line was higher than in the macrophage cell lines RAW264.7 and J774A.1 (approximately 85% vs. 14% in CD4 and LNGFR double-positive cells, as shown in Fig. 3A, lower panels).

Thus, the dual lentiviral vector-based system worked in cell lines in principle. However, we observed some difference of the inducible protein expression in different cell types, with less efficient expression in macrophage cell lines as compared to the 293T cells.

3.2.3. Inducible Expression of the eGFP-Ipr1 Fusion Protein in J774 macrophage cell line and clones—To express the eGFP-Ipr1 fusion protein in macrophage cell line, two lentiviral vectors pHAGE-CMV.rtTA.UBC.dCD4.W and pHAGE-TRE.eGFP-Ipr1.UBC.dLNGFR.W, encoding the rtTA and eGFP-Ipr1 respectively, were co-transduced into J774A.1 cells. About 15% of the double-transduced LNGFR- and hCD4-positive cells became eGFP-positive after the transgene expression was induced with doxycycline. However, the basal and induced expression of the eGFP-Ipr1 transgene varied at individual cell level, which might be explained by different copy numbers and chromosomal integration sites of the vectors.

As discussed above, constitutive expression of the functional Ipr1 protein may negatively impacts macrophage cell growth and in a mixed population may lead to positive selection of cells that express aberrant non-functional forms of the Ipr1 protein. Therefore, to obtain cells in which the eGFP-Ipr1 protein expression is tightly regulated, we cloned the co-transduced J774A.1 cells by limiting dilution. Approximately 400 resulting clones were obtained, split and tested for the eGFP-Ipr1 expression in the presence and absence of doxycycline by microscopy. About 25 clones were selected for further testing using FACS analysis and titration of doxycycline, from which a final set of clones was selected based on undetectable GFP-Ipr1 levels without induction and high levels of inducible expression with a low dose of doxycycline. A typical clone is shown in Fig. 3C.

We observed that even in those selected clones the level of eGFP-Ipr1 expression decreased within 4 days as detected both by FACS (Fig. 3C) and immunoblot using the Ipr1-specific antibodies (Fig. 3B). This decreasing of eGFP-Ipr1 expression was not due to the deactivation of doxycycline since we refreshed the doxycycline-containing medium every day. Also, we observed no decrease of the eGFP protein expression using the same vector encoding eGFP alone as a control. These results indicated that the prolonged expression of the Ipr1 protein in macrophage cell lines was inhibited, most likely, at post-transcriptional level, although the mechanism of this inhibition remains unknown. Nevertheless, the inducible system provided a window between the induction with doxycycline and the Ipr1protein degradation that allowed us to study this protein's function in macrophage cell lines within 2 days post induction.

3.2.4. Inducible Expression of the eGFP-Ipr1 Fusion Gene in primary bone marrow-derived macrophages—For inducible expression of genes in non-transformed primary mouse macrophages, we generated a transgenic mouse strain C3H.TgN (SRA-rtTA)

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that expressed the rtTA protein under the control of a macrophage-specific modified human scavenger receptor-A (SRA) promoter. The SRA promoter controlled transgene expression in CSF-1 differentiated macrophages, but not in bone marrow progenitors (Horvai et al., 1995). The transgene was introduced directly in C3HeB/FeJ mice, which do not express the Ipr1 gene (Pan et al., 2005). To express the eGFP-Ipr1 fusion protein we have developed the following procedure: first, the bone marrow-derived macrophage progenitors were enriched and transduced with lentiviral constructs, next, the transduced cells were positively selected using magnetic cell sorting, expanded and differentiated into macrophages and then, treated with doxycycline to induce the eGFP-Ipr1 gene expression.

To enrich for macrophage progenitors, the C3H.TgN (SRA-rtTA) bone marrow cells were cultured in medium containing recombinant mouse interleukin-3 (rmIL-3) and colony stimulating factor-1 (CSF-1) for three days and depleted of more mature adherent cells. The non-adherent cells were transduced with the lentiviral vectors pHAGE-TRE.eGFP-Ipr1.UBC.dLNGFR.W or pHAGE-TRE.eGFP.UBC.dLNGFR.W, which encoded the GFP-Ipr1 fusion protein or eGFP under the control of doxycycline-inducible promoter (TRE). At m.o.i. of 10, the transduced cells typically represented 5–10% of the population as detected by FACS using LNFGR-specific antibodies (Fig. 4A). The transduced cells were enriched using magnetic beads coated with LNGFR-specific antibodies to 80% after a single round of positive selection (Fig. 4B) and to more than 95% after two rounds of selection (data not shown). The positively selected cells were expanded for 20 days and then differentiated into mature macrophages using the CSF-1 - containing media (Fig. 4C). The proportion of the LNGFR-positive (transduced) cells remained constant in the absence of doxycycline during the whole period of culture. Meanwhile, rapid loss of the LNGFR-positive cells was observed when bone marrow cells were transduced with the lentiviral vector that expressed the *Ipr1* gene constitutively (pHAGE-CMV.Ipr1.UBC.dLNGFP.W) (Fig. 4C). Hence, similar to macrophage cell lines, overexpression of the Ipr1 protein in macrophages progenitors might inhibit their growth. After differentiation into mature macrophages, the expression of both the eGFP-Ipr1 and eGFP proteins was induced and detected by fluorescent microscopy. The eGFP protein was observed mostly in cytoplasm, while the eGFP-Ipr1 fusion protein localized to the nuclei (Fig. 4D).

Thus we demonstrated that using our system, a potentially detrimental gene could be introduced into macrophages, kept silent during the selection and expansion steps, and induced for experimental analysis. We use this approach to study the Ipr1 protein interactions in activated macrophages.

3.3. Identification of proteins that interact with Ipr1 in Activated Macrophages

To identify nuclear proteins that interact with Ipr1 in activated macrophages, we used a clone of J774A.1 cells (clone 21), which was obtained after transduction with lentiviral vectors pHAGE-CMV.rtTA.UBC.dCD4.W and pHAGE-TRE.eGFP-Ipr1.UBC.dLNGFR.W, as described above (Fig. 3). The expression of the GFP-Ipr1 fusion protein was induced with doxycycline (1 μg/ml) for 24 hours. To induce macrophage activation rIFN-γ was added for the last 16 hours of culture to a final concentration of 100 U/mL. Using Western blot analysis we observed that both the endogenous Ipr1 and the GFP-Ipr1 fusion proteins localize to the nucleus of interferon-activated and non-activated macrophages. Therefore, nuclear extracts were isolated from those cells and immunoprecipitated with GFP-specific antibodies coupled to magnetic beads. The precipitated proteins were separated on SDS-PAGE gel and visualized by staining with Coomassie Brilliant Blue. Individual bands were cut from the gel, digested with trypsin and analyzed using mass spectrometry (see Methods for details). Interestingly more Ipr1-interacting proteins were detected after macrophage activation with IFN-γ, as compared to naïve macrophages (Fig. 5A).

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One of the most prominent proteins that interacted with Ipr1 was identified by mass spectroscopy as heat shock cognate protein 70 (Hsc70) (Fig. 5A, band IV). Using co-immunoprecipitation with GFP-specific antibodies and immunoblot analysis with Hsc70-specific antibody, we confirmed the GFP-Ipr1 and Hsc70 protein interactions and demonstrated that this interaction occurred specifically in the nuclei of interferon-activated, but not naïve macrophages (Fig. 5B, lower panel). Using immunoblot of nuclear extracts with Ipr1 specific antibody we detected accumulation of the GFP-Ipr1 fusion protein (Fig. 5C), as well as endogenous Ipr1 (not shown), in the macrophage nuclei upon interferon treatment. Accumulation of the eGFP-Ipr1 fusion protein in clone 21 cells treated with doxycyline and IFN-γ was also demonstrated using FACS analysis: the IFN-γ activated macrophages showed higer percentage of GFP-positive cells and, notably, 7-fold higher main fluorescence intensity as compared to doxycycline-treated, but not activated macrophage cells (Fig. 5D). Meanwhile, the amount of Hsc70 proteins in the nuclei of the interferon-activated and naïve macrophages did not change (Fig. 5B, top panel). Thus the interferon-inducible interaction of the Ipr1 protein with the Hsc70 chaperon may be due to modifications of one or both proteins specific for macrophage activation leading to stabilization of the Ipr1-containing protein complexes.

4. Discussion

Macrophages are among the most versatile and important animal cells (Ross and Auger, 2002; Sasmono and Hume, 2004). However, currently our ability to study these cells using modern molecular approaches is greatly limited as compared to other cell types, because macrophages resist genetic manipulation. Direct plasmid DNA transfection or electroporation is only successful with a few monocyte or macrophage cell lines such as THP-1 or RAW264.7 (Weir and Meltzer, 1993; Hume et al., 2001), which is not suitable for other macrophage cell lines (e.g. J744A.1) and primary macrophages (Heider et al., 2000). Perhaps, this is related to their natural function in tissue homeostasis and host defense, such as recognition and elimination of foreign materials. There was evidence that certain macrophage cell lines (e. g. RAW264.7) had specific defects in inflammatory response to foreign materials i.e. prostaglandin-dependent autoregulation of tumor necrosis factor-alpha secretion upon lipopolysaccharide stimulation (Rouzer et al., 2005). Thus it is beneficial to be able to use different macrophage cell lines and primary cells from different sources.

Here, we presented an improved strategy enabling genetic manipulation of macrophages more efficiently. The major features of our approach include: 1) dual-promoter lentiviral vectors for the inducible expression of the gene-of-interest and constitutive expression of the selectable surface marker; 2) the C3H.TgN (SRA-rtTA) transgenic mouse that enables doxycycline-inducible expression of lentivirally delivered genes-of-interest in primary macrophages. This system is particularly useful when the efficiency of transduction is low and a gene-of-interest is either unstable or exerts adverse effect on target cells. As discussed below, both conditions apply to studies of immunity-related genes in macrophages.

It is important to have independent controls of expression of a selectable surface marker and a gene-of-interest. Therefore, we used dual-promoter lentiviral vectors. Although there were several studies reporting that dual-promoter vectors based on oncoretrovirus backbones showed severe reduction of transgene expression due to promoter interference (Emerman and Temin, 1984; Overell et al., 1988), a similar strategy was successful when using lentiviral vectors (Yu et al., 2003). Thus, Yu and colleagues have achieved efficient and consistent co-expression of two genes in cord-blood CD34+ HSCs or primary endothelial cells (Yu et al., 2003). Our data demonstrate that the dual promoter vectors may be used in macrophage cell lines and primary BMDMs as well.

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To obtain high purity of transduced primary macrophages, we have developed a procedure, in which macrophage progenitors are transduced by lentiviral vectors containing a selectable surface marker expressed under the control of a constitutive UBC promoter and the transduced cells, are enriched using magnetic cell sorting, expanded and differentiated into macrophages. Positive selection using surface markers in case of macrophages has an advantage as compared to drug selection. Since macrophages are phagocytic cells, they ingest dead cells during the drug selection procedure, which affects their growth and behavior. Positive selection using magnetic beads is rapid and avoids the adverse effects associated with drug selection. We have demonstrated that the selectable surface markers used in our vectors are expressed at relatively constant levels during the whole period of observation (up to one month in our studies) and therefore the selection procedure might be repeated several times. Using this strategy we typically obtain 10^8 transduced primary bone marrow-derived macrophages. Because the gene-of-interest is controlled separately by a doxycycline-inducible promoter, it is kept silent during the selection process and does not interfere with macrophage growth.

Hundreds of genes are expressed in activated macrophages in an inducible manner. Their products are involved in host defense displaying pro-inflammatory, immunoregulatory and anti-microbial activities (Ehrt et al., 2001; Schroder et al., 2004; Kota et al., 2006). Some of them exert anti-proliferative or pro-apoptotic effects on macrophages. Thus, macrophages that are genetically manipulated to constitutively express such genes may be under negative selection, i.e. either selectively eliminated or outgrown by the cells that express non-functional genes inactivated by mutations, for example. We have identified the *Ipr1* gene by positional cloning as a candidate gene that controls a macrophage-mediated mechanism of host resistance to intracellular pathogens, MTB and *Listeria monocytogenesis*. This gene encodes an interferon-inducible protein. We observed that similar to some other interferon-inducible proteins, the Ipr1 exerts an anti-proliferative effect on macrophages: cells that expressed functional Ipr1 under the control of a constitutive CMV promoter were rapidly lost during cell expansion. Actually, we have observed cell-cycle arrest at the G2/M transition in macrophages overexpressing eGFP-Ipr1 proteins. We also observed partial deletions in Ipr1 that inactivated this protein in cell lines that were constructed to express Ipr1 constitutively (data not shown).

To address these problems we utilized an inducible system to express the gene-of-interest in macrophages only when desired. We use two methods to express the doxycycline-regulated reverse tetracycline transactivator (rtTA) in target cells. For the macrophage cell lines we use a lentiviral vector that constitutively expresses the rtTA under control of CMV promoter. It can be either used to establish stable cell lines that express the rtTA or simply co-transduced with a lentiviral vector encoding a gene-of-interest. Although utilization of transformed macrophage cell lines *in vitro* is convenient, the analysis of gene function ideally has to be extended to primary macrophages. Therefore, we have developed a transgenic mouse strain C3H.TgN (SRA-rtTA) that enabled us to use the lentiviral system for the inducible gene expression in primary BMDMs. The C3HeB/FeJ mice also serve as a susceptible partner in our genetic analysis of host resistance to tuberculosis, in which four host resistance loci were mapped in addition to the *sst1* (Yan et al., 2006a). Therefore the C3H.TgN (SRA-rtTA) transgenic mice will be useful for the analysis of the *Ipr1* as well as other candidate host resistance genes in primary macrophages *in vitro* and possibly *in vivo*.

Recently, Yan *et al.* have published a macrophage-specific tetracycline-inducible system for *in vivo* expression in mice, which utilizes the c-fms (CSF-1 receptor) promoter/intron regulatory element (Yan et al., 2006b). For inducible expression these mice have to be bred with another transgenic mouse, in which rtTA inducible promoter drives expression of "gene-ofinterest". The c-fms gene promoter is active in macrophages and bone marrow progenitors (Sasmono et al., 2003). Our system is distinct, because the SRA promoter expresses a transgene only in mature macrophages (Horvai et al., 1995) and in bone marrow derived dendritic cells

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(Pan, unpublished observations). Therefore, it is suitable for *in vivo* expression of genes with anti-proliferative effects.

We applied the tetracycline-inducible system, to characterize the Ipr1-interacting proteins in macrophages and observed that macrophage activation with IFN-γ significantly enhanced Ipr1 interactions with nuclear proteins. In additional to transcriptional upregulation of Ipr1 upon macrophage activation with interferons, we also observed accumulation of this protein in the macrophage nuclei and inducible interactions of Ipr1 with molecular chaperon Hsc70. It is possible that interferon signaling enables the Ipr1 and/or Hsc70 protein interactions via phosphorylation of one or both proteins. In addition, interferon might induce the expression of a "bridge" molecule, which connects the Ipr1 and Hsc70 proteins. In either case the observed interactions are dependent on macrophage activation status and might not be detectable in other cell types, although they might be more convenient for co-transfection. These findings highlight the importance of studying host defense-related proteins in a specific cellular environment that is related to their biological function, such as macrophage interactions with pathogenic intracellular bacteria in the case of the Ipr1 protein. Our method permits further analysis of the dynamic Ipr1-containing multiprotein complexes during the course of macrophage activation and infection with virulent strains of *M. tuberculosis* in order to elucidate post-transcriptional modifications, traffic, turnover of the Ipr1 protein in macrophages, as well as its role in innate immunity.

The lentiviral system that we have developed contains a set of components that may allow for comprehensive analysis of macrophage genes both *in vitro* and *in vivo*. Initially, the lentiviral vector expressing a gene-of-interest in inducible manner can be tested using a macrophage cell line, such as J774A.1, as well as in primary BMDM isolated from the C3H.TgN (SRA-rtTA) transgenic mice. Next, the same construct can be used to transduce hematopoietic stem cells isolated from the C3H.TgN (SRA-rtTA) transgenic mice and generate bone marrow chimeras that would express a gene-of-interest in mature macrophages and, possibly, myeloid dendritic cells, after stimulation with doxycycline. Finally, the same lentiviral vector may be used to generate transgenic mice on the C3H.TgN (SRA-rtTA) genetic background (Pfeifer et al., 2002) (Szulc et al., 2006) that would express the gene-of-interest in macrophages after induction with doxycycline. In the future, it may be used in combination with novel systems for tetracycline inducible expression of transgene and/or RNAi (Szulc et al., 2006) and macrophage progenitor expansion (Odegaard et al., 2006; Wang et al., 2006) to make genetic and functional analysis of macrophages more efficient.

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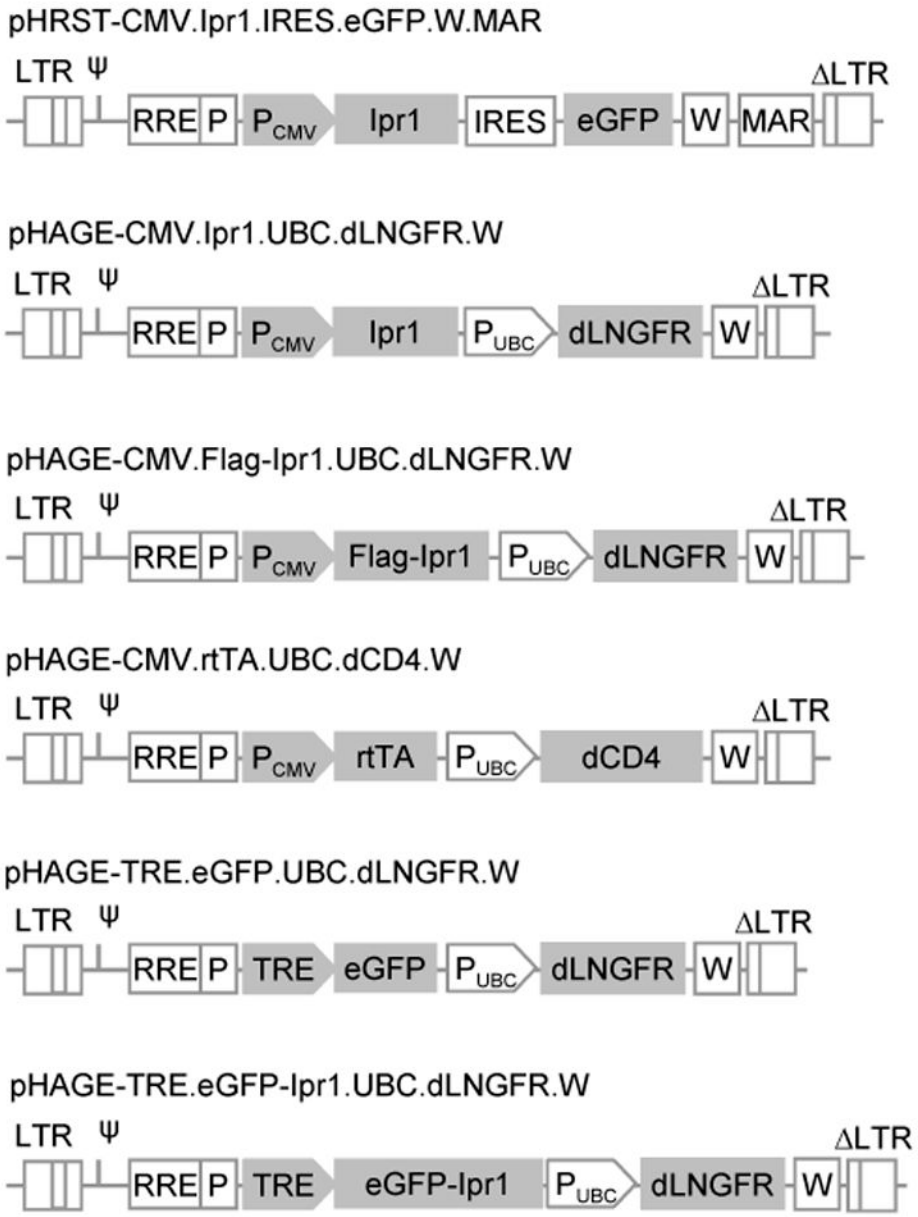

Figure 1.

Schematic diagrams of the pHRST bicistronic and pHAGE dual-promoter lentiviral vectors used in this study. LTR, long terminal repeat; ΔLTR, U3 region-deleted LTR; Ψ, Psi packaging signal; RRE, Rev-responsive element; P, central polypurine tract, W, woodchuck hepatitis virus posttranscriptional regulatory element; MAR, matrix attachment region; P_{CMV}, cytomegalovirus immediate early promoter; P_{UBC}, human ubiquitin C promoter; TRE, tetracycline responsible element; eGFP, enhanced green fluorescent protein; Ipr1, intracellular pathogen resistant gene 1; Flag, FLAG-tag; dLNGFR, truncated human low affinity nerve growth factor receptor; dCD4, truncated human CD4.

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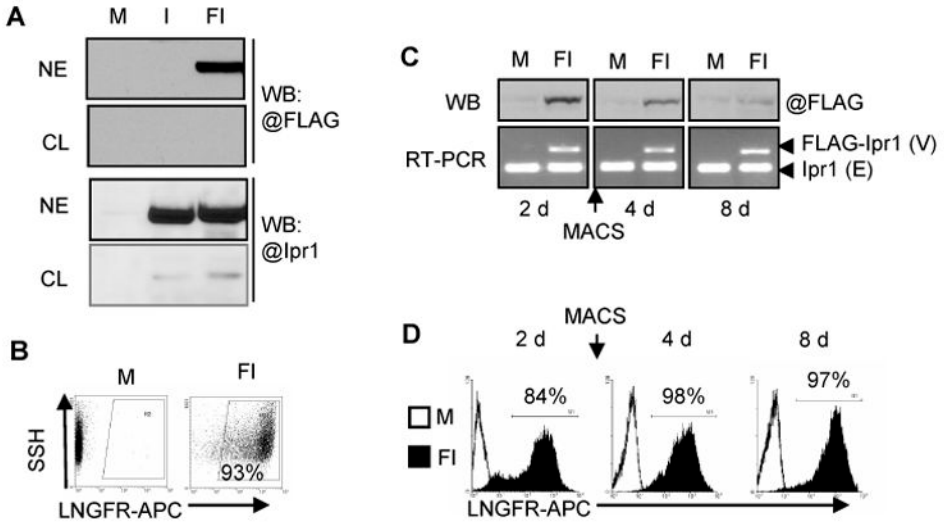

Figure 2.

Constitutive expression of *Ipr1* gene in mouse fibroblast NIH/3T3 and macrophage J774A.1 cell lines using dual-promoter lentiviral vectors. (A) and (B): NIH/3T3 cells were transduced with lentiviral vector pHAGE-CMV.Ipr1.UBC.dLNGFR.W (I) or pHAGE-CMV.Flag-Ipr1.UBC.dLNGFR.W (FI). Seven day post-transduction, expression of Ipr1 and Flag-Ipr1 was detected in nuclear extract (NE) or cytosolic lysate (CL) by immunoblot using anti-FLAG or anti-Ipr1 antibody (panel A), and expression of LNGFR on cell surface was detected by FACS (panel B). (C) and (D): J774A.1 cells were transduced with lentivirus pHAGE-CMV.Flag-Ipr1.UBC.dLNGFR.W (FI). Two day post-transduction, transduced cells were enriched by magnetic cell selection (MACS). Whole cell lysate was prepared at 2 (before MACS), 4 and 8 days post-transduction. Expression of Flag-Ipr1 protein was measured by immunoblot using anti-FLAG antibody. Transcription of vector encoded (V) Flag-Ipr1 and endogenous (E) Ipr1 gene was detected by 3-primer RT-PCR (panel C). The expression of LNGFR was detected at the same interval by FACS (panel D). M, mock transduction.

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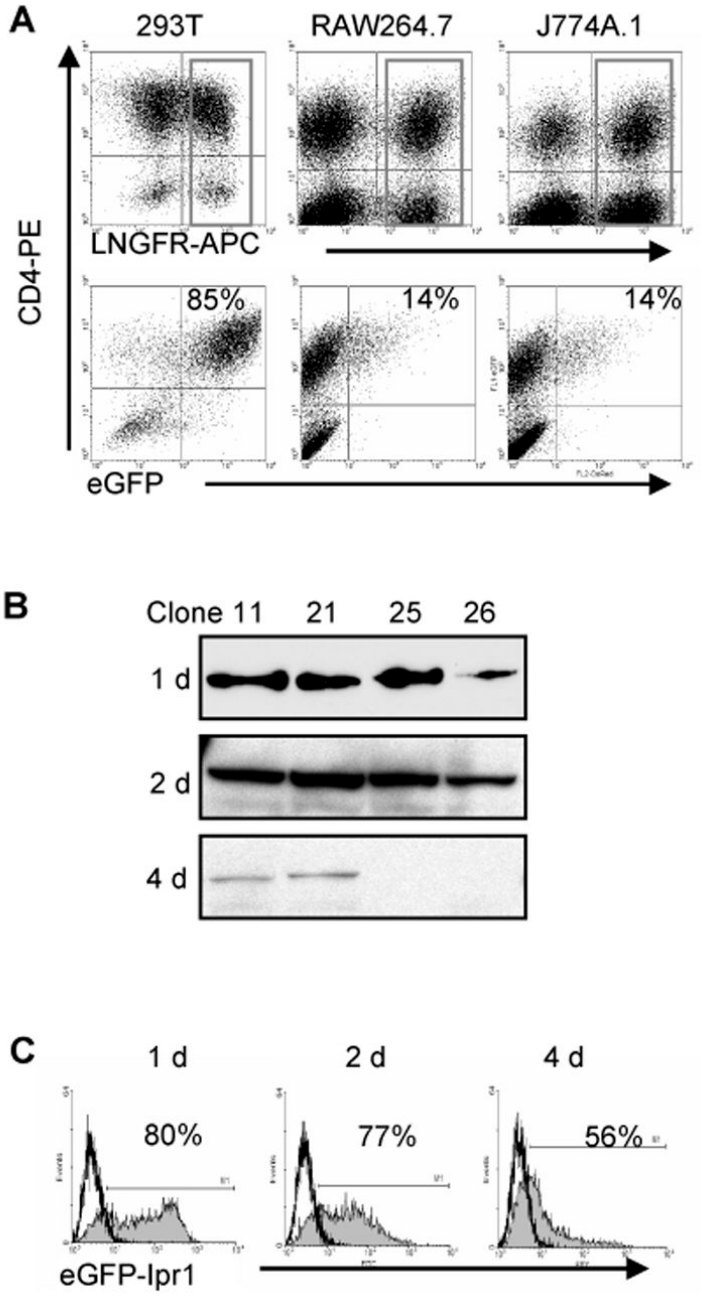

Figure 3.

Lentiviral system for inducible gene expression in macrophage cell lines. (A) 293T, RAW264.7 and J774A.1 cells were co-transduced with two lentiviral vectors pHAGE-CMV.rtTA.UBC.dCD4.W and pHAGE-TRE.eGFP.UBC.dLNGFR.W. One day post transduction, cells were cultured in medium containing 1 μg/mL doxycycline for additional 1 day. The expressions of cells surface markers LNGFR and CD4 (upper panels), as well as eGFP in LNGFR-positive cells (lower panels) were measured by FACS. (B) J774A.1 cells were co-transduced with two lentiviral vectors pHAGE-CMV.rtTA.UBC.dCD4.W and pHAGE-TRE.eGFP-Ipr1.UBC.dLNGFR.W. Individual clones were isolated and tested for induction of eGFP-Ipr1 in medium containing 1 μg/mL doxycycline for additional 1, 2, and 4 days by

immunoblot using Ipr1-specific antibody, (C) The green fluorescence of eGFP-Ipr1 in a typical clone were also measured by FACS.

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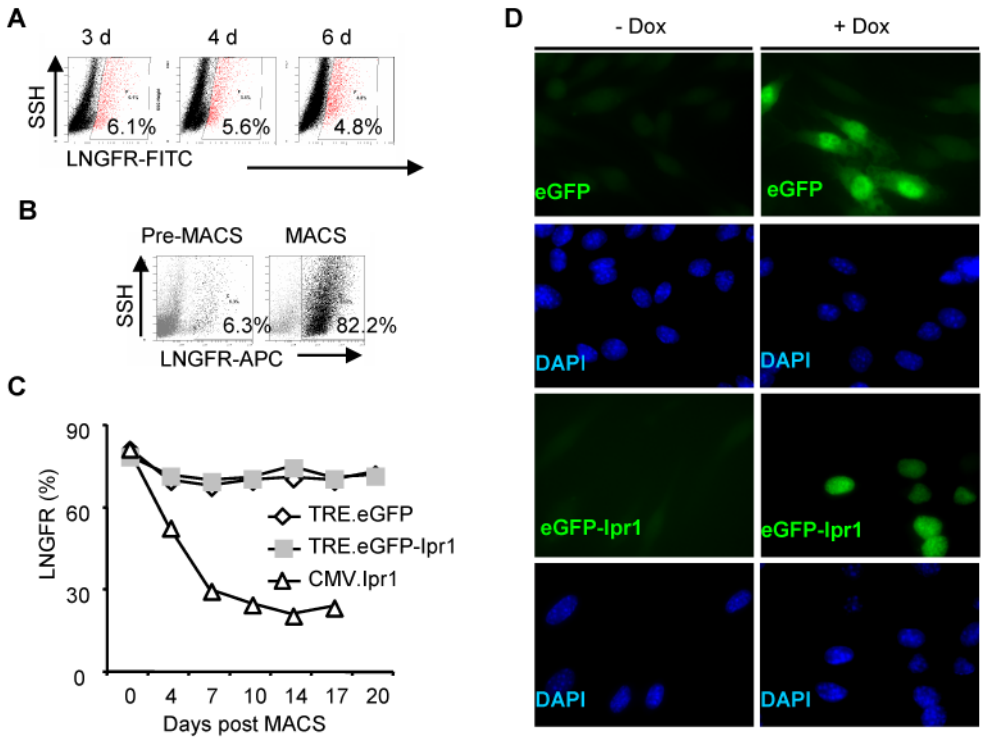

Figure 4.

Lentiviral system for inducible gene expression in BMDMs prepared from C3H.TgN (SRA-rtTA) transgenic mice. (A) The efficiency of transduction was measured by FACS analysis of LNGFR on bone marrow cells transduced with lentiviral vector pHAGE-TRE.eGFP.UBC.dLNGFR.W at 3, 4, and 6 days post bone marrow isolation. All the following lentiviral transductions were performed at 3 days post bone marrow isolation. (B) Enrichment of LNGFR-expressing transduced bone marrow cells. MACS were done 2 days after lentiviral transduction. (C) Bone marrow cells were transduced with lentiviruses pHAGE-TRE.eGFP.UBC.dLNGFR.W, pHAGE-TRE.eGFP-Ipr1.UBC.dLNGFR.W, or pHAGE-CMV.Ipr1.UBC.dLNGFR.W; the proportions of LNGFR-expressing cells were monitored following 20 days after MACS. (D) Microscopy of BMDM cultured from lentiviruses transuded (pHAGE-TRE.eGFP.UBC.dLNGFR.W or pHAGE-TRE.eGFP-Ipr1.UBC.dLNGFR.W), MACS enriched bone marrow cells. −Dox, without doxycycline; +Dox, with 1 μg/mL doxycycline.

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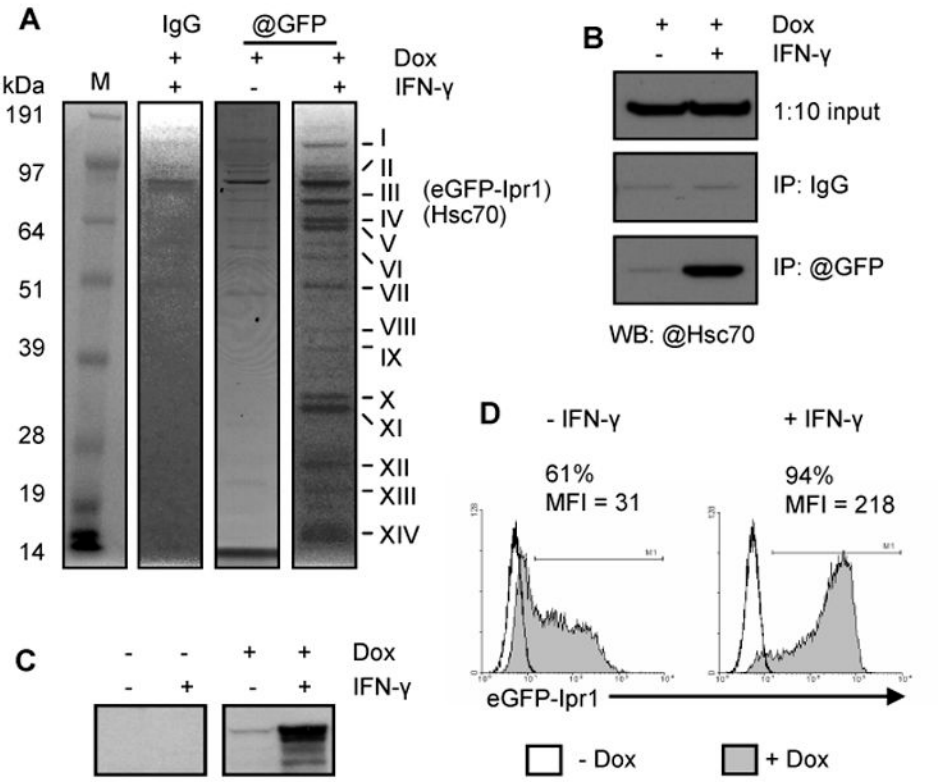

Figure 5.

Characterization of Ipr1-containing protein complexes in macrophage nuclei. (A) Cells of clone 21 of J774A.1 cells, which express eGFP-Ipr1in doxycycline-inducible manner, were cultured in medium containing 1 μg/mL doxycycline with or without rmIFN-γ (100 U/mL). Immunoprecipitation of nuclear extracts was performed using GFP-specific antibodies. Proteins co-precipitated with eGFP-Ipr1 were separated on 10% SDS-PAGE and visualized using Coomassie Brilliant Blue R-250 staining. Bands III and IV were identified as eGFP-Ipr1 and Hsc70, respectively, using Nano-LC tandem mass spectrometry. (B) Western blot of Ipr1-interacting proteins with Hsc70-specific antibodies: nuclear extracts were prepared as in (A), immunoprecipitation (IP) was performed using either anti-GFP or IgG isotype-matched control; eluate from IP and 1:10 amount of nuclear extract (input) were immunoblotted using Hsc70-specific antibodies. (C) Increased amount of eGFP-Ipr1 proteins in the nuclei of IFN-γ activated clone 21 J774A.1 cells was demonstrated by immunoblot using Ipr1-specific antibodies. (D) FACS analysis of eGFP-Ipr1 expression in naïve and IFN-γ - activated clone 21 cells. MFI, mean fluorescence intensity.