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Vaccination with a *piggyBac* plasmid with transgene integration potential leads to sustained antigen expression and CD8⁺ T cell responses

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Abstract

DNA vaccination with plasmid has conventionally involved vectors designed for transient expression of antigens in injected tissues. Next generation plasmids are being developed for site-directed integration of transgenes into safe sites in host genomes and may provide an innovative approach for stable and sustained expression of antigens for vaccination. The goal of this study was to evaluate *in vivo* antigen expression and the generation of cell mediated immunity in mice injected with a non-integrating plasmid compared to a plasmid with integrating potential. Hyperactive *piggyBac* transposase-based integrating vectors (*pmhy*GENIE-3) contained a transgene encoding either eGFP (*pmhy*GENIE-3-eGFP) or luciferase (*pmhy*GENIE-3-GL3), and were compared to transposase-deficient plasmids with the same transgene and DNA backbone. Both non-integrating and integrating plasmids were equivalent at day 1 for protein expression at the site of injection. While protein expression from the non-integrating plasmid was lost by day 14, the *pmhy*GENIE-3 was found to exhibit sustained protein expression up to 28 days post-injection. Vaccination with *pmhy*GENIE-3-eGFP resulted in a robust CD8⁺ T cell response that was threefold higher than that of non-integrating plasmid vaccinations. Additionally we observed in splenocyte restimulation experiments that only the vaccination with *pmhy*GENIE-3-eGFP was characterized by IFN γ producing CD8⁺ T cells. Overall, these findings suggest that plasmids designed to direct integration of transgenes into the host genome are a promising approach for designing DNA vaccines. Robust cell mediated CD8⁺ T cell responses generated using integrating plasmids may provide effective, sustained protection against intracellular pathogens or tumor antigens.

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Conflicts of interest

The authors do not have a commercial or other association that might pose a conflict of interest.

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Keywords

DNA vaccine; cell mediated immunity; tumor; cancer; *piggyBac*; transposase

1. Introduction

DNA vaccination has emerged as a potentially effective means of inoculating a host with one or more genes encoding immunogenic proteins from pathogens or tumors [1–3]. Once the desired genes are introduced into the host cells and gain access to the transcriptional and translational machinery, production of the proteins within the cells allows processing into epitopes and presentation by major histocompatibility complex I and II (MHC-I and -II). By utilizing the host cell to generate relatively high levels of target proteins, a robust cell mediated immune response involving CD8⁺ T cells specific for those particular proteins can be generated. These stimulated effector cells will then recognize and attack cells infected with intracellular pathogens or cancer cells overexpressing certain tumor antigens while ignoring healthy cells [4]. It remains unclear whether the immune system will also eliminate the cells with integrated transgenes. The specificity and nonreplicating properties of DNA vaccines may offer strategic advantages over traditional vaccine approaches such as attenuated live microbes, inactivated pathogens, or purified proteins. While these traditional approaches for developing vaccines have succeeded for conferring protection against many pathogens, new approaches are likely needed for other pathogens such as HIV-1 or for tumor vaccines.

Plasmids used to deliver genes for vaccination have conventionally consisted of non-integrating DNA vectors due to the potential hazards of insertional mutagenesis and unknown positional effects for vectors that integrate randomly [5]. However, the design of safely integrating plasmids involve user-defined directed integration that, if successful, would improve the safety of insertional therapies. While current transposon vector systems are semi-random at best, the goal is to develop plasmids that are designed to insert into loci that are well-suited for gene transfer and defined as genomic safe harbors. Integrations within these sites would thus not be associated with adverse effects such as proto-oncogene activation or tumor suppressor inactivation [6, 7]. For example, user-defined directed transposition to the CCR5 genomic safe harbor was demonstrated in ex vivo cells and single-copy clones harboring targeted integrations were isolated [8]. Integrating plasmids utilize transposons as mobile genetic elements that are capable of self-directed excision and subsequent reintegration within the host genome. Transposases play a key role in the use of these plasmids due to their capacity to recognize and bind to the inverted repeat elements flanking transposons, cut this DNA segment from the donor and reinsert it to the recipient genome. Transposase elements such as *piggyBac*, *Sleeping Beauty* and *Tol2* catalyze these reactions and have shown potential as tools for the stable integration of transgenes when used in the binary plasmid mode [9]. Recent modifications to the transposase and/or the terminal repeats of the transposon have increased their integration efficiency, and/or specificity in ex vivo cell systems, but have not yet achieved the ultimate goal of safe harbor integration in vivo [10–12]. Transgene transmittance to daughter cells was shown in ex vivo cell systems [8], but questions remain regarding the sustained expression of antigen would be achieved in vivo or if antigen expressing cells would be targeted for elimination by the immune system.

If safe integration of desired genes into the host genome can be achieved using these engineered plasmids, they may serve as an invaluable tool for gene delivery in applications such as combating genetic disease, cancer therapy, or vaccination. Standard plasmids containing CMV promoter-driven antigen expression have in some cases demonstrated the

ability to generate expression in some tissues for extended periods, but the goal is to improve expression to more consistently sustained levels that lead to stronger immune responses. New approaches involving minicircle DNA for more sustained transgene expression have led to more effective CD8⁺ T cell responses [13]. Also, the magnitude and the contraction phase of the CD8⁺ T cell response following intradermal DNA immunization was shown to be regulated by the duration rather than the initial exposure to antigen [14]. Cytomegalovirus (CMV) infection, even with a strain limited to a single cycle, drives an inflation of CD8⁺ T cell memory [15], and the development of CMV plasmids delivered intramuscularly have shown sustained expression and may prove to be an effective vaccine vector. The use of plasmids containing the *piggyBac* transposase for vaccines has not been thoroughly investigated. In fact, it is unclear whether vaccination with a plasmid that promotes the stable integration of a gene encoding an immunogenic protein provides stronger cell mediated immunity compared to comparable non-integrating plasmids. In this study, we set out to compare *pmhy*GENIE-3 plasmid, which has integrating potential, to a non-integrating *pmhy*GENIE-3-ΔPB plasmid for their ability to promote sustained GFP and luciferase protein expression and generate CD8⁺ T cell responses in mice. The results of this study will provide the framework for developing safe integrating plasmids as effective vaccine modalities.

2. Materials and methods

2.1 Mice

Female BALB/c mice at 4–8 weeks of age were purchased from Charles River (Hollister, CA, USA) and maintained in a specific pathogen-free animal facility for at least 1 week before experiments. Procedures were performed in accordance with institutional guidelines approved by University of Hawaii Institutional Animal Care and Use Committee (IACUC).

2.2 Plasmids and lipids

Liposomes containing DOTAP and DOPE in a 1:1 molar ratio were prepared as follows. The lipid mixture in chloroform was dried under a stream of nitrogen as a thin layer in a 10-mL round-bottomed tube. The lipid film was hydrated in PBS to give a final concentration of 10 mg/mL. The multilamellar vesicles obtained were then sonicated with Avanti sonicator (Avanti Polar Lipids, Alabaster, AL, USA) and incubated for 10 min with plasmid DNA before subcutaneous (s.c.) injection of 20 μg each of plasmid and lipid per mouse. Mock injections contained liposomes only. The two constructs *pmhy*GENIE-3-eGFP and *pmhy*GENIE-3-GL3 [16] were used as integrating plasmids. In order to construct eGFP and luciferase non-integrating plasmids (*pmhy*GENIE-3-ΔPB-EGFP/*pmhy*GENIE-3-ΔPB-GL3), *piggyback* transposase was excised from *pmhy*GENIE-3 constructs by XhoI restriction digestion and re-ligation. *PiggyBac* catalyzes transposition via a “cut and paste” mechanism involving recognition and binding of *piggyBac* to the terminal repeat elements (TREs) flanking the transposon, excision of this segment from the donor DNA and integration of this DNA into TTAA sites within the host genome [17]. PB integrations displays a semi-random pattern, determined for example by the availability of TTAA sites and the accessibility of chromatin [7].

2.3 PCR analyses of gene integration

Total genomic DNA was extracted from skin of Balb/c mice using the DNeasy Tissue kit (Qiagen, Hilden, Germany). PCR of 100 ng genomic DNA template was performed using the Taq DNA polymerase kit (Qiagen) in a thermal cycler (GeneAmp PCR System 2400, Perkin Elmer, Waltham, MA). The PCR condition was: 2 min at 95°C, 35 cycles of 30s at 95°C, 30s at 61°C, 30s at 72°C and 5 min at 72°C. The sequences of the PCR primers were as follows: eGFP forward: 5'-ACG TAA ACG GCC ACA AGT TC-3' and reverse: 5'-TGC

TCA GGT AGT GGT TGT CG-3', Ampicillin forward: 5'-TTG CCG GGA AGC TAG AGT AA-3' and reverse: 5'-CGA TTT CGG CCT ATT GGT TA-3' and MsrB1 forward: 5'-CAG AGT GGT CTG AAC TTT AGA GC-3' and reverse: 5'-ATC AGC AGA ACC CAG CAA GAA GG-3'. Water was used as a negative control. As positive controls, non-integrating *pmhy*GENIE-3-ΔPB and integrating *pmhy*GENIE-3 were used. After PCR amplification, reaction products (10 μL) were resolved in 1.5% agarose gel stained with SYBR-Green (Invitrogen Life Technologies, Grand Island, NY). MsrB1 amplification served as a control for equal template loading. Quantitative PCR assays were performed by duplex Taqman real-time PCR, to interrogate the presence of the transgene containing piggyBac terminal repeat elements (PB TRE) of *pm*GENIE plasmids. The telomerase reverse transcriptase (*Tert*) gene, which is a known mouse single copy gene, was used as a housekeeping reference. Primers and probes were custom designed (to the 5'-TRE for PB and the 3'-TRE for SB) or pre-made (*Tert*) and were supplied by Applied Biosystems. The primer and probe sequences are as follows: *PB 5TRE Forward* GTG ACA CTT ACC GCA TTG ACA AG, *PB 5TRE Reverse* GCT GTG CAT TTA GGA CAT CTC AGT, *PB Reporter* ACG CCT CAC GGG AGC TC. The *Tert* assay location is chr.13:73778992 on NCBI build 37. It has a 96 bp amplicon that maps within intron 8 of the *Tert* gene. The assays were performed according to the TaqMan copy number assay protocol (Applied Biosystems) using the Life Technologies Quantstudio 12k Flex PCR machine in a 10 μl reaction volume containing 10ng DNA. Five replicates per sample were assayed.

2.4 Fluorescence and luminescence measurements

HEK293 (1×10^6) cells were resuspended in 10 μL of T buffer and transfected with 2 μg each of eGFP and luciferase plasmids in 10 μL tips using a Neon transfection system (Life Technologies, Foster City, CA). Fluorescence or luminescence was monitored over a 24 h period. eGFP-positive cells were detected using an Olympus IX71 inverted fluorescence microscope and counted in 10 random fields at 100× magnification. Bioluminescent signals from luciferase transfected cells were monitored using the IVIS Lumina (Perkin Elmer, Waltham, MA, USA). To assess localization of luciferase transfected cells *in vivo*, after i.p. injections of 0.1 mL of 15 mg/mL D-luciferin, bioluminescence signals of vaccinated mice were evaluated using the IVIS Lumina. Regions of interest were identified around the injection sites and quantified as total photon counts using Living Image™ software. The values were expressed as average of all vaccinated mice for every treatment group. Flow cytometric detection of CD8⁺ T cells expressing intracellular perforin were performed as previously described [18]. APC-anti-CD8 and PE-anti-perforin (BioLegend) were used at a final dilution of 1:500 and 1:200, respectively.

2.5 Measurement of immune responses

Spleen and lymph node cells of immunized mice (1×10^6) were stimulated for 72 h with 10 μg/mL eGFP peptide (200–208 epitope) in the presence of 5 IU/mL interleukin-2 (IL-2). For detection of IFN-γ in the cytoplasm Golgi-plug (BD Biosciences, San Jose, CA, USA) was added 6 hours before staining. Fixation/permeabilization kits were used in combination with CD3-PECy5, CD8-APC, IFN-γ-PE antibodies (eBiosciences, San Diego, CA, USA). Data were collected using the FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Blood (200 μL) was collected from BALB/c immunized mice. PE-labelled H2-D^b pentamer (PENT) presenting the eGFP 200–208 epitope was purchased from Proimmune (Sarasota, FL, USA) and cellular staining protocol performed as recommended by the manufacturer. Briefly, 2×10^6 splenocytes were stained with 10 μL of PENT at room temperature for 10 min, washed and further incubated for 20 min with CD3-FITC, CD8-APC and CD16/32 PE-Cy5 (BD Biosciences). PENT⁺/CD8⁺ cells were analyzed on a FACScalibur Flow Cytometer and analyzed using FlowJo software.

3. Results

3.1 Non-integrating and integrating plasmids promote equivalent transfection efficiency and expression of transgenes into protein in cultured cells

For these studies a *pmhy*GENIE-3 *piggyBac* vector that catalyzes the insertion of a transgene-containing transposon was generated (integrating plasmid, 14 kB) along with a *piggyBac* transposase-deficient version of this plasmid (*pmhy*GENIE-3- Δ PB-eGFP) containing the same transgene and DNA backbone (non-integrating plasmid, 12.3 kB). We first compared the transfection efficiency of cultured cells for the non-integrating and integrating *piggyBac* plasmids containing the transgene encoding eGFP (Fig. 1A). Transfection efficiencies of HEK293 cells with *pmhy*GENIE-3- Δ PB and *pmhy*GENIE-3 *piggyBac* vectors encoding eGFP were similar. We also evaluated protein expression in HEK293 cells transfected with *pmhy*GENIE-3- Δ PB and *pmhy*GENIE-3 *piggyBac* plasmids encoding luciferase (Fig. 1B). Results demonstrated that luciferase expression as measured by luciferase activity in cells transfected with the non-integrating *pmhy*GENIE-3- Δ PB and integrating *pmhy*GENIE-3 *piggyBac* vectors were similar. Overall, the non-integrating and integrating versions of these plasmids were equivalent in the transfection efficiency and transgene expression in cultured cells.

3.2 The integrated eGFP gene is detectable in DNA from injection site

Using *in vivo* vaccination, we next explored whether the *pmhy*GENIE-3 plasmid mediates the insertion of the desired transgene into the host chromosomal DNA. Non-integrating *pmhy*GENIE-3- Δ PB and integrating *pmhy*GENIE-3 encoding eGFP were encapsulated in liposomes and s.c. injected into the hind flanks of mice. Tissue from the injection site was analyzed for the presence of the eGFP gene by PCR on day 28. As shown in Fig. 2A, tissues from mice injected with the *pmhy*GENIE-3-eGFP contained consistently detectable eGFP gene DNA without any detectable plasmid backbone (ampicillin DNA). In contrast, one of three mice injected with *pmhy*GENIE-3- Δ PB-eGFP exhibited both detectable eGFP DNA and plasmid backbone DNA, which suggests the plasmid was lost in the other two mice.

As an additional, more objective assessment of transgene insertion into the mouse genome, DNA extracted from the injection site was treated with the restriction enzyme, Dpn I, which degrades methylated plasmid DNA but not unmethylated chromosomal DNA. Results from qPCR showed that the transgene target was detectable in DNA extracted from mice injected with non-integrating *pmhy*GENIE-3- Δ PB, but the transgene DNA was undetectable after Dpn I treatment (Fig. 2B). In contrast, the target transgene was detected in DNA from mice injected with the integrating *pmhy*GENIE-3 and Dpn I treatment did not alter qPCR detection of this transgene DNA. Overall, these results support the notion that the *piggyBac* transposase successfully directed integration of the eGFP gene into the host genome and the non-integrating plasmid was more likely to lose detectable transgene at the site of injection.

3.3 Immunization with integrating *piggyBac* leads to sustained antigen expression

We next investigated the *in vivo* expression of protein from the non-integrating versus integrating plasmids. The luciferase plasmids were used for this experiment since *in vivo* protein expression is quantifiable using the IVIS imaging system that detects *in vivo* luciferase activity. *pmhy*GENIE-3- Δ PB-GL3 and *pmhy*GENIE-3-GL3 plasmids were encapsulated in liposomes and injected in the hind flank of mice. The following day (Day 1) as well as on Days 14 and 28 the expression of luciferase protein was analyzed using an IVIS imaging system (Fig. 3A). On Day 1 there was equivalent expression of luciferase for *pmhy*GENIE-3- Δ PB-GL3 and *pmhy*GENIE-3-GL3 plasmids that was consistent with the results shown above for cell culture transfection. The luciferase expression decreased over time for both the non-integrating and integrating plasmids, but the *pmhy*GENIE-3-GL3

mediated luciferase expression retained detectable signal up to day 28 (Fig. 3B). This demonstrates a more sustained expression of protein from the integrated luciferase gene compared to expression from a nonintegrated plasmid luciferase gene.

3.4 Vaccination with the integrating plasmid generates more CD8⁺ T cells compared to the non-integrating plasmid

Since eGFP represents a non-self protein that can serve as a vaccine antigen for mice, we utilized the non-integrating and integrating plasmids encoding this protein to compare their potential as vaccine vectors. Importantly, pentamer reagents are available that detect mouse CD8⁺ T cells specific for the eGFP 200–208 epitope. This allowed us to track the frequency of eGFP specific CD8⁺ T cells generated over time in vaccinated mice. Results showed that after both the primary and secondary vaccination with the *pmhy*GENIE-3-EGFP plasmid significantly more eGFP-specific CD8⁺ T cells were detected in the blood of these mice compared to those mice vaccinated with the non-integrating *pmhy*GENIE-3-ΔPB-EGFP plasmid (Fig. 4). The CD8⁺ T cell numbers specific for eGFP followed the classic expansion/contraction pattern as expected, with three-fold more eGFP-specific CD8⁺ T cells at peak response in mice vaccinated with the integrating plasmid compared to those vaccinated with non-integrating plasmid. Note that a secondary boost with the integrating *pmhy*GENIE-3-EGFP plasmid was required for optimal immune responses similar to our previous study using viral vectors [19]. This may have been due to immune responses against the cells expressing antigen. To determine if infiltrating cytotoxic T lymphocytes (CTL) were present in the injection site, cells from the skin tissue at this site were analyzed by flow cytometry for the frequency of CD8⁺perforin⁺ cells (Fig. 5). Injection with the integrating *pmhy*GENIE-3-EGFP plasmid led to higher numbers of infiltrating CTL compared to mock injected and injection with non-integrating *pmhy*GENIE-3-ΔPB-EGFP plasmid. This demonstrates the generation of stronger cell-mediated immune responses using the integrating plasmid and suggests that infiltrating CTL contribute to the eventual decrease in antigen expression exhibited by tissue containing the integrated transgene.

3.5 Vaccination with the integrating plasmid generates functional CD8⁺ T cells that produce IFN γ

We next investigated whether the higher numbers of CD8⁺ T cells generated in the blood of mice vaccinated with the integrating plasmid corresponded to increased function of these cells. Mice were given primary and secondary s.c. injections in the hind flank with liposomes alone (mock injection), 20 μ g of integrating plasmid or 20 μ g non-integrating plasmid as described in the Methods section. Conventional restimulation assays were used to test functionality of CD8⁺ T cells. Specifically, spleens were removed from the eGFP plasmid DNA vaccinated mice on day 28 and splenocytes pulsed with eGFP peptide (200–208 epitope) for three days, and then intracellular staining and flow cytometry was used to measure the level of IFN γ within the CD8⁺ T cells. Results showed that eGFP peptide stimulated IFN γ production only in the splenocytes cultured from the mice vaccinated with the *pmhy*GENIE-3-eGFP plasmid (Fig. 6).

4 Discussion

DNA vaccines have been licensed for use in certain veterinary or aquacultural applications [20, 21]. While safe DNA vaccines approved for use in humans are still under development, strategies have been employed to make these vaccines more safe and efficient with Phase I and II clinical trials based on DNA vaccination protocols currently being conducted [22–24]. The form of DNA used for vaccines may include ‘naked’ DNA or DNA engineered within a viral or plasmid vector. The use of viral vectors such as *Vaccinia virus* and others to deliver antigen genes has been extensively explored and offers promise as a mode of recombinant

live vaccination and cancer therapy [25, 26]. However, the safety of these live recombinant vectors is always a concern as uncontrolled replication can result in complications from vaccination or therapeutic use. Viral vectors that are incapable of replicating in mammalian cells such as the Fowlpox virus have been pursued as safe alternatives [19, 27], but the need for repeated boosts that may lead to neutralization of the viral vectors by circulating antibodies in these secondary inoculations is problematic.

The use of plasmids to encode antigen genes are appealing because they are stable, can be manufactured with relative ease and efficiency and do not require a preservative in final preparation. Another advantage of plasmid DNA vaccines is the potential for maintaining consistency in the plasmid components or backbone as a platform technology, allowing the simple exchange of genes encoding vaccine antigen [2]. Plasmids used in vaccine studies conventionally express protein from extrachromosomal pDNA [2], but since this expression is transient it is expected that this approach would only produce short bursts of protein expression and require multiple injections or 'boosts.' The sustained expression of antigen has been linked to stronger CD8⁺ T cell responses and memory formation [14, 28]. It should be noted that not all nonintegrating plasmids induce transient expression of antigen. In particular, CMV plasmid vectors have been used to express genes at high levels for extended periods of time after delivery [29, 30]. Sustained antigen expression using new generation nonintegrating vectors is also an option, such as the approach involving mini-intron plasmids [13]. Our findings using a different approach suggest that plasmids designed to promote transpositional insertion of the gene to be expressed into the host genome, improve levels of the protein expressed and the efficacy of immunization for generated cell mediated immunity compared to conventional transient expression plasmids. While our data involving PCR analyses of genomic DNA suggest that piggyBac promotes integration of the transgene, more definitive techniques are being developed to confirm stable integration and identify the sites of integration.

While our findings show that a boost with the integrating *pmhy*GENIE-3 plasmid leads to increased CD8⁺ T cells, the use of stably expressed antigen in host cells may be optimized to eliminate the need for boosts. The increased immune response arising from primary and secondary injections are likely due to infiltration of CTL into the injection site tissues shown in our data, and this may lead to destruction of antigen expressing cells. The secondary boost could provide a new wave of transgene expression and perhaps secondary inflammation from the injection process itself that promotes sustained antigen expression, presentation, and antigen-specific CD8⁺ T cell expansion. Currently, we are working on a new version of *pmhy*GENIE-3 plasmid in which HS4 insulators have been included to the transposon. This approach avoids the transgene from being silenced and prolongs antigen expression. The HS4 insulator approach may also be utilized in non-integrating vectors and, until safe integration of transgenes is achieved, may be a more suitable approach. The rapid and robust immune response to vaccination exhibited by hosts with transgene integration is a prerequisite for providing effective immunity against intracellular pathogens or against tumor formation. However, integration also poses a number of challenges for safely inserting the target gene into the genome. Much progress has been made toward the goal of developing *piggyBac* plasmids that direct insertions into safe sites of human genomes [7, 8, 12]. An important feature is the self-inactivating plasmids that contain the transposase and transposon in a single construct [16]. This avoids the requirement of using helper plasmids while reducing the danger of overactive transposase activity leading to overinsertion of the transgene [11]. As these and other safety mechanisms are optimized for *piggyBac* plasmids, their value as vectors for DNA-based vaccination will increase and they may play an important role in the future of immunization against infectious diseases and cancers.

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Abbreviations

eGFP	enhanced green fluorescent protein
Luc	luciferase

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Highlights

- A transposase plasmid (*pmhy*GENIE-3) inserts a transgene into the mouse genome.
- Expression of protein from this integrating plasmid is sustained in vivo.
- Vaccination with the integrating plasmid generates cell mediated immune responses.

Figure 1.

pmhyGENIE-3 and *pmhyGENIE-3-ΔPB* show equivalent in transfection efficiency and protein expression. HEK293 (1×10^6) cells were transfected with 2 μ g of each plasmid, (A) *pmhyGENIE-3-eGFP* and *pmhyGENIE-3-ΔPB-eGFP* or (B) *pmhyGENIE-3-GL3* and *pmhyGENIE-3-ΔPB-GL3*. Cells were monitored over time for percent cells with eGFP signal or luciferase activity as described in the Methods section. Data are shown for 48 h post-transfection and represent mean + S.E.M. (N = 3), means were compared using a student's t-test with significance at *P < 0.05. In both cases, no significant differences were found. Results are representative of two independent experiments.

Figure 2.

Injection with the integrating plasmid encoding eGFP leads to detectable eGFP DNA but not plasmid backbone DNA. Balb/c mice (N = 3/group) were s.c. injected in the hind flank with liposomes (mock), non-integrating *pmhy*GENIE-3-ΔPB-eGFP in liposomes, or integrating *pmhy*GENIE-3-eGFP in liposomes. After 28 days, 25 mg skin was excised from the injection site of each mice, and DNA extracted for PCR to detect the presence of the eGFP transgene or the DNA backbone (ampicillin gene). To demonstrate equivalent extraction of DNA from each tissue, PCR was also performed to amplify a housekeeping enzyme gene methionine sulfoxide reductase (MsrB1). PCR reactions (10 μL) were analyzed by SYBR-Green stained agarose gel electrophoresis. Results demonstrated consistent detection of eGFP DNA in mice injected with the integrating *pmhy*GENIE-3-eGFP vector, with no DNA backbone (ampicillin DNA) detected, while only one of three mice injected with *pmhy*GENIE-3-ΔPB-eGFP exhibited detectable eGFP DNA that was associated with plasmid backbone. Results are representative of two independent experiments.

Figure 3. Injection with *pmhy*GENIE-3-GL3 leads to sustained expression of luciferase protein. (A) Representative images from mice s.c. injected with liposomes alone (mock), *pmhy*GENIE-3- Δ PB-GL3, or integrating *pmhy*GENIE-3-GL3 and luciferase activity monitored over time using an IVIS imaging system. (B) Data are shown for Days 1, 14 and 28 post-injection and represent mean + S.E.M. (N = 5/group). Means were compared using a student's t-test with significance at *P < 0.05.

Figure 4.

Injection with integrating *pmhy*GENIE-3-eGFP leads to higher numbers of specific CD8⁺ T cells compared to *pmhy*GENIE-3-ΔPB-eGFP. (A) Flow cytometric detection of CD8⁺ T cells specific for the eGFP epitope corresponding to amino acids 200–208 was performed using a fluorescent pentamer. (B) Data are shown for blood cells collected at different timepoints after injection and represent mean + S.E.M. (N = 5/group). Means at peaks after first and second injections were compared using a student's t-test with significance at *P < 0.05.

Figure 5.

Injection with *pmhy*GENIE-3-eGFP leads to infiltrating cytotoxic T cells at the site of injection. Skin tissue from injection site was dissociated into single cell suspensions, which were stained for surface CD8 and intracellular perforin and evaluated by flow cytometry (A) A PE-conjugated rat IgG2A isotype control antibody was used to set a gate on those cells staining positive for intracellular perforin. (B) Representative images of flow cytometry data showing CD8⁺perforin⁺ cells detected in the skin tissues from mice injected with the integrating pDNA-GFP. (C) Percent CD8⁺perforin⁺ cells from injection site. Data represent mean + S.E.M. (N = 5/group) and means were compared using a student's t-test with significance at *P < 0.05.

Figure 6.

Injection with *pmhy*GENIE-3-eGFP leads to functional CD8⁺ T cells capable of IFN γ production. (A) Representative data showing flow cytometric detection of intracellular staining for IFN γ in CD8⁺ T cells after pulsing with an irrelevant peptide or with eGFP 200–208 peptide. (B) Data are shown for splenocyte restimulation experiments and represent mean + S.E.M. (N = 5/group), with means compared using a student's t-test and significance at *P < 0.05.