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A short hairpin RNA-based adjuvant targeting NF-κB repressor IκBα promotes migration of dermal dendritic cells to draining lymph nodes and antitumor CTL responses induced by DNA vaccination

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ABSTRACT

DNA vaccination is an attractive approach to elicit tumor-specific cytotoxic CD8$^+$ T lymphocytes (CTL), which can mediate protective immunity against tumors. To initiate CTL responses, antigen-encoding plasmids employed for DNA vaccination need to activate dendritic cells (DC) through the stimulation of DNA-sensing innate immune receptors that converge in the activation of the master transcription factor NF-κB. To this end, NF-κB repressor IκBα needs to be degraded, allowing NF-κB to translocate to the nucleus and transcribe proinflammatory target genes, as well as its repressor IκBα. Therefore, NF-κB activation is self-limited by *de novo* synthesis of IκBa, which sequesters NF-κB in the cytosol. Hence, we tested whether co-delivering a shRNA-based adjuvant able to silence IκBα expression would further promote DNA-induced NFκB activation, DC activation and tumor-protective CTL responses induced by DNA vaccination in a preclinical model. First, an IκBα-targeting shRNA plasmid (shIκBα) was shown to reduce IκBα expression and promote NFκB-driven transcription *in vitro*, as well as up-regulate inflammatory target genes *in vivo*. Then, we showed that intradermal DNA electroporation induced the migration of skin migratory dendritic cells to draining lymph nodes and maturation of dermal dendritic cells (dDC). Interestingly, shIκBα further promoted the migration of mature skin migratory dendritic cells, in particular dDC, which are specialized in antigen cross-presentation and activation of CD8$^+$ T cells. Consistently, mice vaccinated with a plasmid encoding the melanoma-associated antigen tyrosinase-related protein 2 (TRP2) in combination with shIκBα enhanced TRP2-specific CTL responses and reduced the number of lung melanoma foci in mice challenged with intravenous injection of B16F10 cells. Moreover, therapeutic vaccination with pTRP2 and shIκBα delayed the growth of B16F10 melanoma subcutaneous tumors. Our data suggest that adjuvants promoting NF-κB activation represent an attractive strategy to boost DC activation and promote the generation of tumor-protective CTL responses elicited by DNA vaccines.
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1. Introduction

Cytotoxic CD8$^+$ T lymphocyte (CTL)-based immunotherapy has emerged as a new class of cancer treatment [1]. Administration of *ex vivo*-manipulated autologous tumor-specific T cells and blockade of T cell inhibitory signals with monoclonal antibodies have shown objective clinical benefit in patients with melanoma and lung cancer, who have failed to respond to other treatments [2–4]. These studies have demonstrated that CTL can specifically recognize and eliminate cells expressing tumor-associated antigens. DNA vaccination represents a cost-effective strategy to induce antigen-specific CTL immunity by harnessing the superior ability of dendritic cells (DC) to activate naïve CD8$^+$ T cells [5]. Skin contains CD103$^+$ dermal DC, which are specialized in carrying-out antigen cross-presentation to efficiently activate CD8$^+$ T cells [6–8]. Thus, intradermal DNA vaccination represents an attractive approach to induce high levels of antigen-specific cytotoxic CD8$^+$ T cells, which have shown to mediate antitumor immunity in several preclinical models [9–11]. Intradermal electroporation has emerged as a simple, efficient and clinically applicable method for delivering DNA vaccines that greatly enhance plasmid uptake, antigen expression and elicited immune responses [12]. DNA

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electroporation induces a robust production of proinflammatory cytokines and chemokines, which results in the infiltration of innate immune cells that contribute to the induction of the adaptive immune responses [13,14].

For the generation of this proinflammatory milieu, plasmid DNA needs to be recognized by DNA-sensing pattern-recognition receptors expressed by different cells present at the site of vaccination, including DC. We and others have demonstrated that generation of T cell responses elicited by DNA vaccines largely depend on the initial activation of DNA-sensing signaling pathways [15,16]. We have shown that by co-expressing an IκBα mutant that blocks NF-κB activation drastically reduce CTL responses elicited by DNA vaccines [15]. On the other hand, strategies that boost these signaling pathways by co-expressing intracellular adaptor molecules and transcription factors have been shown to enhance DNA vaccine-elicited T cell responses [17,18]. We have previously shown that co-expressing the cytosolic DNA sensor DAI at the vaccination site can further promote the induction of effector and memory CTL as well as $CD4^+$ Th1 responses against tumor-associated antigens [9]. In that study, DAI-enhanced CTL responses was shown to be dependent on NF-κB activation rather than type I IFN signaling. Therefore, NF-κB represents a master transcription factor linking innate and adaptive immune responses. Activation of NF-κB is initiated after degradation of NF-κB repressor IκBα, allowing NF-κB to translocate to the nucleus and transcribe proinflammatory target genes, as well as its own repressor IκBα [19–21]. This signaling pathway is therefore self-limited by *de novo* synthesis of IκBa, which then sequesters NF-κB in the cytosol. Since RNA interference (RNAi) technology can be also used to modulate the type and magnitude of the immune responses by specifically targeting immunosuppressive molecules [22–24], we tested whether co-delivering an shRNA-based adjuvant targeting IκBα expression to enhance DNA-induced NFκB activation, would enhance DC activation and tumor-protective CTL responses induced by DNA vaccination in a preclinical model.

2. Material and methods

2.1. Plasmids and RNAi-based molecules

The pVAX1 plasmid (Invitrogen, Life Technologies) encoding either tyrosinase-related protein 2 (pTRP2) or ovalbumin (pOVA) used for DNA vaccination were previously described [25,26]. DNA oligonucleotides encoding a self-complementary hairpin RNA molecule targeting IκBα (shIκBα, sense: AGCAGACTCCACTCCACTT GGCTGTGATCTCAAGAGG, antisense: ATCACAGCCAAGTGGAGTGG AGTCTGCT) was chemically synthesized and ligated into the cloning site (downstream the U6 promoter) of the self-inactivating lentivirus vector pLL3.7 [27]. Then, the fragment containing the U6 promoter and the shIκBα was sub-cloned into the pVAX1 vector to generate the shIκBα construct. As control, pVAX1 vector encoding the scrambled shIκBα sequence (shCTRL, sense: GCACTACCA GAGCTAACTCAGATAGTACTTCAAGAGA, antisense: GTACTATCTGA GTTAGCTCTGGTAGTGC) was generated. Vectors encoding the IκBα supper-repressor (pIκBα-SR), firefly luciferase under the control of a NF-κB promoter (pNF-κB-luc) and β-galactosidase (pON) were previously described [28]. Plasmids for immunizations were purified using the Midi and GigaPrep Endofree Kit (Macherey Nagel).

2.2. Mice and immunizations

C57BL/6 mice were kept in accordance with the local Animal Bioethics Committee guidelines at Fundación Ciencia & Vida, Santiago, Chile. Mice were anesthetized with sevoflurane 3% and injected intradermally (id) at the lower back using a 29-gauge insulin-grade syringe (Micro-Fine U-100, BD) with 40 μl of PBS containing 40 or 80 μg of plasmid DNA (20 μl each injection site), as previously described [9]. Briefly, electroporation (EP) was performed by placing a parallel needle array electrode (two rows of four 2 mm pins, 1.5 × 4 mm gaps) over the injected bleb to deliver the electric pulses (two 1125 V/cm, 0.05 ms pulses followed by eight 275 V/cm, 10 ms pulses) using the Derma Vax™ DNA Vaccine Skin Delivery System (Cyto Pulse Sciences, Inc.). Mice were immunized once or two times, prime and boost two weeks apart. Mice untreated or vaccinated with empty vectors were also included as controls.

2.3. Reverse transcription and quantitative real time PCR

Total RNA was isolated using Trizol reagent (Thermo Fisher Scientific) from vaccinated skin taken 24 h after DNA vaccination. cDNA was prepared using 500 ng of RNA, 500 ng of random primers (Thermo Fisher Scientific), 1 μL of dNTP 10 μM (Thermo Fisher Scientific), 1 μL of RNaseOUT™ (Thermo Fisher Scientific), 5X Green GoTaq® Reaction Buffer (Promega) and 1 μL of M-MLV Reverse Transcriptase (Promega). Transcript levels were determined by quantitative real-time PCR (Brilliant II SYBR® Green QPCR Master Mix, Agilent Technologies) using a two-step cycling program (1 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 62–64 °C) and normalized to the ribosomal protein S29 (*Rps29*) housekeeping gene. Primers used are the following: *Tnfa* fwd: 5′AAATGGGCTTTCCGAATTCA3′; *Tnfa* rev: 5′CAGGGAAGAATC TGGAAAGGT3′; *Ifna* fwd: 5′TGCAACCCTCCTAGACTCATTCT3′; *Ifna* rev: 5′CCAGCAGGGCGTCTTCCT3′; *Il6* fwd: 5′AGGATACCACTCCCAA CAGACCT3′; *Il6* rev: 5′CAAGTGCATCATCGTTGTTCATAC3′; *Rps29* fwd: 5′GAGCCGACTCGTTCCTTT3′; *Rps29* rev: 5′TGTTCAGCCCGT ATTTGC3′.

2.4. Antibodies for flow cytometry

Monoclonal antibodies specific for mouse CD11c-PeCy7 (Clone N418), CD11c-BV510 (Clone N418), CD8-PeCy7 (Clone 53–6,7), CD8-BV421 (Clone 53–6,7), CD8-APC/Cy7 (Clone 53–6,7), CD207-APC (Clone 4C7), CD207-PE (Clone 4C7), CD11b-APC/Cy7 (CloneM1/70), CD11b-FITC (Clone M1/70), CD103-PerCP (Clone 2E7), CD103-BV421 (Clone 2E7), XCR1-APC (Clone ZET), MHC class II (I-A/I-E)-PerCP (Clone M5/114.15.2), MHC class II (I-A/I-E)-APC/Cy7 (Clone M5/114.15.2), CD3-PerCP (Clone 17 A2), CD3-BV510 (Clone 17 A2), CD80-APC (Clone 16–10 A1), IFN-γ-PE (Clone XMG1.2), TNF-α-BV421 (Clone MP6XT22), and viability dye Zombie Aqua, were obtained from Biolegend (San Diego, CA, USA). Non-specific binding was blocked by mouse Fc receptor blocking (Biolegend clone 93). Samples were analyzed in a FACSCanto II cytometer (BD Bioscience) and the data analyzed using FlowJo version 6.4.7 (Tree Star, Inc.).

2.5. Analysis of skin migratory dendritic cells at draining lymph nodes

Draining lymph nodes from mice vaccinated with different vectors were mechanically disaggregated and treated with collagenase IV (Gibco, Carlsbad, CA, USA) 5 mg/mL and DNAse 5 mg/mL (Applichem, Darmastdat, Germany) in PBS-FBS 2% 30 min at 37 °C in a shaker bath. DC phenotype was evaluated by flow cytometry using specific antibodies for CD11c, XCR1, CD103, CD207, CD8, CD11b, MHC class II and CD80.

2.6. Detection of TRP2-specific $CD8^+$ T cells

$TRP2_{(180–188)}$-specific $CD8^+$ T cells in peripheral blood, from immunized mice were evaluated by *ex vivo* stimulation with $TRP2_{(180–188)}$ (SVYDFFVWL) and $OVA_{(257–264)}$ (SIINFEKL) as control

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