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Use of Cre-adenovirus and CAR transgenic mice for efficient deletion of genes in post-thymic T cells

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Abstract

Conditional gene deletion using lineage-specific transgenic expression of Cre has been useful for defining the role of specific gene products in mice in vivo. However, this technology has had limitations for studies of peripheral T cell biology, since the T-lineage promoters commonly used are active early in thymic development. As such, T cell development can be altered by the resulting genetic alterations, thus limiting the interpretation of the data in post-thymic T cell studies. Thus, new strategies are needed to delete targeted genes directly in peripheral T lymphocytes. The availability of transgenic mice expressing the CAR in the T cell compartment enabled testing of Cre-mediated recombination using an adenoviral vector in naïve peripheral T cells in vitro, even without cellular activation. Using Rosa26R reporter×CAR transgenic mice, we describe conditions by which Cre-mediated deletion of targeted genes can be achieved with primary T cells in vitro. These cells can also be adoptively transferred into defined recipient mice for study in vivo. We use conditional PTEN-deficient mice as proof of concept to confirm the value of this technique for deleting a negative regulator of T cell activation. This technology should be broadly applicable for studies of T cell specific gene deletion to gain understanding of function in the post-thymic T cell compartment.

Keywords

Adenoviral transduction; Cre; CAR; T cells

1. Introduction

Conditional deletion via Cre-loxP technology has proved to be a powerful approach for assessing the function of genes of interest in specific mouse tissues in vivo. The Cre recombinase is encoded by the *cre* gene of bacteriophage P1 (Hamilton and Abremski, 1984). Cre specifically recognizes a 34-bp site on the P1 genome called loxP and efficiently catalyzes reciprocal conservative DNA recombination between pairs of loxP sites, resulting in excision of the DNA between the loxP sites (Nagy, 2000). Because neither accessory host factors nor DNA topological structures appear to be required for efficient Cre-mediated DNA recombination, it has become an important tool for genomic manipulation in eukaryotic cells. Using tissue- or cell-specific promoters driving Cre expression combined

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with a loxP-flanked gene allele of interest enables the study of selected genes in a specific tissue or at a particular developmental stage (Logan et al., 2002; de Boer et al., 2003; Belteki et al., 2005).

Obtaining gene segment deletion in post-thymic T cells for studies on the functional impact of the absence of that gene on T cell-based immune responses in the periphery has remained a challenge with this technology. The major dilemma using T cell lineage-specific Lck (Gu et al., 1994) or CD2 (Wilson et al., 2002) promoter/enhancer cassettes to drive Cre expression is that deletion of the gene of interest occurs in thymocytes, at the development stage of T-lineage cells. Thus, conditional knockout mice for various genes have been invaluable for implicating those gene products in thymic development (Hagenbeek et al., 2004). However, with thymic development altered, it becomes difficult to establish the exact contribution of a particular gene in post-thymic T cells, for example selectively in the naïve, effector, or memory states. Thus, development of a new technology to target specific Cre-mediated deletion in post-thymic T cells would be extremely useful for these types of experimental questions.

To pursue this end, we have taken advantage of CAR Tg mice that express CAR in the T cell lineage off an Lck promoter/CD2 enhancer cassette (Wan et al., 2000). When crossed with mice having a specific gene segment targeted by flanking loxP sites, a Cre-adenovirus should be capable of mediating deletion of that gene segment in vitro, either in resting or activated T cells. We utilized the Rosa26R reporter mouse as a model to test this notion, and report strategies for efficient gene deletion in peripheral T cells using this approach. The simplicity and efficiency of this system should make it a useful tool for genetic manipulation of post-thymic T cells for mechanistic studies, both in vitro and following adoptive transfer in vivo.

2. Materials and methods

2.1. Mice and cells

CAR Tg mice expressing the extracellular domain of the CAR under control of the Lck promoter/CD2 enhancer were generated as described by Wan et al. (2000). The R26R mice, with a Rosa promoter driving the β -gal transgene, were kindly provided by Dr A. Imamoto (University of Chicago) (Zambrowicz et al., 1997). PTEN/loxP-targeted mice were kindly provided by Dr Tak Mak (Ontario Cancer Institute) (Suzuki et al., 2001). CAR Tg mice were interbred with R26R mice and with PTEN/loxP-targeted mice. All mice were maintained under specific pathogen-free conditions in a barrier facility at the University of Chicago according to approved protocols and NIH guidelines. CAR Tg Th1 cells were generated following OVA immunization, and were maintained by weekly passage as previously reported (Gajewski et al., 1989).

2.2. Adenoviral vectors

An adenoviral vector containing the gene expression unit of *cre* (Ad-Cre) and an adenoviral vector without a coding cDNA (EV) were generated as described by Marks et al. (2003). The recombinant adenoviral vectors containing a human UbC promoter were generated according to a two-cosmid system protocol as described by Wang et al. (2000).

2.3. Adenoviral transduction of CAR Tg T cells

Briefly, CAR Tg T cell clones were harvested and purified by centrifugation over Ficoll-Hypaque, washed twice, and resuspended in DMEM supplemented with 2% FCS at 2×10^7 /ml. They were then mixed with an equal volume of DMEM containing 2% FCS and the desired MOI of Ad particles and incubated at 37 °C for 1 h. The cell/virus mixture was then

transferred to a 10-cm tissue cell culture dish, incubated overnight at 37 °C in an 8% CO₂ atmosphere, then washed and used for the experiments. Naïve CAR Tg T cells were isolated by negative selection using total T cell enrichment cocktail purchased from Stemcell Technologies Inc (Vancouver, BC). The cells were then transduced as described above. For the naive cells, additional culture for 3–10 days was performed in the presence of rmIL-7 (1 ng/ml; BD Pharmingen, San Jose, CA) in some experiments. Additional rmIL-7 was added to the cell culture every 3 days. The cells were then harvested on the required day and used for the experiments.

2.4. T cell stimulation

The antibodies (1 µg/ml) were immobilized onto sheep-anti-mouse IgG pre-coated beads (Dynal, Oslo, Norway) by overnight incubation at 4 °C in bead-binding buffer (0.5% bovine serum albumin (BSA, Sigma, Inc., St. Louis, MO) in Ca²⁺, Mg²⁺-free Dulbecco's phosphate-buffered saline (DPBS, Gibco-BRL, Gaithersburg, MD). For stimulation, T cells were incubated with the antibody-coated beads for the required time periods at 37 °C at a 5:1 bead to T cell ratio.

2.5. Flow cytometric analysis

Flow cytometry was performed on a FACScan I instrument with Cellquest software. Briefly, 1×10⁶ cells were stained for anti-CD3-PE (purchased from BD Pharmingen, San Jose, CA). Cells were then washed and resuspended in 0.1 ml of FACS buffer (PBS with 2% FCS and 0.1% NaN₃). An equal volume of 2 mM FDG (purchased from Molecular Probes, Eugene, OR) in distilled water was added and the cells were incubated at 37 °C for 1 min. The reaction was stopped by adding 0.8 ml of ice-cold FACS buffer. Cells were incubated on ice for 15–45 min before analysis.

2.6. Cytokine ELISAs

IL-2 production was analyzed by ELISA using NUNC Immunosorp 96-well plates and antibody pairs from Pharmingen, as described previously (Griffin et al., 2000). Plates were read with a Packard (Perkin Elmer Life Sciences) plate reader using Softmax Pro software.

2.7. Western blot analysis

Briefly, cells were lysed for 30 min on ice, the lysates were then centrifuged for 10 min at 12,000 rpm as described by Rivas et al. (2001). The supernatants were transferred to a new tube, and then 5× reducing sample buffer was added to each sample. Samples were heated for 5 min at 95 °C and separated on 10% SDS-PAGE gels (BioRad Lab, Hercules, CA). After transferring onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA), Western blotting was performed. The anti-Cre Ab was purchased from Novagen (San Diego, CA), the anti-PTEN Ab was purchased from Cell Signaling Technology (Beverly, MA), and the anti-total-ERK mAb was from Zymed (South San Francisco, CA).

2.8. In vivo transfer of T cells

The T cells were washed three times and resuspended in Dulbecco's PBS. The cells were then adoptively transferred into mice by injection into the retro-orbital venous plexus of anesthetized mice (Brown et al., 2006).

3. Results

3.1. Adenoviral mediated transduction induces Cre expression in CAR Tg T cells

An adenoviral vector encoding Cre protein driven by the human ubiquitin C promoter (Marks et al., 2003; Tripathi et al., 2006) was generated and used to deliver the *cre* gene into

CAR Tg T cells. Previously we showed that CAR Tg T cells were efficiently transducible using adenoviral-mediated gene transfer and that the ubiquitin C promoter was particularly active in resting T cells (Wan et al., 2000; Marks et al., 2003; Tripathi et al., 2006). For the present study, we transduced cultured CAR Tg CD4⁺ Th1 T cell clones with increasing MOIs of adeno-Cre. Compared with empty virus-transduced T cells, an increased amount of Cre recombinase protein was detected with increasing MOI by Western blotting (Figure 1a). In order to determine whether purified primary T cells could be transduced similarly, we purified total naïve T cells from CAR Tg mice and transduced them with adeno-Cre. As shown in Fig. 1b, a comparable expression level of the Cre recombinase was detected in naïve T cells, but a higher MOI was required. Thus, a detectable level of Cre recombinase expression was achieved in both CD4⁺ Th1 T cell clones and in naïve primary T cells after adeno-Cre transduction. An MOI of 200–500 was selected for most subsequent experiments.

3.2. Cre-mediated gene deletion in CAR-Rosa26R T cells

To test the enzymatic activity of transduced Cre, we intercrossed CAR Tg mice with Rosa26R mice (Soriano, 1999). The Rosa26R mice have a Rosa promoter driving β -gal transgene expression, but a neomycin gene flanked by loxP sites is inserted between the promoter and the β -gal gene. In the presence of Cre recombinase, the insulator sequence is deleted through DNA recombination and β -gal activity becomes detectable, thus serving as a useful reporter system for monitoring Cre activity. We first purified total T cells from CAR Tg $\times$ Rosa26R reporter mice and transduced them with adeno-Cre. After overnight transduction, transduced T cells were activated with soluble anti-CD3 antibody in the presence of irradiated T-depleted splenocytes plus IL-2 for 3 days. β -gal activity was monitored by flow cytometry. As shown in Fig. 2a, the majority of adeno-Cre transduced CAR reporter T cells showed β -gal activity as indicated using cell permeable substrate FDG (Rowland et al., 1999), suggesting effective Cre-mediated gene segment deletion in the majority of adeno-Cre transduced T cells.

It was then of interest to attempt the same Cre-mediated deletion in T cells without concomitant cellular activation. We therefore primed CAR Tg $\times$ Rosa26R T cells with anti-CD3 plus irradiated T-depleted B6 splenocytes and IL-2 for 3 days, transduced them with adeno-Cre during overnight culture, and analyzed for FDG fluorescence by flow cytometry on the next day. However, this approach resulted in only a minority of transduced cells expressing β -gal activity (Fig. 2b). Similarly, when naïve splenic CAR Tg $\times$ Rosa26R T cells were transduced overnight with adeno-Cre without any T cell activation, only minimal β -gal expression was achieved (Fig. 2c). Thus, these straightforward approaches did not efficiently induce Cre-mediated recombination in resting T cells.

3.3. Cre-mediated gene deletion in primary T cells requires extended time but not cell cycle progression

Our ultimate goal was to support Cre-mediated deletion in quiescent T cells, yet our preliminary results suggested that activated but not resting T cells might be successfully manipulated with this approach. One possible explanation for this phenomenon was that perhaps Cre activity in T cells might depend on cell cycle progression to be effective. To test this hypothesis, we transduced and stimulated splenic T cells as above, in the presence or absence of aphidicolin to block DNA synthesis. As shown in Fig. 3a, aphidicolin potently inhibited T cell proliferation as measured by thymidine incorporation. We then tested whether cell cycle inhibition would abolish Cre activity. On the contrary, a similar percentage of cells displaying FDG fluorescence was detected in primed adeno-Cre transduced CAR T cells, regardless of the presence of aphidicolin (Fig. 3b), suggesting that cell cycle progression is not needed for Cre-mediated recombination in peripheral T cells.

Another possible explanation for the failure of Cre-mediated deletion in naïve T cells was inadequate time. However, naïve unstimulated murine T cells do not survive well for more than 24 h in vitro. Recombinant IL-7 was used to better support their survival for prolonged culture in vitro. Purified CAR Tg×Rosa26R splenic T cells were transduced with either empty virus or adeno-Cre, kept in culture for 3 days with IL-7 (1 ng/ml) to maintain viability, and analyzed for Cre activity by FDG FACS. As shown in Fig. 4, efficient Cre-mediated expression was detected in the majority of cells by this time point. These results suggest that an extended time period is required to support efficient Cre-mediated targeted deletion and consequent altered gene expression.

3.4. Cre-mediated gene deleted T cells can be maintained following transfer in vivo

Although in vitro transduction with adeno-Cre would be useful for studying the functional effects of gene deletion on T cell properties in vitro, it is also desirable to achieve similar results with an approach applicable for in vivo experimentation. In order to determine whether Cre-transduced T cells would persist in vivo, we transduced CAR Tg×Rosa26R T cells followed by culture in the presence of IL-7 for 3 days, verified recombination efficiency by FACS analysis (Fig. 5a), then adoptively transferred transduced cells into recipient mice by intravenous injection. We utilized P14 TCR Tg×RAG1^{-/-} mice as hosts, both to prevent homeostatic proliferation of the transferred cells (Brown et al., 2006) and to prevent immune-mediated rejection of T cells expressing both Cre and β-gal proteins. We also chose conditions in which only a subset of T cells was successfully transduced, so that we could monitor the fate of both β-gal⁺ and β-gal⁻ populations. Prior to transfer in vivo, approximately 45% of transduced cells were positive for FDG FACS. Following analysis of splenocytes from recipient mice that had received transduced T cells 7 days earlier, approximately 30% of T cells continued to show β-gal activity (Fig. 5b). Thus, persistent expression of Cre and β-gal proteins does not significantly limit the durability of transduced T cells for at least 1 week in vivo.

3.5. Cre-mediated deletion of PTEN expression in naïve splenic T cells in vitro

It was of interest to apply this strategy utilizing adeno-Cre on deletion of a gene relevant for T cell activation. PTEN is a phosphatase which dephosphorylates PIP₃ to regenerate PIP₂. Conditional deletion of PTEN within the T cell compartment leads to a lymphoproliferative disorder as well as autoimmunity (Buckler et al., 2006). We deleted PTEN in peripheral splenic T cells using adeno-Cre and investigated IL-2 production to determine whether heightened TCR signaling would be observed. Purified total T cells from CAR/PTEN-flox homozygous mice were transduced with either empty vector or adeno-Cre. In order to allow additional time for loss of PTEN protein that was already present in the T cells before Cre-mediated *pten* gene deletion, we cultured transduced T cells in the presence of IL-7 for 9 days. The cells were then either lysed and investigated for Cre and PTEN expression or stimulated with anti-CD3/anti-CD28-coated beads and assessed for IL-2 production. As shown in Fig. 6a, Cre protein expression was detected after adeno-Cre transduction. As expected, PTEN protein expression was substantially reduced in adeno-Cre-transduced T cells compared to empty vector-transduced T cells. The Cre-mediated deletion likely happened on both alleles as the ratio of PTEN versus endogenous Erk1,2 in adeno-Cre-transduced T cells was more than 70% lower than in empty vector-transduced T cells (Fig. 6b). To confirm whether T cells with diminished PTEN levels would have a functional consequence, cells were collected and stimulated for assessment of IL-2 production. As shown in Fig. 6c, T cells transduced with adeno-Cre showed increased IL-2 production, consistent with the reported negative regulatory role for PTEN in TCR/CD28-mediated signaling.

To verify that Cre-mediated deletion occurs on both alleles, we immunized the CAR/PTEN-flox homozygous mice with OVA plus complete Freund's adjuvant, and derived Th1 T cell clones. These cells could be kept in culture for longer periods of time to confirm complete loss of PTEN protein. As shown in Fig. 6d, PTEN protein was completely undetectable in CAR/PTEN-flox T cells transduced with adeno-Cre when analyzed 14 days after transduction. Thus, these results confirm the utility of adeno-Cre transduction to delete expression of a relevant gene in peripheral T cells with functional consequence.

4. Discussion

Our present study demonstrates that mature peripheral T lymphocytes can be transduced with an adeno-Cre vector and induce the enzymatic recombination of targeted DNA in vitro. This strategy allows the conditional and inducible modification of the post-thymic T cell compartment, thus providing a new tool to study gene function in mature peripheral T lymphocytes.

It has been shown previously that site-specific recombination by Cre recombinase requires no additional proteins and gives a simple recombination product. Since Cre acts virtually stoichiometrically and each of the 13-bp inverted repeats of the loxP site binds a single Cre monomer, the number of functional Cre molecules required for DNA synapsis and recombination in *E. coli* is very low (Hamilton and Abremski, 1984). In eukaryotic cells, slightly higher concentrations of Cre may be required compared to *E. coli*. The methods used for introduction of the Cre recombinase into cells in vitro have varied greatly and include electroporation or calcium phosphate-based transfection. However, these strategies are not effective at high efficiency expression in primary murine T cells. Utilizing a Cre retrovirus could also be considered, but requires T cell activation to induce cell cycle progression and thus cannot be used to study the resting naïve state. Recombinant Cre protein expressed as a cell-permeable fusion peptide has also been examined in vitro and ex vivo (Jo et al., 2001), but the efficiency of gene deletion is lower than that achieved using adenoviral transduction. The main solution for lineage/cell type-specific excision is the use of established transgenic lines expressing Cre under the control of a promoter with the required specificity. However, this strategy has the limitations described earlier when considering gene function in peripheral T cells. Thus, the strategy of intercrossing mice bearing loxP-targeted gene segments to CAR Tg mice and utilizing adeno-Cre in vitro has added a new technology that should have wide applicability for specific types of experiment. It should be noted that the amount of time needed for in vitro culture of the cells following transduction will likely vary with the half-life of the mature protein encoded by the targeted gene. Interesting genes to target at precise T cell differentiation states include: the lipid phosphatase PTEN, the absence of which gives rise to profound spontaneous T cell proliferation, autoimmunity, and T cell lymphoma when deleted centrally (Sakai et al., 1998; Di Cristofano et al., 1999); IL-7R (Cosmi et al., 2004), which is essential for optimal survival of peripheral naïve T cells (Peschon et al., 1994; Munitic et al., 2004; Purton et al., 2007; Seki et al., 2007); and signaling molecules in the Ras pathway, such as RasGRP1, which when deleted in the thymus fail to give rise to peripheral T cells (Dower et al., 2000), thus precluding functional studies in the periphery. Deletion of these or multiple other loxP-targeted genes in post thymic T cells should provide critical information regarding their function in the peripheral immune response.

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Abbreviations

CAR	Coxsackie and adenovirus receptor
Cre	cyclization recombination
loxP	locus of X-over of P1
OVA	ovalbumin
FDG	fluorescein di- β -D-galactopyranoside

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Fig. 1. Adenoviral-mediated Cre expression in CAR Tg T cells. (a) Western blot analysis of cultured CAR Tg CD4⁺ Th1 T cell clone transduced with either EV or Adeno-Cre. (b) Western blotting of splenic T cells isolated from CAR Tg mice transduced with EV or Adeno-Cre. Similar results were observed in at least two independent experiments.

Fig. 2.

Cre-mediated deletion is achieved in primed T cells after adeno-Cre transfection. (a) Freshly isolated T cells from CAR Tg×Rosa26R reporter mice were transduced with either EV or Adeno-Cre and incubated overnight. Transduced cells were then primed in culture for 3 days, and β -gal activity was analyzed by flow cytometry. (b) The same T cells were separated and primed in culture for 3 days, then transduced with either EV or Adeno-Cre, and β -gal activity was analyzed by flow cytometry on the next day. (c) T cells from CAR Tg×Rosa26R reporter mice were isolated and transduced, and β -gal activity was analyzed by flow cytometry on the next day. Similar results were seen in at least two experiments.

Fig. 3.

Cre-mediated deletion is not cell cycle dependant. (a) CAR Tg×Rosa26R reporter T cells were isolated, transduced, and primed in culture for 3 days with or without the presence of the cell cycle blocker, aphidicolin. T cell proliferation was measured by thymidine incorporation. (b) CAR Tg×Rosa26R reporter were treated as in (a) and flow cytometry was performed to analyze β-gal activity.

Fig. 4. Extended cell culture period is needed for Cre-mediated deletion. Freshly isolated CAR Tg×Rosa26R reporter T cells were transduced with either EV or Adeno-Cre and incubated overnight. Transduced T cells were maintained in culture for 3 days in the presence of 1 ng/ml IL-7, and β -gal activity was analyzed by flow cytometry.

Fig. 5.

Cre-mediated deletion is maintained in vivo. CAR Tg×Rosa26R reporter T cells were isolated, transduced, and maintained in culture for 3 days at the presence of IL-7. (a) β -gal activity was assessed by flow cytometry prior to in vivo transfer. (b) Transduced T cells were adoptively transferred into P14 TCR Tg RAG1^{-/-} mice intravenously. One week later, T cells were purified and β -gal activity was analyzed by flow cytometry.

Fig. 6.

Cre-mediated deletion of PTEN expression in peripheral T cells. CAR Tg mice were intercrossed with conditional PTEN gene-targeted mice. Splenic T cells were isolated, transduced with either empty vector or adeno-Cre, and maintained in culture for 9 days in IL-7. (a) Western blot analysis was performed for Cre and PTEN expression. (b) Transduced cells were stimulated overnight with beads conjugated with anti-CD3 mAb (0.001 μg/ml) and anti-CD28 mAb (1 μg/ml) and IL-2 production was measured by ELISA. *, undetectable. (c) Western blots were scanned and densitometry was performed to determine the ratio of PTEN to ERK signal. (d) Th1 clones were derived from CAR/PTEN-flox mice and adeno-Cre-mediated deletion was performed as for primary T cells, with culture for 14 days prior to Western blot analysis. Similar results were observed in three independent experiments.