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High Efficiency Ex Vivo Gene Transfer to Primary Murine B Cells Using Plasmid or Viral Vectors

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Abstract

Primary autologous B-lymphocytes, following *ex vivo* gene transfer and re-implantation, have been successfully utilized to prevent autoimmune disease and adaptive responses to therapeutic proteins in several animal models. However, efficient gene transfer to primary B cells requires use of retroviral vectors, which increase the risk of insertional mutagenesis. Here, we evaluated several alternative gene transfer approaches. Resting splenic B cells were purified and activated with LPS, and *ex vivo* GFP gene transfer was performed by means of nucleofection, lipofectamine, adenoviral infection, or murine retroviral infection. The Adenoviral (Ad) vectors were added to B cell cultures with or without calcium phosphate precipitation. For transfection and nucleofection, naked plasmid DNA was utilized. Nucleofection technology represents a modified electroporation technique for effective transfer of nucleic acids to the nucleus and thus enhances the efficiency of transfer particularly for primary cells. Efficiency of *ex vivo* gene transfer was determined by flow cytometry using GFP, CD19, and a vital dye as markers. Nucleofection yielded the highest level of gene transfer with 60-65% of B cells being GFP+. Efficiencies were 30-35% for retrovirus, 20% for Ad5/11, 15% for Ad5/35, and 5% for lipofectamine-mediated transfection. Calcium phosphate precipitation increased efficiencies for Ad vectors to 30% (Ad5/11) and 25% (Ad5/35). Lipofectamin caused the greatest cell death at 80%, followed by nucleofection (35%), and viral vector (10-15% in each case). For all methods, gene transfer efficiencies were nearly identical for B cells from C57BL/6 or C3H/HeOuJ mice. In conclusion, recent advances in gene transfer technologies provide alternatives to retroviral vectors for primary B cells. If stable gene transfer is desired, non-integrating vector systems may be combined with transposon- or phage integrase-based systems or future site-specific systems to achieve integration into the host B cell genome.

Keywords: B cells; Transfection; *Ex vivo* gene transfer; Plasmid DNA; Adenovirus; Retrovirus

Introduction

B-lymphocytes play a crucial role in adaptive immune responses as producers of immunoglobulins and as antigen presenting cells. In addition, cancers such as B cell lymphomas can result from malignant B cells. The ability to genetically modify B cells is desirable for several reasons. Efficient gene transfer to primary B cells has the potential to induce immune responses against tumors, thereby allowing development of novel therapeutic strategies for treatment of cancer [1-4]. *Ex vivo* modified B cells can produce antibodies against tumor antigens and initiate antibody-mediated cell death. B cell derived cancers can also be more effectively targeted by monoclonal antibodies when target receptors are expressed by modified B cells [5-9].

Conversely, B cells can be genetically modified to down-regulate immune responses [10-14]. For example, B cell-mediated gene therapy induced tolerance in several animal models of autoimmune diseases and in the treatment of a murine model of the genetic disease hemophilia A [10]. In these studies, murine B cells transduced *ex vivo* with retroviral vectors expressing peptide–IgG fusion proteins become efficient tolerogenic antigen presenting cells (APCs) *in vivo* after re-implantation [11-13]. Hypo-responsiveness specific to several protein antigens encoded in the peptide-IgG fusion has been achieved by this approach [15-19]. Finally, *in vivo* and *ex vivo* expression of functional proteins or regulatory RNAs or gene markers could be useful for basic immuno- or cancer biology studies.

Unfortunately, ineffective gene transfer to primary B cells has hampered conducting these types of studies and approaches discussed above in mouse models [20-22]. The only method effective thus far relies on the use of retroviral vectors. These integrating vectors provide stable but limited levels of transgene expression and pose a risk of insertional mutagenesis [23]. Therefore, it is desirable to develop

alternative vectors for B cell-mediated gene therapy. Efficient infection of primary lymphocytes with adenoviral vectors based on serotypes Ad5/35 and 5/11 has been reported [1]. Nucleofection is a non-viral, electroporation-based method that facilitates DNA transfer to nucleus and has been developed in recent years for improved transfer of naked DNA to primary cells [24]. In this study, we describe several viral and non-viral methods for effective *ex vivo* gene transfer to primary murine B cells.

Material and Methods

Mice

C3H/HeOuJ and C57BL/6 mice were purchased from Jackson laboratories. All animals were used at 6-8 weeks of age and housed in pathogen-free environment at the University of Florida. C3H/HeOuJ were selected over the more commonly used C3H/HeJ because this particular C3H strain is not TLR4$^{-/-}$ and therefore can be stimulated with LPS.

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Cell culture

Mouse spleens were isolated from C3H/HeOuJ and C57/BL6, and single cell suspension of splenocytes were prepared using 70-nm cell strainer in the MACS buffer (PBS supplemented with 1% FBS and 2 mM EDTA). Resting B cells were isolated using a negative selection kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Purified B cells were cultured with RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine, 50μM 2-mercaptoethanol, 100μM nonessential amino acids, 1 mM sodium pyruvate, and 200 μM ITS (sigma-Aldrich). B cells were pre-stimulated with 20μg/ml bacterial LPS (*Escherichia coli* 055:B5; Sigma-Aldrich) for 36 hrs before gene transfer.

Retroviral preparation

The retroviral vector MSCV-CMV-GFP was obtained from Dr. David Scott (University of Maryland, Baltimore, MD) and packaged in the GPE-86 cell line. Preparations with a titer of more than 1x10^6 colony-forming units (CFUs)/mL were used for transduction of B cells. To generate packaging cell lines that stably produce retrovirus, 293T cells were co-transfected with MSCV-CMV-GFP, the helper plasmid pSR-G, and pEQPAM3e by standard calcium phosphate precipitation. The supernatant was collected 48 hours after transfection, filtered, and frozen at -80°C. To confirm transfection, the 293T cells were analyzed for green fluorescent protein (GFP) expression. Subsequently, the retroviral supernatants were used to stably transduce the GPE-86 packaging cell line. The GPE-86 cell line is stably transfected with gag, pol and env helper gene to eliminate the risk of existing replicative elements in the packaged retrovirus. Retroviral titers were determined by GFP expression in NIH3T3 cells (a murine fibroblast cell line). Specifically, NIH3T3 cells were treated with dilutions of retroviral supernatant and sorted for GFP signal after 72hrs and the titer calculated by Poisson analysis. Preparations with a titer of more than 1x10^6 colony-forming units (CFUs)/mL were used for transduction of B cells.

Retroviral transduction

MSCV-CMV-GFP retrovirus was packaged in the GPE-86 cell line using an ecotropic murine capsid. Supernatant from packaging cells were filtered and added to LPS-stimulated B cells in a 24-well plate, followed by centrifuging for 30min at 300g to improve retroviral transduction. B cells were cultured in the presence of 6μg/ml polybrene and 20μg/ml LPS, and evaluated for GFP signal after 48hrs.

Adenoviral infection

Adenoviral stock was a gift from Dr. Andre Lieber (University of Washington, Seattle, WA). Adenoviral stocks were freshly prepared by infection of HEK-293 cells. Once cytopathic effect was evident 30 hrs after infections, cells were harvested and washed. Virus was released by 3 cycles of freeze-thaw. Adenoviral particles were purified by a discontinuous CsCl gradient followed by a continuous CsCl gradient to completely separate infectious from defective viral particles. The viral preparations were dialyzed against Tris 20mM pH 8, 2.5% glycerol, and 25mM NaCl, concentrated using Amicon Ultra-4 MWCO 30000 concentrators (Millipore, Ville St. Laurent, QC, Canada), and sterilized by filtration through 0.22-μm Millex-GV filters (Millipore). The viral titers of both preparations (Ad5/11-GFP and Ad5/F35-GFP) were determined by DNA optic density measurement. Adenovirus 5/11 and 5/35 were added to 24-well plates containing LPS-stimulated B cells at a final MOI of 100 particles per cell. The plate was centrifuged for 15min at 300g to transiently co-precipitate B cell and viral particles in order to increase infection efficiency. To increase adenoviral gene transfer to B cells, indicated amounts of vector were mixed in to final volume of 500μl of Eagles minimal essential medium. Next, a second tube was prepared with 498μl Eagles minimal essential medium supplemented
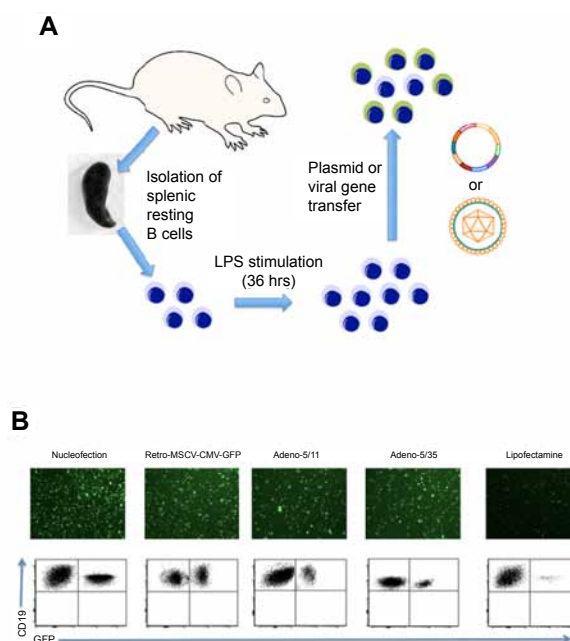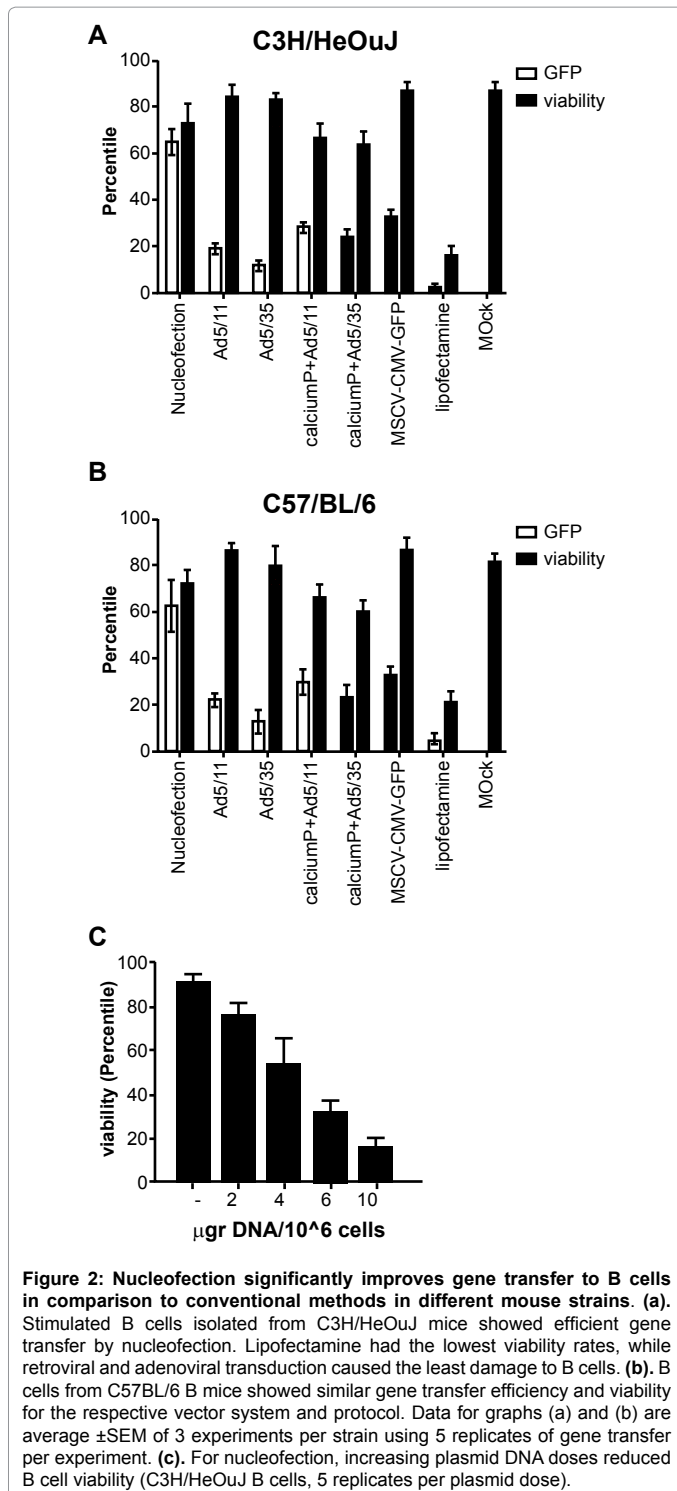

Figure 1: Nucleofection has the highest gene transfer efficiency compared to other viral and non-viral methods. Live images were captured from stimulated B cells after multiple gene transfers carrying GFP protein. Nucleofection with pMax-GFP plasmid, retroviral MSCV-GFP transduction, Adenovirus-GFP (serotypes 5/11 and 5/35) transduction, and pMax-GFP lipofectamine transfection are shown in the upper row at (40X fluorescent optical zoom). The lower row depicts flow cytometry dot plots for the corresponding gene transfer methods. In each plot, the left axis shows CD19 stain and the right axis GFP signal intensity.

Figure 2: Nucleofection significantly improves gene transfer to B cells in comparison to conventional methods in different mouse strains. **(a).** Stimulated B cells isolated from C3H/HeOuJ mice showed efficient gene transfer by nucleofection. Lipofectamine had the lowest viability rates, while retroviral and adenoviral transduction caused the least damage to B cells. **(b).** B cells from C57BL/6 B mice showed similar gene transfer efficiency and viability for the respective vector system and protocol. Data for graphs (a) and (b) are average ±SEM of 3 experiments per strain using 5 replicates of gene transfer per experiment. **(c).** For nucleofection, increasing plasmid DNA doses reduced B cell viability (C3H/HeOuJ B cells, 5 replicates per plasmid dose).

Amaxa nucleofection and lipofectamine transfection

Plasmid pMax-GFP (3.4 kbp) encoding a GFP expression cassette driven by the CMV I.E. enhancer/promoter promoter (Lonza group Ltd.) was used for nucleofection and for lipofectamine transfection. Activated B cells were collected and resuspended in Amaxa nucleofection media and transferred to nucleofector cuvettes. Nucleofection was performed with 2µg of pMax-GFP per 10^6 cells using the Amaxa nucleofector II device and Z-001 program. Nucleofected B cells were rescued in B cell culture media and incubated further for GFP expression analysis.

Conventional Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used for transfection of B cells per manufacturer's instructions. Briefly, max-GFP plasmid was incubated with lipofectamine in B cell media without FBS and B cells were resuspended in the transfection mixture according to manufacturer's guidelines and incubated for 4 hrs before adding fresh complete B cell media.

Flow cytometry and fluorescent microscopy

Cultured B cells were collected 48 hrs after gene delivery followed by antibody staining using anti-murine CD19 (1D3) conjugated to APC-Cy7 (BD Pharmingen, San Jose, CA). B cells were incubated with antibody at 4°C for 30 min, and 7-AAD, a cell viability marker, was added to samples before proceeding to FACS sorting using BD LSR II (BD Biosciences, San Jose, CA). Sorting results were analyzed with FCSExpress 5.1, and statistical analysis was performed with Prism 10.4 (GraphPad Software, La Jolla, CA). To acquire GFP signal, an inverted fluorescent microscope (Leica Camera, Allendale, NJ) was used to take live images of B cells. High-resolution pictures were processed with Openlab imaging software into publishing format.

Results and Discussion

Nucleofection yields high efficiency of gene transfer

We compared several standard viral gene delivery techniques, including adenoviral and retroviral transduction, as well as non-viral methods such as conventional lipofectamine and a novel nucleofection procedure. Each set of experiments was repeated three times to validate the results and assure reproducibility. Upon examination of GFP expression after *ex vivo* gene transfer to primary activated murine B cells, nucleofection was found to yield the highest and earliest detectable GFP signal (as early as 4 hrs post gene transfer) (Figure 1). In both of the C3H/HeOuJ and C57BL/6 mice, nucleofection yielded a high efficiency of gene transfer (65-75% of B cells were GFP+), which was 3- to 5-fold higher in comparison to adenovirus serotype 5/11 (19-25%) and 5/35 (12-18%) gene transfer and 2-fold higher than retroviral (MSCV-CMV-GFP) gene transfer (24-38%) (Figure 2a,b). Nucleofection was on average 13-fold more effective compared to lipofectamine. Retroviral transduction efficiency was comparable to previous reports by others [11,16,20,21]. However, nucleofection efficiency was improved by ~10 times in comparison to previously published studies, where the investigators used human T cell nucleofection reagents and protocols to nucleofect murine B cells because neither B cell- nor mouse-specific were available at the time [24]. We also attempted to use nucleofection for resting B cells. GFP gene transfer efficiency was approximately 70% of that achieved with LPS-stimulated B cells; however, cell viability was reduced by 50% at 24 hrs (data not shown).

Calcium phosphate precipitation increases adenoviral gene transfer to B cells

Others have previously shown that calcium phosphate precipitation

with 2µl of 2M $CaCl_2$ (ProFection, calcium-phosphate mammalian transfection system). After light vortexing, the contents of the calcium-containing tube were added to the vector-containing tube, lightly vortexed, and incubated at room temperature for 30 min. The resultant mixture contained a final concentration of approximately 6mM Ca^{2+}. A volume of 250µl of the complex was added to each well of a 24-well plate. The plate was centrifuged for 15 min at 300g and incubated for 1 h followed by aspiration and addition of fresh culture media.

of adenoviral particles onto target cells can improve transduction of hepatopoietic cells [25]. In our study with primary B cell, Incorporation of this additional step in the gene transfer protocol improved the transduction efficiency to 25-36% for serotype 5/11 and to 20-25% for 5/35 (Figure 2a,b). However, paired t-test failed to show a significant enhancing effect of calcium precipitation on transduction efficiency of serotype 5/35. In contrast, serotype 5/11 transduction efficiency was significantly improved after adding calcium treatment. Serotypes 5/11 and 5/35 are based on human serotype 5, but contain fiber knob domains from serotypes 11 or 35, respectively [26-28]. These fiber knob domains bind to the complement receptor CD46 for internalization into the target cells [29,30]. This is in contrast to serotype 5, which predominantly uses the CAR receptor. Human hematopoietic cells express high CD46 levels, which may explain why of 5/11 and 5/35 serotypes were effective in gene transfer to B cells [27,28]. However, CD46 expression is thought to be much more limited in mouse cells, suggesting that other paths toward viral entry may be at play in primary murine B cells. For example, serotypes 5/11 and 5/35 shows slower release from the endosome, which facilitates viral DNA trafficking to the nucleus.

A concern with *in vitro* gene transfer, in particular when using transfection reagents, is the potential for cytotoxicity resulting from cell damage and induction of apoptosis. However, approximately 70% percent of B cells were viable after nucleofection, while only approximately 20% of B cells survived use of lipofectamine reagent (Figure 2a,b). It is well established that the cytotoxic effects of cationic liposomes include cell shrinking, reduced mitosis, and vacuolization of the cytoplasm, which will induce apoptosis [31].

While the nucleofection procedure by itself had no negative effect on B cell viability within the 48-hr time frame of observation, increasing the amount of plasmid DNA caused fatal toxicity to the B cells in a plasmid dose-dependent manner (Figure 2c). Therefore, use of limited plasmid DNA doses is essential for preserving B cell viability. Adenoviral and retroviral infection did not decrease B cell survival significantly in comparison to B cells cultured without gene transfer, although a mild increase in toxicity (15-20% decrease in viability) was observed after calcium precipitation of adenovirus, most likely due to calcium influx into B cells (Figure 2a,b).

Implications for B cell gene transfer

Functional *ex vivo* analyses of transgenes in primary B lymphocytes have been severely hampered by the lack of an efficient and rapid gene delivery method [20-22]. Here we report that nucleofection technology allows efficient transfection and transgene expression within hours in primary B cells of two widely used mouse strains. In a side-by-side comparison, nucleofection was superior in its effectiveness compared to other nonviral and viral gene delivery methods. Nucleofection is a novel, electroporation-based method using a karyophilic solution, which, in principle, allows for an efficient nuclear delivery of plasmid DNA. Our study demonstrates that the most recently developed reagents specific for murie B cells confer high efficiency of gene transfer, which was substantially higher than in earlier reports [24]. Enriched media and an optimized activation protocol likely facilitated efficient nucleofection and transgene expression.

Currently, gene transfer for therapeutic purposes relies largely on viral vectors. However, retroviral vectors typically suffer from low titers, a requirement for active cell division to achieve integration, and oncogenic potential due to insertional mutagenesis [32]. While adenoviral vectors can be produced at high titers and transfer genes to both dividing and nondividing cells, the transient nature of transgene expression and immunogenicity are major drawbacks [33]. Plasmid vectors can yield high levels of transgene expression per transfected cell, but expression is transient due to lack of integration. However, non-viral integrating systems such as those based on transposons and bacteriophage integrases as well as site-specific or gene correction methods (such as zinc finger nucleases) are progressing [33]. For instance, PhiC31 integrase catalyzes short sequence integration between homologous bacteriophage binding site in plasmid DNA and pseudo-binding sequences in mammalian genomic DNA. The Calos lab and others have established PhiC31 integrated gene expression in different cell lines and stem cells [34-37]. Several transposon systems including sleeping beauty, Tol2, and piggyBac effectively cut and paste DNA fragments flanked by terminal repeat sequences into repetitive genomic loci. *In vitro* gene transfer to HSCs, T cells, and CD19⁺ lymphoid malignancies along with the successful *in vivo* application of transposons has been reported [38-41]. Additionally, transposon and other non-viral integrase systems have been able to transfer large donor DNA fragments up to 14 kbp, in contrast to integrating viral vectors, which typically have more limited packaging capacities.

These systems may allow for development of future nucleofection-based protocols for stable gene transfer or gene correction. Finally, re-implantation of gene-modfied primary B cells with the different methods described here will help define which vector/method combination is most useful for the purpose of immune modulation or tolerance induction.

In conclusion, we describe nucleofection and alternative technologies as effective gene delivery methods for primary murine B cells. The absence of a simple, fast, and efficient transfection method for B cells has until now restricted many studies to immortalized cell lines. Efficient gene delivery, such as by nucleofection, could provide critical information in primary *ex vivo* B cell cultures before time-consuming knockout or knock-down *in vivo* approaches and may aid in the advancement of therapeutic gene delivery approaches.

Author statement

BM and IZ performed experiments. All authors analyzed data. RWH and OC supervised the studies. BM, BKS, and RWH wrote the manuscript. None of the authors have a financial conflict for these studies.

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