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An adenoviral vector for probing promoter activity in primary immune cells

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Abstract

Functional analysis of the DNA regulatory regions that control gene expression has largely been performed through transient transfection of promoter–reporter constructs into transformed cells. However, transformed cells are often poor models of primary cells. To directly analyze DNA regulatory regions in primary cells, we generated a novel adenoviral luciferase reporter vector, *pShuttle-luciferase-GFP* (pSLUG) that contains a promoterless luciferase cassette (with an upstream cloning site) for probing promoter activity, and a GFP expression cassette that allows for the identification of transduced cells. Recombinant adenoviruses generated from this vector can transduce a wide range of primary immune cells with high efficiency, including human macrophages, dendritic cells and T cells; and mouse T cells transgenic for the coxsackie and adenoviral receptor (CAR). In primary T cells, we show inducible nuclear factor of activated T cells (NF-AT) activity using a recombinant pSLUG adenovirus containing a consensus NF-AT promoter. We further show inducible IL-12/23 p40 promoter activity in primary macrophages and dendritic cells using a recombinant pSLUG adenovirus containing the proximal human IL-12/23 p40 promoter. The pSLUG system promises to be a powerful tool for the analysis of DNA regulatory regions in diverse types of primary immune cells.

Keywords

T cell; Macrophage; Dendritic cell; Transcription; Promoter

1. Introduction

Functional analysis of the DNA regulatory regions that control gene expression has largely been performed through transient transfection of transformed cells. The reasons for this approach are practical and obvious: the ability to obtain large numbers of homogeneous cells, and ease of gene transduction. However, transformed cells are often poor models for primary cells. Firstly, they exhibit constitutive proliferation. Secondly, they often have demonstrable defects in cell signaling. These related properties can both alter gene expression. For example, the human T cell line, Jurkat, widely used for promoter analysis, lacks expression of the tumor suppressor, phosphatase and tensin homologue (PTEN), and therefore exhibits constitutive phosphatidylinositol-3-kinase signaling (Shan et al., 2000). Perhaps as a consequence of altered PTEN expression, Ras-mediated ERK activation also varies between primary T cells and Jurkat T cells (Li et al., 1999; Marks et al., 2003). Similarly, the mouse macrophage cell line RAW264.7, also widely used for promoter analysis, exhibits very different responsiveness to microbial and T cell-derived stimuli compared with primary macrophages, (Rao, 2001; Galindo et al., 2004; Rouzer et al., 2005). Very little functional analysis has been done on DNA regulatory regions in dendritic cells (DC) because of the lack of available DC lines and the resistance of primary DC to transfection. Thus, for cells of the immune system, promoter-reporter studies have, perforce, been carried out under situations that provide a wealth of data, the physiological relevance of which remains open to question.

Recent technical developments have facilitated gene delivery into hard-to-transfect cells such as T cells and macrophages. Retroviral transduction has been used to facilitate DNA delivery into T cells. However, transduction by retroviruses is dependent upon active replication by the target cell; for non-replicating cells such as macrophages, retroviral transduction is not an efficient means of DNA delivery. Adenoviral transduction has been used to deliver DNA into macrophages and DC with relatively high efficiency (Wan et al., 1997; Yuan et al., 2000). However, primary mouse T cells are not easily transduced with adenoviruses. To circumvent this issue, transgenic mice were recently made that express the coxsackie and adenoviral receptor (CAR) under the control of the CD2 promoter (Wan et al., 2000). T cells from CAR transgenic (CAR Tg) mice are readily transduced by adenoviruses (Wan et al., 2000), and recent studies have shown that this is an efficient way to deliver dominant negative signaling molecules to interfere with signal transduction events in vitro and in vivo (Marks et al., 2003; Wan and DeGregori, 2003). Thus, recombinant adenoviruses (rAdV) are a useful tool for highly efficient DNA delivery into multiple types of immune cells.

Recently, several groups have also used rAdV as a tool to probe gene promoter activity in different types of non-immune cells (Zheng et al., 2001; Stirland et al., 2003; Wilkins et al., 2004), suggesting that rAdV could also readily be used to probe promoter activity in primary immune cells. We thus developed a novel adenoviral vector, *pShuttle-luciferase-GFP* (pSLUG), that allows for the generation of rAdV that are highly efficient transducers of multiple primary immune cell types. Transduction of primary mouse or human T cells with pSLUG AdV containing a consensus NF-AT reporter revealed inducible NF-AT promoter activity. Transduction of primary human macrophages and DC with pSLUG AdV containing the proximal human IL-12/23p40 promoter revealed inducible IL-12/23p40 promoter activity. These data demonstrate the potential usefulness of pSLUG-derived vectors for probing DNA regulatory elements in primary immune cells.

2. Materials and methods

2.1. Generation of adenoviral luciferase shuttle vector

An adenoviral shuttle vector was created which contains a promoterless luciferase reporter gene and an enhanced Green Fluorescent Protein (eGFP) expression cassette. A *HindIII/SalI*

fragment from pGL3 basic vector (Promega) containing luciferase cDNA was cloned into the *HindIII/SalI* sites of pShuttle (He et al., 1998) (Stratagene, La Jolla, CA) to create pShuttle-luciferase. A cassette containing eGFP cDNA driven by the human ubiquitin c promoter was isolated from the adenoviral expression plasmid pShUbP-UbP-GFP (a kind gift from Dr. James Degregori, University of Colorado) via *BglII* digestion and was cloned into the *BglII* site of pShuttle-luciferase.

2.2. Generation of pSLUG-NF-AT promoter and pSLUG-IL-12/23p40 promoter vectors

A DNA clone containing a 234bp fragment containing three tandem copies of a consensus NF-AT binding site upstream of a minimal thymidine kinase promoter was a kind gift from Dr. Jeff Molkentin (Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA) (Fiedler et al., 2002). This promoter fragment was excised with *BsmI*, blunted with T4 DNA polymerase (NEB) and ligated into the *EcoRV* site of pSLUG. Recombinants were screened by restriction digest and potential clones verified by DNA sequencing. A 3.3Kb fragment of the proximal human IL-12/23 p40 promoter (Ma et al., 1996) was cloned in both orientations into the *XhoI* site of pSLUG. Recombinants were screened by restriction digest and potential clones were verified by DNA sequencing.

2.3. Generation of recombinant adenoviruses

All adenoviruses were prepared using the pAdEasy system described previously (He et al., 1998). Recombinant adenoviral DNA was generated by homologous recombination in *E. coli*. Briefly, BJ5183 *E. coli* containing the adenoviral DNA backbone pAdEasy-1 (Stratagene, La Jolla, CA) was transformed with *PmeI* linearized pSLUG plasmid DNA and selected on Kanamycin LB agar plates. Recombinant clones were confirmed by restriction digest and DNA sequencing. To generate high titer stocks, E1A- and E1B-transformed HEK293 (ATCC) cells were transfected with recombinant adenoviral DNA using CaPO_4 transfection as described (Hildeman et al., 2002). 7–10days after transfection, HEK-293 cells were harvested and viral seed stock was obtained by 4–5 freeze / thaw cycles in an ethanol / dry ice bath followed by quick thawing at 37°C. A primary stock was generated by infecting an increased number of HEK-293 cells with a fraction of the seed stock. Viral particles were purified using a CsCl gradient purification method (Wan et al., 2000). After dialysis with 10% glycerol, 10mM Tris-HCl (pH 7.5–8.0) and 1mM MgCl_2 the viral stock was aliquoted and stored at –70°C. pSLUG-3.3p40 and pSLUG-reverse 3.3p40 AdV stocks were tested for endotoxin contamination by the limulus amebocyte lysate assay (BioWhittaker); endotoxin levels were <0.03EU/ml. The titer of each virus was determined by infecting 2×10^5 HEK-293 cells with various dilutions of adenoviral stock and assessing the percentage of GFP⁺ cells by flow cytometry. Viral infectious units were determined using the following equation: $(2 \times 10^5) (\% \text{ GFP}^+ \text{ cells}) / (\text{dilution of virus})(\text{ml of infectious inoculum}) = \text{infectious units/ml}$. Stocks generally had between 10^9 and 10^{11} infectious units/ml.

2.4. Adenoviral transduction of primary T cells, dendritic cells and macrophages

CAR Tg mice, described previously (Wan et al., 2000), were bred in the Cincinnati Children's Hospital Medical Center (CCHMC) Vivarium. Animal protocols were approved by the CCHMC Institutional Animal Care and Use Committee. Single cell suspensions were generated from spleens of CAR Tg mice by passage through 100µm nylon mesh (BD Falcon). Red blood cells were removed by short incubation with hypotonic ammonium chloride buffer, and T cells were purified using a pan T cell isolation kit (Miltenyi Biotec). T cells were consistently >90% pure as assessed by flow cytometry. CAR Tg T-cells were activated with antibodies to CD3 (2µg/ml) (145-2C11) and CD28 (2µg/ml) overnight (12–20h) at 37°C followed by washing with Kappler/Marrack complete tumor medium (CTM, s-MEM containing 100U/ml penicillin, 100µg/ml streptomycin, 2-mercaptoethanol, sodium pyruvate,

nonessential amino acids, and 5% FBS). T cells were infected with adenoviruses at the indicated MOI for 1h at 37°C in CTM media, washed twice with CTM to remove the non-adherent viral particles, and cultured for 24h at 37°C in 10% CO₂ at a concentration of 10⁶ cells/ml.

For human T cell purification, PBMC were separated on lymphocyte separation medium (Ficoll-Hypaque, Amersham, Piscataway, NJ) and resuspended at 10⁷/ml in complete medium (RPMI 1640 containing 100U/ml penicillin, 100µg/ml streptomycin, 2mM glutamine and 5mM HEPES; all from Gibco Life Technologies, Gaithersburg, MD). T cells were purified from PBMC by negative selection. After a first step of plastic adherence (45min at 37°C), non-adherent cells were treated with a cocktail of lytic antibodies and complement, according to the manufacturer's instructions (T-Qwick, One-Lambda, Los Angeles, CA). T cells were >90% pure, as assessed by flow cytometry. T cells were stimulated (or not, as a control) overnight with magnetic beads coated with antibodies to CD3 and CD28 (T cell Expander, Dynal) in 12-well plates in complete medium supplemented with 10% FBS. Preliminary experiments had determined the optimal concentration to be 2.5µl beads/10⁶ T cells; this concentration was used throughout the study. After incubation, stimulating beads were removed using a magnet. Purified T cells were washed twice with media and infected with the virus as described above for CAR Tg T cells.

Human monocyte-derived macrophages generated with GM-CSF (GM-Mac) or with M-CSF (M-Mac), along with monocyte-derived DC, were differentiated as described previously (Karp et al., 1998; Chougnnet et al., 1999). Briefly, monocyte-derived GM-Mac and M-Mac were generated by plating monocytes isolated by counter-current elutriation at a density of 4 × 10⁶ cells per well in 6-well plates in RPMI-1640 containing 50ng/ml gentamicin and 2mM glutamine. After an initial step of plastic adherence (2h at 37°C), the medium was replaced with complete medium (RPMI-1640 containing 10% FBS, 50ng/ml gentamicin, 2mM glutamine and 10mM HEPES buffer) along with 500U/ml GM-CSF or 50ng/ml M-CSF. Every other day half the medium was replaced with cytokine containing media. DC were generated by plating elutriated monocytes at 2 × 10⁶ cells per well in 6-well plates in RPMI-1640 containing 50ng/ml gentamicin and 2mM glutamine. After adherence on plastic, the medium was replaced with complete medium (RPMI-1640 containing 10% FBS, 50ng/ml gentamicin, 2mM glutamine and 10mM HEPES buffer) along with 500U/ml GM-CSF and 1000U/ml IL-4. Every other day, half of the medium was replaced with fresh complete media containing GM-CSF and IL-4. Differentiation of monocytes to DC and macrophages was confirmed by flow cytometry. DC expressed CD1a, CD83, CD80, CD86 and HLA-DR^{high}, and were negative for CD14. Macrophages expressed CD14 and CD16. These differentiation protocols had previously been shown to yield functionally mature DC (mixed leukocyte reaction) and macrophages (phagocytosis of *Leishmania major* promastigotes) [data not shown]. After 7 days, DC and macrophages were harvested, counted, and infected with rAdV. Macrophages were harvested using 10mM EDTA in PBS. 2h later, cells were washed and resuspended in cytokine supplemented complete medium, and plated at 2×10⁵ cells/well in 96-well tissue culture plates.

2.5. Flow cytometry

Human and mouse T cells were stained with species-specific, fluorescently labeled antibodies against CD4 and CD8 (BD Pharmingen, Mountain View, CA) in FACS buffer (balanced salt solution containing 10% FBS and 5mM sodium azide) for 40min at 4°C. The cells were then washed twice before being fixed in FACS buffer containing 4% paraformaldehyde. T cells were subsequently washed twice prior to flow cytometry. Macrophages and DC were harvested and washed twice prior to flow cytometry. Transduction efficiency was quantified in all cell types by measuring the percentage of GFP⁺ cells using a FACSCalibur flow cytometer (Becton Dickinson). A minimum of 10⁵ events was collected. Intracellular IL-2 staining was performed

on T cells stimulated with PMA and ionomycin in the presence of brefeldin A (10µg/ml) followed by staining cells with fluorescently labeled antibodies to CD4 and CD8 (BD-Pharmingen). After surface staining, cells were fixed with 2% formaldehyde for 45min, permeabilized with FACS buffer containing 0.015% saponin (Sigma-Aldrich, Chicago, IL), and stained with fluorescently labeled antibody to IL-2 (BD Pharmingen). Data involving IL-2 production in T cells was acquired with a FACSCalibur flow cytometer and analyzed using CellQuest software.

2.6. Quantitation of IL-2 and IL-12/23p40

HT-2 cells, a generous gift from Dr. Philippa Marrack (Howard Hughes Medical Institute, Denver, CO), were used to quantify the amount of mouse IL-2 in culture supernatants as described (Leibson et al., 1981). Briefly, either naïve or anti-CD3/CD28-activated CAR Tg T cells were cultured with media alone or were transduced with varying amounts of pSLUG-NF-ATf AdV. Twenty hours later, supernatants were harvested and transferred to wells containing HT-2 cells that had been washed extensively to remove exogenous IL-2. HT-2 cell survival 20h later was determined by an MTT assay as described (Leibson et al., 1981). A standard curve was generated using rmIL-2 (R&D Systems, Minneapolis, MN). Human IL-12/23p40 protein concentrations in culture supernatants were quantitated by ELISA (Opti-EIA kit; BD Biosciences).

2.7. Luciferase assays

Twenty hours after adenoviral transduction, T cells were stimulated with PMA (50ng/ml, Sigma, St. Louis, MO) and ionomycin (1µg/ml, Sigma, St. Louis, MO) for 5h at 37°C. Cells were then washed twice with PBS, and luciferase activity was measured in cell lysates using the Bright-glo luciferase assay kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, cells were washed twice with PBS and lysed with 1×lysis buffer. After centrifugation of the lysate, 20µl of supernatant was added to 100µl of luciferin substrate (Promega) and assayed in a luminometer. Four hours after rAdV infection, human monocyte-derived macrophages and DC were stimulated with *Staphylococcus aureus* Cowan strain (SAC) (0.0075% wt/vol) for 8h. Cells were then lysed, and luciferase activity was measured in cell lysates using a luminometer.

2.8. Electroporation of Jurkat T cells

Jurkat cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. 3×10^6 Jurkat cells were electroporated with 15µg of pSLUG-NF-ATf plasmid DNA (180V/960µF, Gene-Pulser, Bio-Rad). 18h after electroporation, cells were activated with PMA and ionomycin for 6h, and luciferase activity was assessed as described above. Transfection efficiency was determined by measuring the percentage of GFP⁺ cells using flow cytometry.

3. Results

3.1. Generation of recombinant promoterless luciferase adenoviral vector

rAdV containing DNA regulatory regions upstream of a luciferase reporter have been used to monitor gene expression in cardiomyocytes, primary pituitary cells, and salivary gland epithelial cells (Zheng et al., 2001; Stirland et al., 2003; Wilkins et al., 2004). To determine whether such rAdV would be useful for analyzing gene expression in immune cells, we developed a rAdV that is based on the pAdEasy system (Stratagene) (He et al., 1998). This rAdV vector, pSLUG, was created by insertion of luciferase cDNA from pGL3basic (Promega, Madison, WI) into pShuttle (He et al., 1998)(Fig. 1). A short multicloning site containing the unique restriction sites *KpnI*, *XhoI* and *EcoRV* allows for cloning of DNA regulatory regions

upstream of the promoterless luciferase cDNA cassette. To track transduced cells, a human ubiquitin C promoter-driven eGFP cassette was inserted downstream of the promoterless luciferase cassette (Fig. 1). Using homologous recombination in *E. coli*, pSLUG can accept another 3.1–3.3Kb of DNA using the pAdEasy-1 Adenoviral backbone, and another 5.5–5.7Kb of DNA using the pAdEasy-2 Adenoviral backbone, to generate rAdV(He et al., 1998). Thus, pSLUG allows for insertion of large regions of DNA upstream of the luciferase cassette and subsequent generation of rAdV, and should allow for probing of promoter activity in primary cells using adenoviral transduction.

3.2. pSLUG adenoviruses can infect a broad range of primary mouse and human immune cells

rAdV have been used for gene transduction in multiple cell types, including macrophages (Foxwell et al., 1998), DC (Offringa et al., 2005) and T cells (Huang et al., 1995; Wan et al., 2000). We determined the efficiency with which pSLUG-AdV infect primary immune cells. First, we took advantage of CAR Tg mice, which express CAR under the control of the CD2 promoter and display significant expression of CAR in both CD4⁺ and CD8⁺ T cells (Wan et al., 2000). Primary resting T cells from CAR Tg mice were readily transduced with pSLUG-AdV, and the percentage of GFP⁺ T cells increased in a dose-dependent manner relative to the amount of input virus (Fig. 2A,B). When measured 24h after transduction, up to 60–70% of CD4⁺ and CD8⁺ CAR Tg T cells were GFP⁺ (Fig. 2A,B). Further, we tested the transduction efficiency in primary human T cells. Primary resting human T cells were not amenable to AdV transduction; <1% of the T cells were GFP⁺ following pSLUG-AdV transduction, even at very high MOI (data not shown). However, activation of human T cells substantially increased pSLUG-AdV transduction efficiency. Although the amount of virus required to transduce human T cells was much higher than for CAR Tg T cells, human T cells were readily transduced with pSLUG-AdV, with transduction efficiencies ranging from 10% to 25%, depending upon the MOI (Fig. 2C). Thus, both mouse and human T cells were readily transduced with pSLUG-AdV.

To determine the transduction efficiency of pSLUG-AdV in other immune cells, we examined human monocyte-derived GM-CSF macrophages (GM-Mac), monocyte-derived M-CSF macrophages (M-Mac) and monocyte-derived DC. GM-Mac, M-Mac, and DC were infected with pSLUG-AdV across a range of MOI (0–300). In all 3 cell types, a dose-dependent increase in the percentage of GFP⁺ cells was observed with increasing pSLUG-AdV MOI (Fig. 2D–F). Transduction efficiency reached 90% in GM-Mac; 100% in M-Mac and 60% in DC, demonstrating the high infectivity of these cell types for adenoviruses (Fig. 2D–F). We note that rAdV transduction of M-Mac was very efficient, because a higher percentage of GFP⁺ cells was observed at lower MOI compared to the other cell types (Fig. 2D–F). Adenoviral transduction itself did not cause increased cell death or survival of macrophages and dendritic cells as measured by propidium iodide staining of transduced cells (data not shown). Thus, pSLUG-AdV were quite efficient at transducing primary human GM-Mac, M-Mac, and DC.

3.3. Inducible NF-AT activity in primary mouse and human T cells

We next determined if pSLUG-AdV could be used to detect promoter activity in primary T cells. To do this, we cloned a 234bp DNA fragment containing 3 tandem copies of a consensus NF-AT binding site along with a minimal TK promoter directly upstream of the luciferase cDNA in pSLUG. As a control, a separate vector was generated in which the orientation of this promoter fragment was reversed. Thus, two pSLUG-AdV were made, one having the NF-AT/TK promoter sequences in the forward orientation (NF-ATf) and a second having the NF-AT/TK promoter sequences in the reverse orientation (NF-ATr). rAdV were made from each of the vectors, used to transduce primary CAR Tg T cells, and luciferase activity measured with and without stimulation. Without stimulation, very little luciferase activity was detected

in primary CAR Tg T cells transduced with either NF-ATf or NF-ATr AdV (Fig. 3A). However, following stimulation with PMA and ionomycin *in vitro*, luciferase activity in T cells transduced with NF-ATf AdV increased 7-fold, while luciferase activity in T cells transduced with NF-ATr AdV did not increase significantly (Fig. 3A). This increase was not due to a lack of transduction of T cells with the NF-ATr AdV, because similar percentages of GFP⁺ T cells were observed with either rAdV (Fig. 3A, histogram panels). Similar to CAR Tg T cells, we observed inducible NF-AT activity in primary human CD4⁺ T cells transduced with NF-ATf AdV, but not with NF-ATr AdV (Fig. 3B). Moreover, confirming the specificity of the inducible NF-AT activity in transduced T cells, we observed that such activity was significantly decreased by pre-culture with cyclosporin A (Fig. 3C). Thus, recombinant pSLUG-AdV detected inducible NF-AT promoter activity in both mouse and human T cells.

The stimulated increases in NF-AT activity that we observed in primary T cells were not as large as those previously reported using T cell lines (Shaw et al., 1988; Wu et al., 1995). To determine whether this was an effect of the construct we were using or a property of the cell type (primary vs. cell line), we examined inducible NF-AT activity in Jurkat T cells using the pSLUG-NF-AT shuttle plasmid. We found a roughly 2-fold increase in NF-AT activity in PMA/ionomycin-stimulated Jurkat T cells transfected (via electroporation) with the pSLUG-NF-ATf plasmid (data not shown). It should also be noted that, using a similar 3-copy NF-AT promoter-reporter in transgenic mice, another group has similarly shown a roughly 10-fold induction of luciferase activity in primary T cells (Aune and Flavell, 1997). Moreover, increasing the number of copies of the NF-AT reporter sequence used in our studies results in substantially higher fold increases in inducible NF-AT activity (Wilkins et al., 2004). Thus, it is likely that this minimal NF-AT consensus sequence is not as active as other NF-AT promoters previously used. Nonetheless, it demonstrates that this system may be useful to study reporter activity of minimally active promoters (e.g., CD40L and Bcl-2).

We also tested NF-AT promoter inducibility across a range of MOI with pSLUG-NF-ATf. Even at low doses of virus (MOI = 25), we found a nearly 5-fold induction of NF-AT activity (Fig. 4A). At higher doses of virus, we found a nearly 8-fold induction of NF-AT activity in stimulated T cells (Fig. 4A). Next, we examined whether or not viral transduction affected regulation of the endogenous IL-2 gene, because the consensus NF-AT site was derived from the IL-2 promoter. First, we tested whether pSLUG rAdV transduction of naïve or previously activated T cells led to detectable IL-2 production. In naïve T cells, transduction with pSLUG-NF-ATf did not induce production of IL-2 (data not shown). In previously activated T cells, there was a slight decrease in IL-2 production in cells that were transduced with virus compared to T cells that were not transduced (Fig. 4B). This decrease in IL-2 production is likely due to the modestly increased death of T cells caused by viral transduction (data not shown). Second, we assessed IL-2 production at the single cell level in AdV transduced T cells. CAR Tg T cells were transduced with NF-ATf AdV and, 24h later, were activated with PMA and ionomycin for 5h. After culture, IL-2 production was measured by intracellular immunofluorescent staining and flow cytometric analysis. Notably, similar percentages of IL-2⁺ cells were observed in both CD4⁺ GFP⁺ (transduced) and CD4⁺ GFP⁻ (non-transduced) T cell populations (Fig. 4C). Further, the amount of IL-2 produced per T cell (as measured by mean fluorescence intensity) was similar for both groups (CD4⁺ GFP⁺, MFI = 57.7 ± 1.7; CD4⁺ GFP⁻, MFI = 57.0 ± 1.2). Thus, although we cannot rule out potential non-specific effects of adenoviral transduction on T cells, transduction did not affect basal IL-2 production and only slightly affected IL-2 production following re-stimulation *in vitro*.

3.4. Inducible IL-12/23p40 promoter activity in primary DC and macrophages

To determine whether pSLUG-AdV could be used to probe promoter activity in macrophages and DC, we cloned 3.3Kb of genomic DNA spanning the proximal human IL-12/23p40

promoter into pSLUG, in both orientations, generating pSLUG-p40f-AdV and pSLUG-p40r-AdV. To induce promoter activity, we stimulated cells with *Staphylococcus aureus* Cowan strain 1 (SAC), a potent inducer of IL-12/23p40 transcription (D'Andrea et al., 1992). GM-Mac and DC from individual donors were infected with both AdV at an MOI of 50 (GM-Mac) or 200 (DC). Following infection, cells were washed, incubated for 4h, and stimulated with SAC for 8h. Luciferase activity was measured in cell lysates. Robust, SAC-stimulable luciferase activity was detected in pSLUG-p40f-AdV infected macrophages (Fig. 5A) and DC (Fig. 5B). As expected, SAC-stimulable luciferase activity was not evident in macrophages and DC infected with the control pSLUG-p40r-AdV (data not shown). Under the conditions of adenovirus infection employed in these experiments, no effects on expression of the endogenous IL-12/23p40 were observed. That is, as assessed by direct measurement of IL-12/23p40 levels in culture supernatants by ELISA, adenoviral infection led neither to the induction of IL-12/23p40 production in unstimulated macrophages or DC, nor to alterations in SAC-induced IL-12/23p40 production (data not shown). Critical parameters for assuring a lack of effect of adenovirus infection on expression of the endogenous gene, revealed by preliminary optimization experiments, included: (a) washing the cells to remove unbound virus after infection; and (b) stimulation with SAC within 4–6h after adenoviral infection. Thus, transduction with pSLUG-p40f-AdV allows for functional study of the IL-12/23p40 promoter in primary macrophages and DC.

4. Discussion

Although much has been learned about gene regulation through the use of transformed cells, it is clear that such cells are not always representative of primary cells. Thus, new techniques are required for the assessment of promoter activity in primary cells. Here we describe a novel adenoviral vector that can be used to monitor gene expression in multiple types of primary immune cells, including macrophages, DC, and T cells. While other groups have used rAdV to probe gene expression in other cell types, this appears to be the first report of rAdV being used for such a purpose in diverse types of immune cells.

RAW264.7 cells have been widely used to probe promoter activity. This mouse macrophage-like cell line was derived from pristane-elicited murine peritoneal macrophages transformed with Abelson leukemia virus (Raschke et al., 1978). While a mouse cell line, it has been used to study both murine and human promoters, including the human IL-12/23p40 promoter (Ma et al., 1996; Plevy et al., 1997; Wang et al., 2000). Even though RAW264.7 cells resemble murine macrophages in many aspects, there are important differences as well. Optimal p40 production from RAW264.7 cells following LPS stimulation needs an additional signal by DMSO (Ma et al., 1996); no such stimulation is needed for primary macrophages. More generally, a variety of studies have delineated important differences in signaling and gene transcription between RAW264.7 cells and primary macrophages (Russell et al., 1980; Rao, 2001; Hunt et al., 2002; Galindo et al., 2004; Rouzer et al., 2005). Such studies question the physiological relevance of extrapolating data from myeloid cell lines to primary myeloid cells. The use of adenoviral vector systems should facilitate probing of promoter regulation in primary immune cells; the cells that are physiologically most relevant for such studies. An added bonus of the rAdV system is remarkably high efficiency of transduction compared to that achieved by electroporation. Higher transduction efficiency should help avoid the potential bias induced by preferential transduction of subpopulations of cells that may differ functionally from the bulk of cells that are not amenable to transfection. It also should give a better handle on the potential effects of the transduction process on regulation of the endogenous gene being studied.

Another useful aspect of pSLUG is the ability to compare DNA regulatory regions between multiple cell types using the same vector. These types of studies may identify cis-acting

elements responsible for tissue-specific and/or cell-type specific expression of certain genes. This could be quite useful for testing the involvement of cis-acting elements that are operative in CD4⁺ versus CD8⁺ T cells during thymic selection or post-thymic activation of these two cell types. For instance, one study has shown that CD4⁺ T cell production of IFN- γ requires STAT4 signaling while CD8⁺ T cell production of IFN- γ is STAT-4 independent (Carter and Murphy, 1999). rAdV could be used to test differential utilization of STAT4 DNA binding regions within the IFN- γ gene between CD4⁺ and CD8⁺ T cells. Similarly, for cells as diverse as macrophages and T cells, pSLUG may be useful for identification of differentially used cis-acting elements that control expression of genes common to these different cell types.

rAdV may also be useful to monitor gene expression in vivo. Current methods of analysis of DNA regulatory regions in vivo involve technically challenging techniques such as, creation of “knock-in” reporter mice (Mohrs et al., 2001), transgenic reporter mice (DiLella et al., 1988), or in vivo DNA footprinting (Becker et al., 2001). The use of pSLUG-derived rAdV may offer a more simple strategy in which primary cells could be removed, transduced with rAdV, adoptively transferred into recipient mice, and tracked in vivo for gene expression. Although not for the purpose of probing promoter activity, a recent study showed the feasibility of such a rAdV transduction and adoptive transfer system (Wan and DeGregori, 2003). The recent development of in vivo imaging techniques, such as two photon microscopy and biophotonic imaging have allowed for monitoring the trafficking of T cell responses in vivo (Bousso, 2004; Malstrom et al., 2004). Thus, one use for pSLUG rAdV that we envision is a transduction and adoptive transfer approach combined with imaging technology to monitor gene expression in vivo. Such analysis should allow for analysis of gene expression in space and time during immune responses in vivo.

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Abbreviations

CAR	coxsackie and adenoviral receptor
eGFP	enhanced Green Fluorescent Protein
GM-Mac	monocyte-derived macrophages generated with GM-CSF
M-Mac	monocyte-derived macrophages generated with M-CSF
pSLUG	pShuttle-luciferase-GFP
PTEN	phosphatase and tensin homologue
rAdV	recombinant adenovirus
SAC	<i>Staphylococcus aureus</i> Cowan strain I.

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Fig. 1.

Map of pSLUG (pShuttle-*Luciferase-GFP*) Adenoviral vector. pSLUG was developed from the pShuttle vector described previously (He et al., 1998). pSLUG contains a Kanamycin^r cassette for antibiotic selection. A polylinker containing unique *XhoI*, *KpnI* and *EcoRV* sites is available for cloning of DNA regulatory regions upstream of a promoterless luciferase cDNA cassette that contains a bovine growth hormone polyadenylation signal. A cassette containing the human ubiquitin C promoter driving expression of eGFP along with a BGH/SV40 polyadenylation signal was cloned from the plasmid pShUbp-Ubp (Wan et al., 2000).

Fig. 2. pSLUG Adenoviruses transduce primary mouse and human immune cells with high efficiency. (A) Mouse CD4⁺ T cells (CAR Tg); (B) mouse CD8⁺ T cells (CAR Tg); (C) human T cells; (D) human monocyte-derived macrophages (M-CSF); (E) human monocyte-derived macrophages (GM-CSF); (F) human monocyte-derived DC. Cells were infected with the indicated MOI of pSLUG AdV. 48h later, the transduction efficiency was measured by quantifying the percentage of GFP⁺ cells using flow cytometry. Results show the percentage ($\pm$ SD) of cells that are GFP⁺. Results are representative of at least 3 independent experiments with similar results.

Fig. 3.

Inducible NF-AT promoter activity in primary T cells. (A) Splenocytes from individual CAR Tg mice ($N=3$) were harvested and activated in vitro with antibodies to CD3 and CD28. (B) Purified human peripheral blood T cells were harvested and activated in vitro with anti-CD3/anti-CD28 coated beads. Cells were then infected with either NF-ATf AdV or with NF-ATr AdV (MOI = 300IU/cell for CAR Tg T cells; 10^3 IU/cell for human T cells). 24–48h after infection, cells were cultured in media alone or in media containing PMA and ionomycin for 5h. Background luciferase activity is shown in control T cells that were not infected with AdV. Five hours after infection, T cells were lysed and analyzed for luciferase activity, shown in relative luciferase units $\pm$ SD. Flow cytometric histograms to the right of the bar graph in panel A shows the percentage of CD4⁺ T cells infected with either NF-ATf AdV (upper panel) or NF-ATr AdV (lower panel) that are GFP⁺. This experiment is representative of three independent experiments with similar results. (C) NF-AT activity is blocked by cyclosporine (CsA). T cells from CAR Tg mice were treated as described in A above and, prior to mitogenic stimulation, CsA was added and luciferase activity was measured 6h after mitogenic stimulation. Statistically significant differences are indicated by * symbols (* denotes $p<0.026$; ** denotes $p<0.007$; *** denotes $p<0.001$; Student's 2-sample *t*-test Minitab for Windows).

Fig. 4.

Effects of adenoviral transduction of luciferase activity and IL-2 production. (A) Purified CAR Tg spleen cells were activated in vitro with antibodies to CD3 and CD28 for 24h, then transduced or not with various doses of pSLUG-NF-ATf. Twenty hours after transduction, cells were stimulated (closed squares) or not (open squares) with PMA and ionomycin, and luciferase activity was measured 5h later. The relative luciferase light units $\pm$ S.D. are shown. Numbers above individual data points indicate the fold induction. (B) Purified CAR Tg spleen cells were activated in vitro with antibodies to CD3 and CD28 for 24h, then were transduced or not with various doses of pSLUG-NF-ATf. Twenty hours after transduction supernatants were harvested and IL-2 levels were quantified using an HT-2 assay as described (Leibson et al., 1981). The mean concentration of IL-2 (pg/ml) $\pm$ S.D. is shown. (C) Splenocytes from CAR Tg mice ($N = 3$) were harvested and activated in vitro with antibodies to CD3 and CD28. Cells were then infected with NF-ATf AdV (MOI = 300IU/cell). Twenty hours after infection, cells were cultured with media alone or with media containing PMA and ionomycin for 5h in the presence of brefeldin A. Cells were then stained with antibodies to CD4 and CD8, followed by intracellular staining for IL-2. The dot plot shows CD4 staining (y-axis) versus GFP (x-axis) and CD4⁺ GFP⁻ (R2) versus CD4⁺ GFP⁺ (R3) cells were gated and sent to histograms displaying the IL-2 signal on the x-axis. Results show the percent of CD4⁺ GFP⁻ versus CD4⁺ GFP⁺ cells that are IL-2 $\pm$ SD. Similar results were obtained for CD8⁺ T cells (data not shown).

Fig. 5.

Inducible IL-12/23p40 promoter activity in primary human monocyte-derived macrophages and DC. (A) Inducible IL-12/23p40 promoter activity in monocyte-derived macrophages. GM-Mac, derived from 4 different donors were infected with pSLUG-p40f AdV at an MOI = 50. Cells were stimulated in triplicate with SAC (filled bars) or with media alone (open bars). 8h later, cells were lysed and analyzed for luciferase activity. Transduction efficiency ranged from 20% to 38% (data not shown). (B) Inducible IL-12/23p40 promoter activity in monocyte-derived DC. Monocyte-derived DC from 2 different donors were infected with pSLUG-p40f AdV at an MOI = 200. Cells were stimulated with SAC (filled bars) or with media alone (open bars) 8h later, cells were lysed and analyzed for luciferase activity. NS denotes no stimulation. Results show the fold increase in luciferase activity which was generated by dividing the raw data from SAC samples for each donor by the NS samples for each donor $\pm$ S.D.