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Role of TLR3 in the immunogenicity of replicon plasmid-based vaccines

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SUMMARY

Replicon plasmids encoding an alphavirus RNA replicase constitute an alternative to conventional DNA plasmids with promise for DNA vaccination in humans. Replicase activity amplifies the levels of transgene mRNA through a copying process involving double-stranded (ds) RNA intermediates, which contribute to vaccine immunogenicity by activating innate antiviral responses. Toll-like receptor (TLR) 3 is a dsRNA innate immune receptor expressed by antigen-presenting dendritic cells (DC). Here, we test the hypothesis that TLR3 is necessary for the immunogenicity of replicon plasmid based DNA vaccines. We show that mouse $CD8\alpha^+$ DC phagocytose dying replicon plasmid-transfected cells in vitro and are activated in a TLR3-dependent fashion by dsRNA present within those cells. However, we find that cytotoxic T cell responses to a replicon plasmid intramuscular vaccine are not diminished in the absence of TLR3 in vivo. Our results underscore the potential role of TLR3 in mediating immune activation by dsRNA-bearing replicon plasmid transfected cells and indicate that other innate sensing pathways can compensate for TLR3 absence in vivo.

Keywords

dendritic cells; toll-like receptors; double-stranded RNA; replicon vaccines

INTRODUCTION

Most currently available vaccines work by eliciting antibody responses and are relatively inefficient at inducing cellular immunity. Yet, the latter is vital for resistance to a wide panel of pathogens, including *Mycobacterium tuberculosis* and the malaria parasite, as well as

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being a critical element of anti-tumor immunotherapy. Thus, there is a need for improved vaccination approaches that elicit potent $CD4^+$ and $CD8^+$ T cell responses1. In mice, naked plasmid DNA immunization is very effective at promoting cytotoxic T lymphocyte (CTL) responses yet DNA vaccines have yielded less impressive results in human trials2,3. This may be due in part to the fact that in humans DNA vaccines are administered in a small volume. Experimental studies in mice have shown that DNA vaccines lose immunogenicity when the volume injected is reduced, suggesting that local tissue damage and consequent inflammation are critical for vaccine efficacy4. Because equivalent levels of tissue damage would be unacceptable in a clinical setting, other strategies must be explored to increase the immunogenicity of DNA vaccines.

One promising approach has been the development of replicon-based DNA vaccines, which in some studies are superior to conventional DNA vaccines at eliciting immune responses. Replicon plasmids encode an alphavirus replicase, an RNA-dependent RNA polymerase that copies and, thereby, greatly amplifies the plasmid-encoded transgene RNA5. Amplification of the transgene RNA allows greater levels of antigen expression and this was initially thought to account for the increased immunogenicity of replicon-based plasmids6,7. However, it is now clear that these vaccines effectively activate the innate arm of the immune system, which dictates the subsequent adaptive response8. Indeed, the immunogenicity of replicon plasmid-based DNA vaccines is dependent on the induction of type I interferons (IFN-α/β), a hallmark of innate virus detection9. Thus, the potency of replicon-based DNA vaccines may be due to the fact that they mimic virus infection.

The alphavirus replicases act through formation of double stranded (ds) RNA intermediates, which are a potent inducers of innate antiviral responses10. DsRNA can also directly activate dendritic cells (DC) to allow coupling of innate and adaptive immunity11. Toll-like receptor 3 (TLR3) was the first receptor described to couple detection of dsRNA to transcription of innate response genes, including those encoding the type I IFNs12-14. In the mouse, the $CD8\alpha^+$ subset of DC expresses the highest levels of TLR3 and, therefore, has the ability to respond readily to dsRNA15. We have previously shown that $CD8\alpha^+$ DC can ingest material from dying cells and use TLR3 to detect dsRNA associated with virus infection16. The triggering of TLR3 in this setting promotes $CD8\alpha^+$ DC activation and leads to cross-priming of T cells specific for antigens present within the virally-infected cells.

DsRNA is generated within cells transfected with replicon-based plasmids, leading to activation of the 2′-5′-oligoadenylate antiviral pathway, which culminates in induction of apoptosis8. Thus, we hypothesized that TLR3-mediated activation of DC by replicon-transfected cells bearing dsRNA might underlie the adaptive immune response to replicon-based DNA vaccines. Here we show that mouse $CD8\alpha^+$ DC are activated via a TLR3-dependent pathway by exposure to replicon plasmid-transfected cells. However, TLR3 is not required for the immunogenicity of replicon-based DNA vaccination in vivo.

RESULTS

Induction of apoptosis in cells transfected with replicon vector

VERO cells were transfected by electroporation with equal amounts of a Sindbis virus-based replicon plasmid (pSIN-GFP) or a conventional plasmid (pEGFP), both encoding green fluorescent protein (GFP). Six hours after transfection, 15% of replicon-transfected and 54% of cells transfected with the conventional control plasmid expressed high levels of GFP (Fig. 1A). While cells expressing GFP encoded by the conventional plasmid showed a broad distribution of GFP fluorescence levels, replicon plasmid-transfected cells showed a high proportion of cells with high levels of GFP (Fig. 1A). This difference in transgene

expression is most likely attributable to the replicon-driven amplification of the transgene RNA, as described previously17.

To determine the level of apoptosis induced by transgene expression, we measured the frequency of annexin V^+ $DAPI^-$ cells in VERO cell cultures 6 hours post electroporation. Among the GFP^- cell populations, only 3-5% of cells were annexin V^+ and $DAPI^-$ irrespective of whether they were electroporated with replicon or conventional plasmid DNA (Fig. 1A). In the population transfected with the conventional plasmid, the GFP^+ cells showed a similarly low basal level of apoptotic cells (Fig. 1A). In contrast, at least 25% of GFP^+ cells in the population transfected with replicon plasmid showed signs of apoptosis (annexin V^+ and $DAPI^-$). The percentage of cells expressing high levels of GFP correlated with the frequency of apoptotic cells and increased with the dose of replicon plasmid used for transfection (Fig. 1B and C). In contrast, there was no correlation between dose of DNA and induction of apoptosis in cells transfected with the conventional plasmid (Fig. 1B). Thus, cells expressing a replicon plasmid-encoded transgene but not those transfected with conventional plasmid readily undergo apoptosis, as previously noted18.

Expression of replicon-encoded transgenes is accompanied by the production of dsRNA

Next we measured the levels of dsRNA in VERO cells transfected with either plasmid. Six hours post electroporation, 11% of replicon plasmid-transfected VERO cells expressed GFP. All GFP^+ VERO cells could be stained for dsRNA while the GFP^- cell population remained negative (Fig. 2). The level of dsRNA in GFP-expressing replicon-transfected cells was similar to the level detected in VERO cells transfected with polyI:C, a commonly used synthetic dsRNA (Fig. 2). 41% of VERO cells transfected with 10μg of conventional plasmid DNA expressed GFP, but none of the GFP-expressing cells nor any of the GFP^- cells stained positive for dsRNA even when a higher dose of DNA was used for electroporation (Fig. 2 and data not shown). Thus, dsRNA accumulates exclusively in transgene-expressing cells transfected with replicon plasmid DNA and is not produced in cells transfected with a conventional plasmid.

Uptake of transfected cells by splenic DC

We next compared the uptake of VERO cells transfected with replicon plasmid or conventional plasmid DNA by DC. Six hours after electroporation, VERO cells were labeled with the membrane dye PKH26 and were co-cultured with CD11c-enriched splenic cells. Uptake of PKH26-labelled cellular material by $CD8\alpha^+$ DC was determined by flow cytometry19. 72% and 64% of $CD8\alpha^+$ DC stained positive for the membrane dye PKH26 after incubation with VERO cells transfected with pSIN-GFP and pEGFP, respectively (Fig. 3 A). The dye transfer reflected true uptake of cells or cell debris by DC as it was blocked by latrunculin, which prevents actin-dependent phagocytic and macropinocytic processes (data not shown; see reference 19). In multiple experiments, no significant differences were seen in uptake of replicon plasmid-versus conventional plasmid-transfected VERO cells (Fig. 3B). Thus, $CD8\alpha^+$ DC have equal access to cellular material from replicon plasmid- or conventional plasmid-transfected VERO cells despite the increased frequency of apoptosis in the former population.

DC activation by replicon plasmid transfected VERO cells

Splenocytes enriched for $CD8\alpha^+$ DC were co-cultured with VERO cells transfected with replicon and conventional plasmid DNA and DC activation was monitored by production of IL-616. VERO cells transfected with the conventional plasmid DNA did not induce DC activation at any of the ratios tested (Fig. 4A). In contrast, dose-dependent activation was seen when the DC were incubated with VERO cells that had been transfected with the replicon-based plasmid (Fig. 4A). However, the response was weaker than that to poly I:C-

transfected cells, which served as a positive control (Fig. 4A). To test whether this was due to the fact that only 10-15% of VERO cells were actually transfected with replicon plasmid, we sorted GFP^+ VERO cells and repeated the co-culture experiments. When the GFP^+ fraction of replicon plasmid-transfected VERO cells was used for the co-culture experiment, DC activation as measured by IL-6 induction exceeded that observed with polyI:C-associated VERO cells (Fig. 4B). The equivalent GFP^+ fraction from conventional plasmid transfected cells was not stimulatory (Fig. 4B). Sorted GFP^- cells from replicon plasmid or conventional plasmid-transfected samples also did not induce IL-6 (data not shown). Thus, the ability of DNA transfected cells to induce DC activation correlates with their dsRNA content.

DC activation by replicon-plasmid-transfected cells is TLR3-dependent

To test whether activation of $CD8\alpha^+$ DC in response to ingested material from replicon plasmid-transfected cells was mediated by recognition of dsRNA associated with the cellular material, activation of wild type $CD8\alpha^+$ DC was compared to activation of $CD8\alpha^+$ DC from TLR3-deficient mice. TLR3-deficient $CD8\alpha^+$ DC were unable to respond to replicon plasmid-transfected VERO cells (Fig. 4C). As expected, such DC also failed to be activated by polyI:C-transfected VERO cells or by soluble polyI:C, whereas their response to CpG 1668 oligonucleotide, which triggers TLR9, was unaffected (Fig. 4C). Thus, TLR3-mediated recognition of cell-associated dsRNA allows $CD8\alpha^+$ DC to respond to cells transfected with replicon plasmid DNA.

As the expression of the replicon plasmid-endoded transgene induced apoptosis (Fig. 1A and C) and because cell death could be sufficient to promote DC activation, we normalized for the level of apoptosis by irradiating cells prior to co-culture with DC. To ensure efficient trangene expression, VERO cells were cultured for 12 hours post electroporation. Cells then were UV-irradiated followed by another 12 hours of cell culture to allow the cells to undergo irradiation-induced apoptosis / secondary necrosis. 12 hours post irradiation, the frequency of Annexin V-positive cells in replicon versus conventional plasmid-electroporated samples was identical (data not shown). When the UV-irradiated cells were subsequently co-cultured with splenic DC, a slight increase in IL-6 was observed in response to cells electroporated with conventional plasmid, but the levels of cytokine were lower than in response to replicon plasmid-transfected cells (Fig. 4D). Thus induction of cell death in cells expressing conventional plasmid DNA might increase their stimulatory ability but this falls markedly short of that observed for cells electroporated with replicon plasmid. This can probably be attributed to the presence of dsRNA in the latter.

$CD8a^+$ T cell priming in response to DNA vaccination is TLR3-independent

Because activation of $CD8\alpha^+$ DC by replicon-expressing cells was TLR3-dependent, we wondered whether the immunogenicity of replicon vector-based DNA vaccines also depended on TLR3. We immunized wild type and TLR3-deficient mice intramuscularly with replicon or conventional plasmid vectors expressing the model antigen ovalbumin (OVA) and used anti-CD40 antibody as adjuvant to increase the immune response. Three weeks later, we analysed the number of OVA specific $CD8^+$ T cells in the spleen and in vivo cytoxicity against OVA peptide-pulsed targets. Despite a high variability within groups, overall numbers of OVA specific $CD8^+$ T cells were not significantly different for replicon and conventional plasmid-vaccinated mice (Fig. 5A). In vivo cytotoxic activity against OVA was also similar between WT and $TLR3^{-/-}$ mice independent of the type of plasmid used for vaccination (Fig. 5B). The same result was obtained in mice vaccinated in the absence of anti-CD40 antibody (data not shown). Thus while activation of $CD8\alpha^+$ DC in response to replicon-expressing cells in vitro is dependent on TLR3, in vivo CTL priming upon intramuscular injection of replicon DNA is TLR3-independent.

DISCUSSION

In this study, we tested the hypothesis that the immunogenicity of replicon-based plasmid vectors used for DNA vaccination is mediated by TLR3-dependent activation of cells of the innate immune system such as DC. We show that dsRNA, the putative agonist ligand for TLR3, is generated within cells transfected with replicon plasmid vector and that co-culture of such cells with $CD8\alpha^+$ DC, the primary mouse APC involved in crosspriming20, leads to TLR3-dependent DC activation. However, when mice were vaccinated with replicon plasmid via the intramuscular route, we found that CTL responses directed against the protein encoded by the transgene were independent of TLR3. The discrepancy between the TLR3-dependence of replicon plasmid-mediated DC activation in vitro and the redundancy of TLR3 for immunization with replicon plasmids in vivo highlights the complex nature of innate recognition of dsRNA in vivo.

Despite the repeated involvement of $CD8\alpha^+$ DC in most models of crosspriming20, the observation that induction of CTL responses by intramuscular injection of replicon plasmid DNA is independent of TLR3 suggests that $CD8\alpha^+$ DC may be dispensable for the response to replicon vaccination. This might indicate that such DC do not have access to replicon-transfected cells in vivo. We focused on intramuscular vaccination as this is the route most frequently used for DNA vaccination, particularly in humans. Skin delivery of replicon could conceivably allow greater involvement of $CD8\alpha^+$ DC present in draining lymph nodes. Alternatively, it is possible that $CD8\alpha^+$ DC are involved even in responses to intramuscular replicon vaccinations but that other receptors substitute for TLR3 in $CD8\alpha^+$ DC activation. In this regard, IFN-α/β produced by the transfected cells themselves could potentially activate DC in *trans*, promoting cross-priming, as previously suggested21.

A more likely alternative is that the response to replicon-based plasmids in vivo reflects not only cross-priming by DC of material acquired from heterologous transfected cells but also endogenous antigen presentation by DC that were directly transfected in vivo, as observed for DNA vaccination by conventional plasmids22-24. In that situation, TLR3 would not be expected to operate and DC activation would therefore ensue from triggering of other innate receptors. Notably, DNA has the potential to trigger TLR9 but, interestingly, conventional plasmid DNA vaccines are still immunogenic in TLR9-deficient mice25,26. In fact, conventional plasmid DNA vaccines appear to work independently of most TLRs as no reduction in vaccine efficiency is observed in $MyD88^{-/-}$ mice25. However, because TLR3 uses the adaptor TRIF rather than MyD88, it remained possible that TLR3 was required. The present study formally excludes a requirement for TLR3 in mediating the immunogenicity of either conventional or replicon DNA vaccines. Thus, TLRs appear largely redundant in immune responses to DNA vaccination.

All cells possess several non-TLR cytoplasmic receptors for dsRNA that activate cell intrinsic antiviral pathways11. These include the 2′-5′-oligoadenylate synthetase system, which acts to induce cleavage of cellular RNAs and/or induce apoptosis in virus infected cells11. Notably, cells transfected with replicon plasmid DNA activate the 2′-5′-oligoadenylate production and mice deficient in RNAseL, a mediator of the 2′-5′-oligoadenylate antiviral effect, display reduced CTL responses to replicon plasmid vaccination8. However, RNAseL alone does not lead to the induction of IFN-α/β, which is also essential for the immunogenicity of replicon-based DNA vaccines27. It is therefore likely that vaccination with replicon plasmid DNA leads to activation of one or another of the recently-discovered IFN-α/β-inducing cytoplasmic sensors for viral nucleic acids11. These include the RNA helicases MDA5 and RIG-I and the DNA sensor, DAI, all of which induce IFN-α/β gene transcription via a pathway involving TANK-binding kinase-1 (TBK1)28-32. Interestingly, although DAI is dispensable, TBK1 is essential for immune

responses to DNA vaccination with a conventional plasmid vector33. These observations suggest that MDA5 and/or RIG-I mediated immune activation might mediate the efficacy of DNA vaccines with replicon-based or conventional plasmids.

MATERIALS AND METHODS

Cells

African green monkey kidney (VERO) fibroblasts were grown in serum-free SF-VPM medium (Invitrogen, Paisley, UK).).

C57BL/6 mice were obtained from Charles River. TLR3$^{-/-}$ and C57BL/6 SJL-CD45.1 congenic mice were bred at Cancer Research UK. Splenocytes were prepared by digestion with Liberase CI and DNaseI (both from Roche, Welwyn Garden City, Hertfordshire, UK). CD11c-enriched cells were prepared using anti-CD11c MACS beads (Miltenyi, Bergisch Gladbach, Germany) according to manufacturer's instructions and cells were separated on an AUTOMACS apparatus (Miltenyi). Sorted CD8α^{+} DC were purified from splenic suspensions as described34.

Plasmid vectors

The following plasmid vectors were used: pEGFP-C1 was purchased from Clontech and pcDNA3-ΔOVA was cloned as described35. The replicon vectors were obtained from Chiron Corporation. They encode Sindbis replicase, which drives the replication of the encoded transgene and are derived from pSIN1.5-βgal. The GFP-encoding replicon pSIN1.5-EGFP (pSIN-GFP) has been described17, while the sequence of ovalbumin missing the leader sequence was excised from pcDNA3-ΔOVA and cloned into pSIN1.5 replicon vector.

In vitro transfection

VERO cells were harvested, resupended in electroporation buffer containing plasmid DNA and electroporated at 300mV and 150μF36. Cells were cultured for 6 hours before transgene expression and apoptosis induction were analysed. Cells were used for in vitro DC co-cultures or in vivo vaccination 6 hours after electroporation. Cells transfected with GFP-encoding replicon or conventional plasmid were used unsorted or after sorting of GFP^{+} and GFP^{-} cells on a MoFlo cell sorter. As controls, cells were electroporated with 10μg of poly I:C (GE Healthcare, Chaltfont St. Giles, Buckinghamshire, UK) at 300mV and 150μF. Poly I:C-electroporated cells were used directly for staining of dsRNA and for in vitro co-culture with splenic DC.

Apoptosis

VERO cells were harvested and resuspended in Annexin V binding buffer containing Annexin V conjugated to Alexa647 (Invitrogen). Stained cells were analysed by flow cytometry on a FACScalibur (BD Biosciences, Oxford, UK) after addition of propidiumiodide. For induction of apoptosis, VERO cells were irradiated in a UV cosslinker with a dose of 15μJ/cm^2 12 hours post electroporation. Cells were cultured an additional 12 hours to allow the cells to undergo apoptosis / secondary necrosis and were subsequently analysed by Annexin V staining and co-cultured with splenic DC.

Immunostaining

VERO cells were fixed in 4% paraformaldehyde and subsequently stained with anti-dsRNA antibody (clone K1; English & Scientific Consulting Bt., Szirák, Hungary) in the presence of saponin to allow for cell permeabilisation. Cells were then stained with a biotinylated rat

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anti-mouse secondary antibody (Jackson ImmunoResearch, from Stratech, Newmarket Suffolk, UK) followed by incubation with fluorescently labeled streptavidin (BD Biosciences). Samples were analysed by flow cytometry on a FACScalibur (BD Biosciences).

Uptake assay

Transfected and mock-treated VERO cells were labeled with PKH26 and co-cultured with CD11c-enriched splenic DC for 4 hours at a ratio of 5:1. Uptake of material from PKH26-labeled VERO cells by $CD8\alpha^+$ splenic DC was determined by flow cytometry by gating on $CD11c^+$ $CD8\alpha^+$ cells. This gating excluded free VERO cells (data not shown).

In vitro activation assay

VERO cells were co-cultured with sorted splenic $CD8\alpha^+$ DC at ratios of 1:5, 1:1 and 5:1. As controls for DC activation, splenic DC were cultured in the presence of 100μg/ml polyI:C and 0.5μg/ml CpG 1668 oligonucleotide (MWG, Edersberg, Germany). After over night culture, levels of IL-6 in the co-culture supernatant were determined by sandwich ELISA using anti-mouse IL-6 antibodies from BD Biosciences (clones MP5-20F3 and MP5-32C11 as capture and detection antibody, respectively).

Vaccination

For DNA vaccination, C57BL/6 and $TLR3^{-/-}$ mice were injected intramuscularly with 50μg of replicon and conventional plasmid DNA. The amount of plasmid DNA was chosen to allow for equivalent levels of T cell expansion and CTL induction. In some experiments, mice were additionally injected with 50μg of anti-CD40 antibody (clone FGK-45; BD Biosciences) intraperitoneally. Three weeks after vaccination, peptide-pulsed (200ng of SIINFEKL) and unpulsed splenocytes labeled with different amounts of CFSE were injected intravenously to determine antigen-specific target cell killing. The next day, splenocytes were isolated by DNAse and liberase digestion and the frequencies of peptide-loaded and unloaded CFSE-stained target cells were determined by flow cytometry. Antigen-specific target cell killing was calculated using the following formula: $(1\text{-}\%CFSE_{peptide}/\%CFSE_{no\ peptide})\times 100$. In addition, splenocytes from vaccinated mice were stained with $H\text{-}2K^b$/SIINFEKL pentamers (ProImmune Ltd, Oxford, UK), anti-CD8α and anti-Thy1.2 antibodies (both BD Biosciences) and the frequency of antigen-specific CD8 T cells was determined by flow cytometry. Absolute numbers of $pentamer^+$ cells were determined by addition of a fixed number of Calibrite beads (BD Biosciences) to the samples prior to flow cytometric acquisition.

Statistical analysis

Data were analysed using a two-tailed Student's t test (Prism program; Graphpad software, La Jolla, USA). Differences were considered significant at a p-value <0.05.

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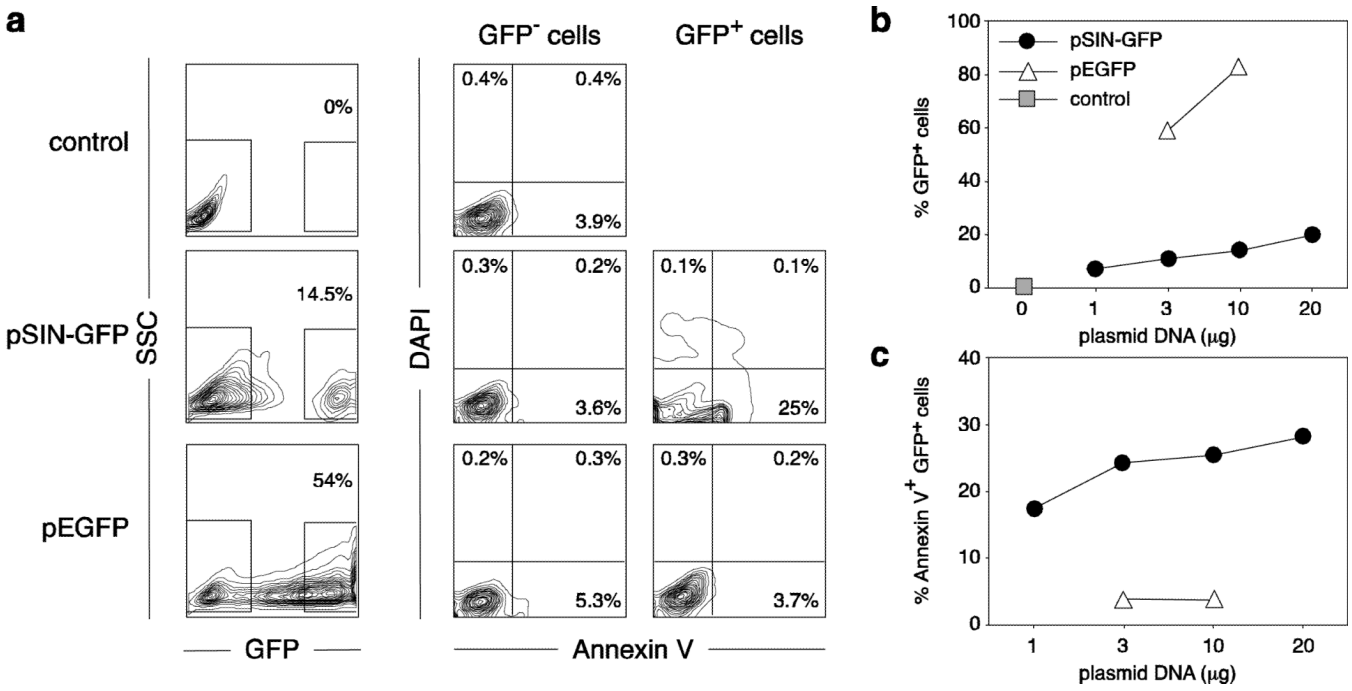

Fig. 1. Replicon vectors induce apoptosis in transfected cells

VERO cells were transfected with the replicon plasmid pSIN-GFP or the conventional plasmid pEGFP by electroporation (10μg plasmid DNA per 1×10^7 cells). Control cells were electroporated in the absence of plasmid DNA. (a, b) GFP expression was analysed by flow cytometry 6 hours after electroporation. Cells were gated as shown in (a) discriminating between GFP^- cells and cells expressing high levels of GFP. (a, c) The percentage of apoptotic cells was determined by staining with fluorescently labeled Annexin V and DAPI. The results are representative of three independent experiments.

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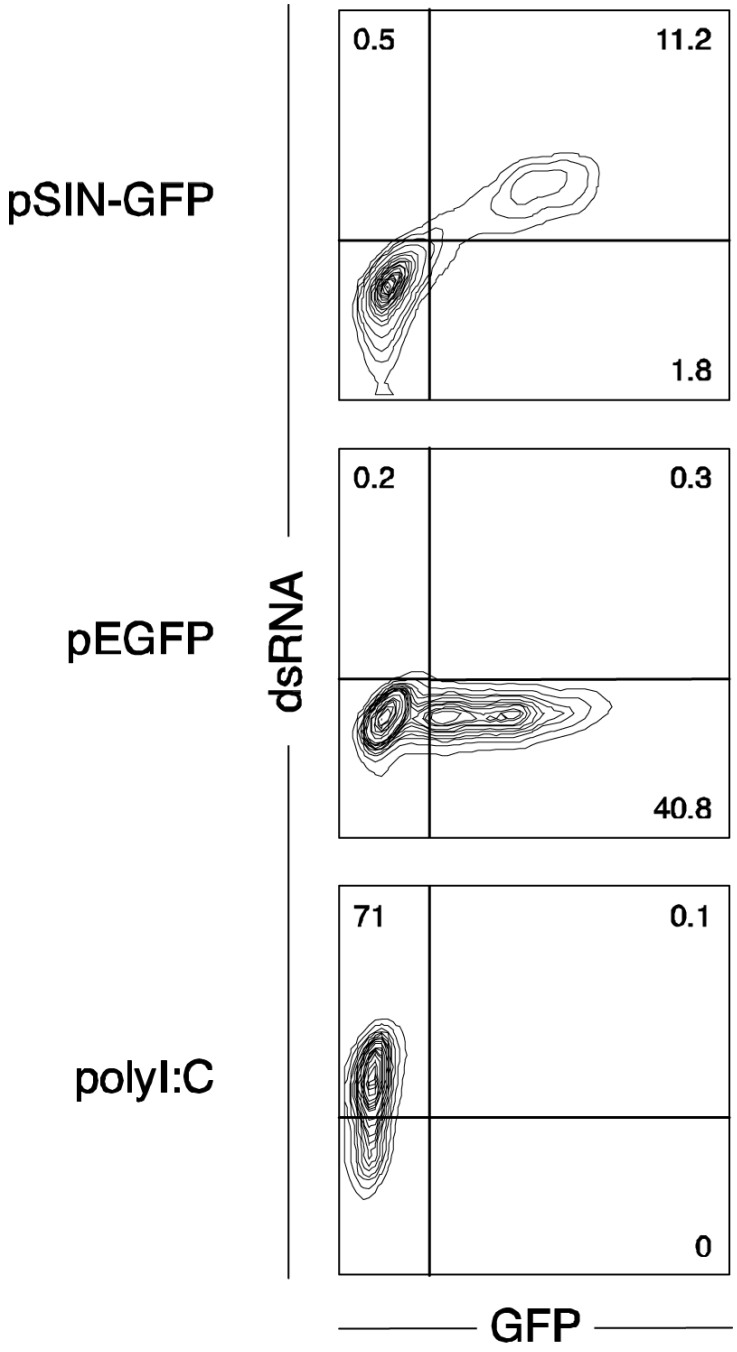

Fig. 2. Cells transfected with a replicon vector contain dsRNA

VERO cells were transfected with 20μg of the replicon plasmid pSIN-GFP or 10μg of the conventional plasmid pEGFP by electroporation and cultured for 6 hours. As a positive control, cells were loaded with 40μg of polyI:C by electroporation immediately before staining. Cells were stained with an anti-dsRNA antibody and analysed by flow cytometry. The data are representative of three independent experiments.

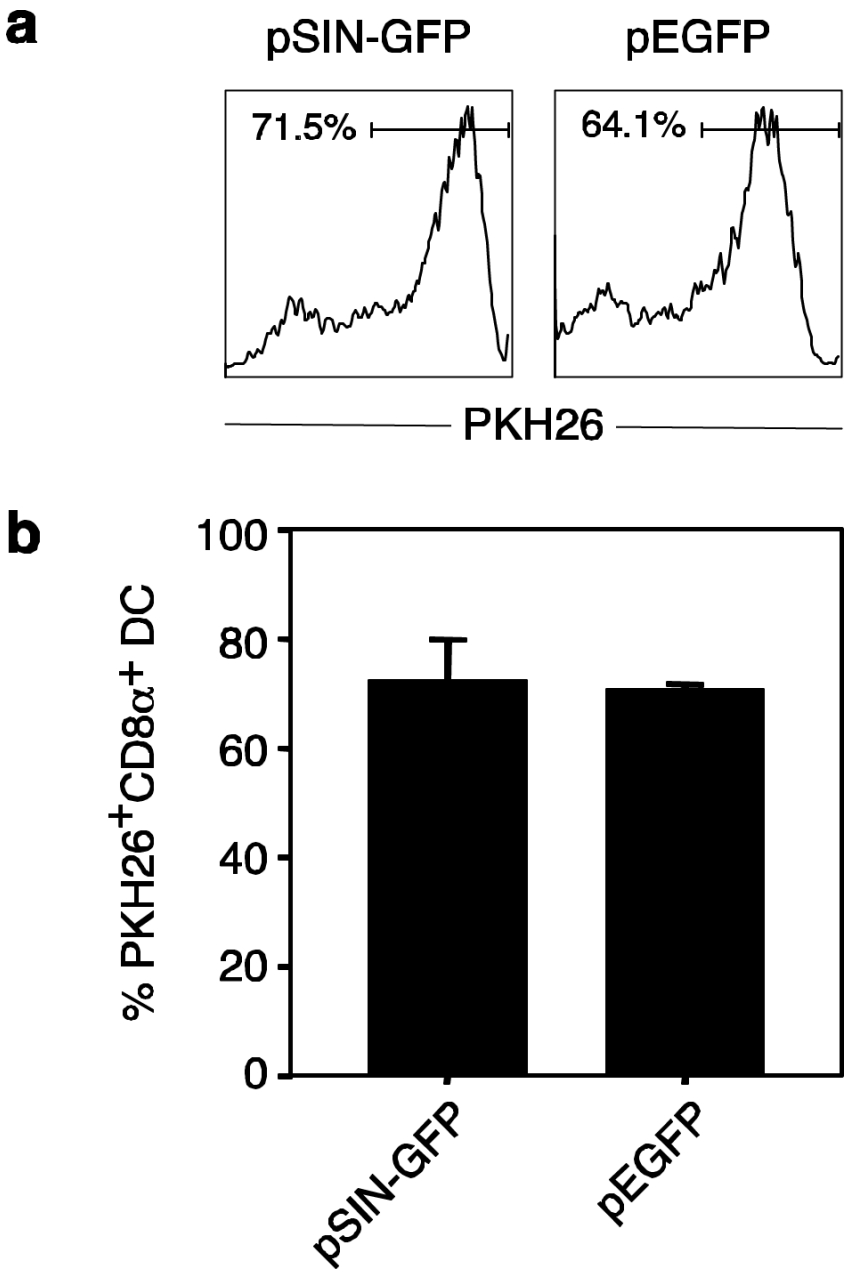

Fig. 3. Uptake of transfected cells by splenic DC

VERO cells were transfected with the replicon plasmid pSIN-GFP or the conventional plasmid pEGFP by electroporation and cultured for 6 hours. Cells were labeled with PKH26 and co-cultured with CD11c-enriched splenic DC for 4 hours. Uptake of material from PKH26-labeled VERO cells was determined by flow cytometry gating on $CD11c^+$ $CD8\alpha^+$ splenic DC. (a) Representative data from one of two independent experiments. (b) Pooled data from the two experiments showing the frequency of $PKH26^+$ $CD8\alpha^+$ splenic DC. No significant difference was observed between uptake of conventional or replicon plasmid transfected cells ($p>0.05$, two tailed student's t test).

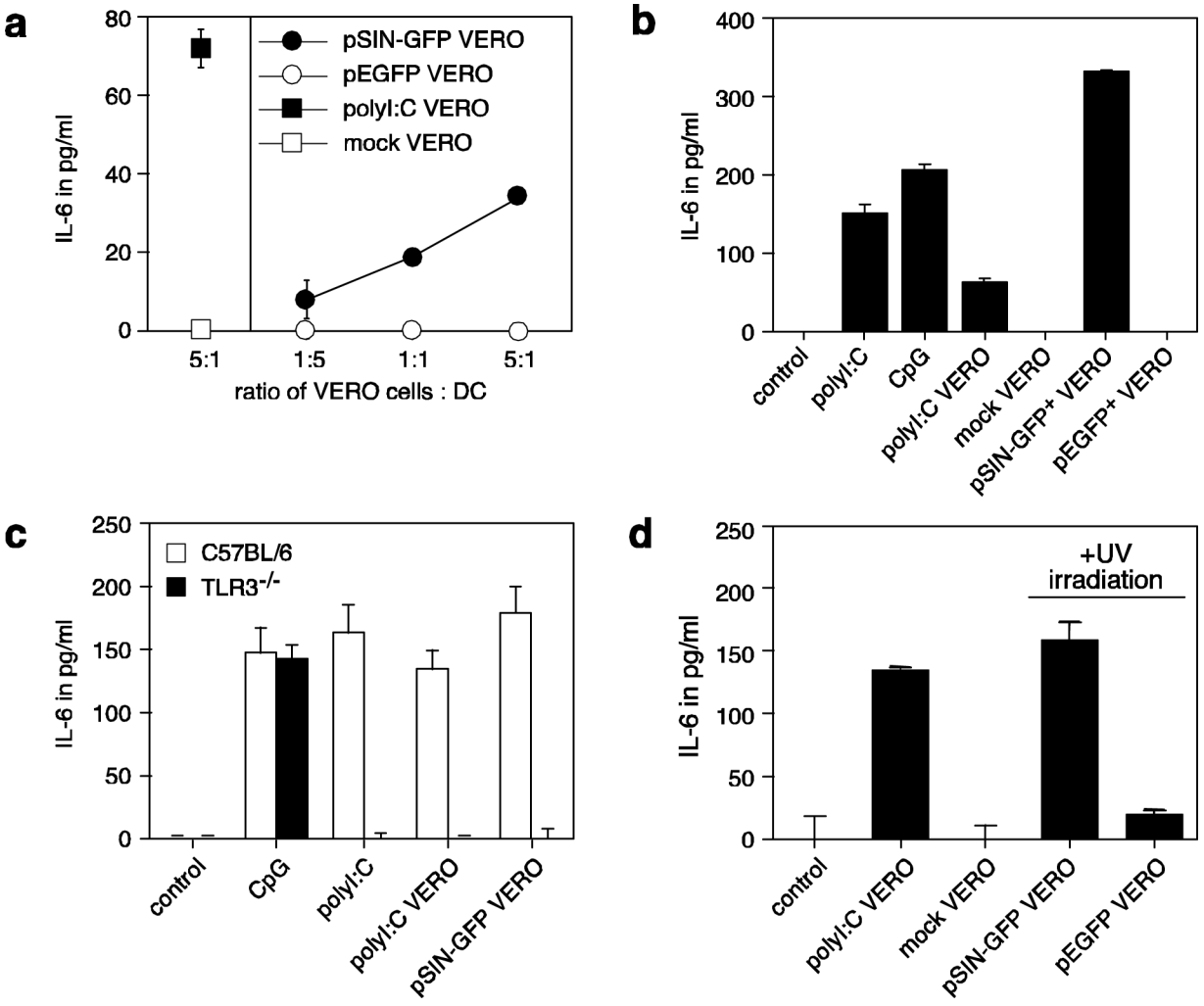

Fig. 4. In vitro activation of DC induced by replicon-transfected cell is TLR3-dependent

VERO cells were transfected with the replicon plasmid pSIN-GFP or the conventional plasmid pEGFP by electroporation and cultured for 6 hours. Total transfected cells (a, c) or sorted GFP^+ cells (b) tested. In (d) transfected VERO cells were further treated with UV irradiation and allowed to undergo cell death before culture with DC. Control cells were electroporated in the presence or absence of polyI:C. Cells were co-cultured with CD8α-enriched splenic DC from wild type mice (a-c) or $TLR3^{-/-}$ mice (c) overnight and the induction of IL-6 was measured by sandwich ELISA. The ratio of VERO cells : DC was 5:1 (b-d) or as indicated (a). As control, DC were cultured alone or in the presence of polyI:C or CpG. The standard deviations are depicted and the data are representative of three independent experiments.

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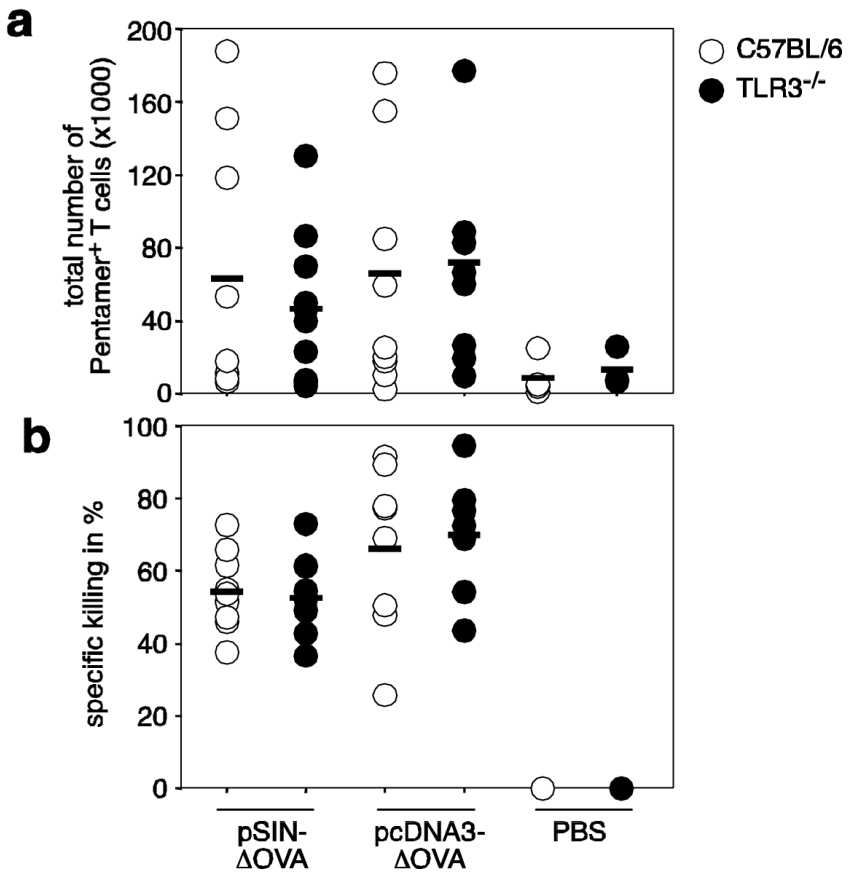

Fig. 5. The induction of CTL responses in vivo takes place independent of TLR3-mediated activation

C57BL/6 and TLR3$^{-/-}$ mice were vaccinated with 50μg of replicon (pSIN-ΔOVA) or conventional plasmid (pcDNA3-ΔOVA) DNA intramuscularly. Anti-CD40 antibody was injected i.p. to boost immunization. Expansion of antigen-specific T cells was determined by Pentamer staining (a) and antigen-specific killing of target cells was assessed by in vivo CTL assay (b) 21 days after vaccination. The results represent pooled data from two independent experiments.

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