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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.

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

peptide (1 μg/ml) for 8 h. Brefeldin A-containing GolgiPlug (BD Biosciences) was added during the last 6 h. Surface and intracellular staining was performed using Cytofix/Cytoperm™ fixation/permeabilization solution set (BD Biosciences) according to the manufacturer's instructions. Monoclonal antibodies anti-mouse CD8α, CD3, IFN-γ and TNF-α were used for flow cytometry analysis. Non-specific binding was blocked by adding unconjugated rat anti-mouse CD16/CD32 antibody (mouse BD Fc block, BD Biosciences).

2.7. Tumor challenge experiments

Tumor challenge was performed by injecting either 2.5×10^5 B16F10 melanoma cells subcutaneously in the flank for the formation of solid tumors, or 4×10^5 B16F10 melanoma cells intravenously for the induction of metastatic foci in the lungs. The growth of solid tumors was monitored by measuring perpendicular tumor diameters with calipers, whereas lung metastasis was quantified by counting the number of melanoma foci on day 21 after cell inoculation. Mice were sacrificed when moribund or when the mean tumor diameter ≥15 mm, according to the approved ethical protocol.

2.8. Statistical analysis

Statistical analysis was performed with the Graphpad Prism software (Graphpad Software Inc.) using Mann-Whitney unpaired *t* test between relevant groups.

3. Results

3.1. shIκBα silences the expression of the NFκB inhibitor IκBα

A plasmid encoding a shRNA targeting IκBα (shIκBα) was tested to reduce IκBα protein expression in B16F10 cells co-transfected with a vector encoding the IκBα-super repressor (pIκBαSR), which carries mutations that prevent its phosphorylation and subsequent degradation [29], allowing the accumulation of IκBα protein to be readily detectable by western blot. A significant reduction in IκBα expression levels was detected in cells transfected with shIκBα as compared to shCTRL that was used as control (Supplementary Fig. 1A). Concomitantly, shIκBα was shown to reduce IκBα mRNA levels (Supplementary Fig. 1B). Importantly, shIκBα was able to promote NFκB-driven transcription as compared to a control scrambled shRNA (shCTRL), either in the absence or presence of TNF-α added to the culture to stimulate NFκB activation (Supplementary Fig. 1C). To test whether shIκBα would enhance the expression of target genes with a known role in promoting DC activation *in vivo*, mice were intradermally electroporated with shIκBα, shCTRL and a plasmid encoding the model antigen ovalbumin (pOVA) as control. Then, RNA was isolated from electroporated skin obtained 24 h later and analyzed by RT-qPCR. As observed in Fig. 1, DNA electroporation with either pOVA or shCTRL enhanced the expression of IFN-α (∼4-fold) and IL-6 (∼150-fold) as compared to skin from naïve mice. Interestingly, shIκBα further promoted the expression of these transcripts up to ∼8- and 250-fold, respectively. Transcript levels for TNF-α were almost not affected by DNA electroporation. Based on these results, we tested shIκBα as potential adjuvant for DNA vaccination.

3.2. shIκBα promotes migration of mature dermal dendritic cells (dDC)

To address the time after DNA vaccination at which skin-derived migratory dendritic cells (mDC) arrived to the draining lymph nodes, mice were intradermally electroporated with pOVA, and mDC were analyzed, based on the high expression of MHC class II and the presence of CD11c, in inguinal lymph nodes at different time points. mDC accumulated in vaccinated skin-draining lymph nodes one day after DNA electroporation (Fig. 2A). Hence, to test our hypothesis, mice were intradermally electroporated with shIκBα, shCTRL or pOVA, and skin-derived mDC were analyzed one day later. DNA vaccination with pOVA or shCTRL induced the migration of mDC as compared to the unvaccinated naïve group (Fig. 2B and C). Interestingly, intradermal electroporation with shIκBα further promoted migration of mDC to draining lymph nodes, as shown in Fig. 2B and C. To address specifically which skin-derived mDC sub-population was migrating to the lymph nodes, Langerhans cells (LC) and dermal dendritic cells (dDC) were analyzed based on the expression of CD11b, CD207, CD103 and XCR1 (as shown in Fig. 3A and B). We found that migration of both LC (CD11b+CD207+CD103-XCR1-) and dDC (CD11b-CD207+CD103+XCR1+) was promoted after DNA vaccination in comparison to naïve mice (Fig. 3C and D). Remarkably, intradermal electroporation with shIκBα further enhanced the migration of mature dDC (Fig. 3D), which are specialized in cross-presentation of antigens and activation of $CD8^+$ T cells. Also, shIκBα promoted the recruitment of monocytes to draining lymph nodes (data not shown) which is concordant with increased inflammation in this group. Moreover, DNA vaccination induced the maturation, measured as CD80 upregulation, of dDC but not LC present at skin-draining lymph nodes (Fig. 3E and F). Both LC and dDC expressed

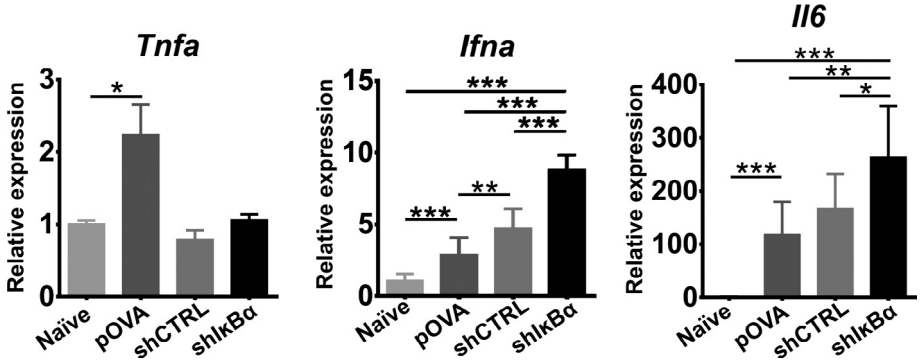

Fig. 1. Cytokine expression in the skin after intradermal DNA vaccination. C57BL/6 mice were intradermally electroporated with 80 μg of pOVA, shCTRL and shIκBα and vaccinated skin was excised 24 h later. Skin from naïve mice was used as control. RNA was isolated and cDNA prepared by reverse transcription. Then, *Tnfa*, *Ifna* and *Il6* transcript levels were measured by qPCR and normalized to the 30 S ribosomal protein subunit (*Rps29*) housekeeping gene. Transcript levels were expressed relative to naïve mice. Bars are the mean plus SEM. Results were obtained using three mice per group. Statistical analysis was performed using Mann Whitney unpaired *t* test. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

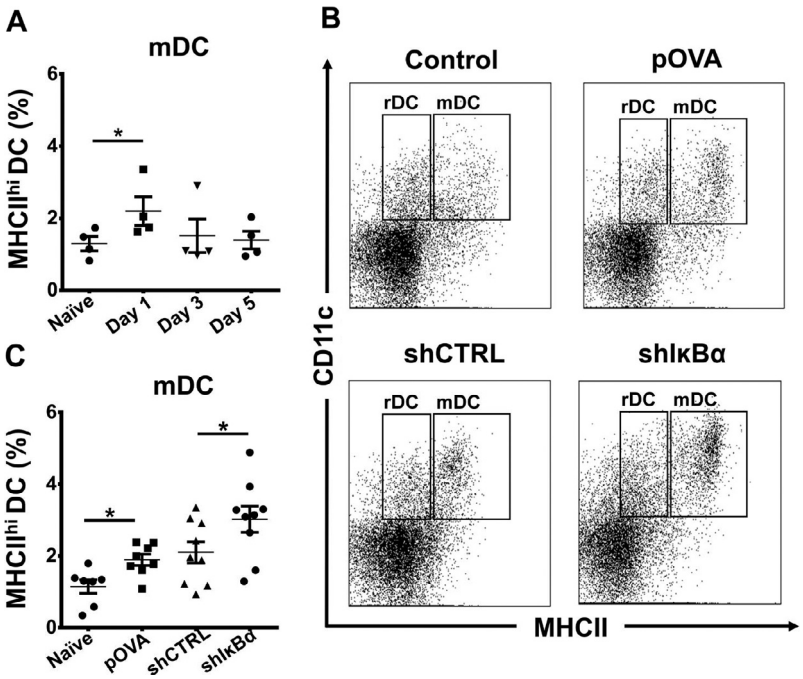

Fig. 2. Analysis of migratory dendritic cells in draining lymph nodes after DNA vaccination. C57BL/6 mice (n = 4–9) were intradermally electroporated with plasmid DNA and migratory dendritic cells (mDC) were analyzed in inguinal lymph nodes by flow cytometry and defined as $CD11c^{+}MHCII^{hi}$. **A.** Quantification of mDC during a time course analysis (1, 3 and 5 days) after DNA electroporation with pOVA. **B-C.** Analysis of mDC in draining lymph nodes one day after DNA electroporation with pOVA, shCTRL and shIκBα. Representative dot plots (B) and quantification (C) of mDC for each condition are shown. Bars are the mean ± SEM. Results are from two independent experiments. Statistical analysis was performed using Mann-Whitney unpaired *t* test. $^{*}p < 0.05$.

high levels of CCR7 and MHC class II, but their levels remained unaffected by DNA vaccination (data not shown).

3.3. Co-administration of shIκBα along with a DNA vaccine increases TRP2-specific CTL responses and protection against melanoma tumor models

To test whether shIκBα is able to promote CTL responses induced by DNA vaccination *in vivo*, mice were vaccinated twice with a plasmid encoding the melanoma-associated antigen tyrosinase-related protein 2 (pTRP2) in combination with shIκBα, shCTRL or an empty plasmid. CTL responses were evaluated in peripheral blood by *in vitro* peptide stimulation followed by intracellular cytokine staining two weeks after last vaccination. As hypothesized, shIκBα adjuvant enhanced TRP2-specific CTL responses induced by DNA vaccination as compared with shCTRL and empty plasmid (Fig. 4A and B). To test the potential of shIκBα to promote vaccination-induced antitumor effects, vaccinated mice were intravenously injected with B16F10 melanoma cells and three weeks after, pulmonary metastatic melanoma foci were enumerated. A significant decrease in the number of melanoma foci in lungs was observed in mice vaccinated with pTRP2 and shIκBα combination compared to mice vaccinated with pTRP2 and shCTRL or empty pVAX (Fig. 5A and B). These results indicate that shIκBα is able to increase CTL immunity generated by a DNA vaccine encoding the tumor antigen TRP2. We evaluated whether the combination of pTRP2 and shIκBα had the capability to suppress tumor growth in a therapeutic setting of minimal residual disease. One day before vaccination mice were subcutaneously inoculated with B16F10 melanoma cells and tumor growth was registered. A significant delay in tumor growth of mice vaccinated with pTRP2 and shIκBα compared to pTRP2 and shCTRL combination was observed (Fig. 5C). The delayed tumor growth led to significant reduction in tumor weight in pTRP2 plus shIκBα vaccinated mice (Fig. 5D). Altogether, these results led us to conclude that shIκBα acts as an efficient adjuvant that promotes migration of mature dDC and antigen-specific $CD8^{+}$ T cell responses able to target tumor burden and metastatic spreading of melanoma.

4. Discussion

DNA vaccination holds promise for generating long-lasting $CD8^{+}$ T cell responses able to eradicate established and disseminated tumors. Harnessing DNA-sensing signaling pathways of innate immunity that mediate the induction of T cell immunity represents a unique opportunity to develop potent adjuvant strategies that unleash the full potential of DNA vaccines against cancer [30]. Plasmid DNA can stimulate several innate immune receptors that converge in the activation of NF-κB, a master transcription factor that links innate and adaptive immune responses [15]. In the present study, we demonstrated that a shRNA targeting the NF-κB repressor, IκBα, can be used as an adjuvant to promote tumor-protective CTL responses elicited by DNA vaccination. First, we showed that intradermal DNA vaccination assisted by electroporation induces the migration of skin-derived DC, both epidermal LC and mature dDC, to draining lymph nodes. Remarkably, shIκBα was able to further promote the accumulation of mature dDC in the vaccinated-skin draining lymph nodes as early as 24 h after vaccination. These results suggest that U6 promoter-driven shIκBα molecules are rapidly expressed, becoming readily available to silence IκBα and promote NFκB activation. Indeed, it has been shown that intradermal electroporation induces an efficient plasmid uptake and rapid expression of plasmid-encoded genes, which can become detectable within the first hour [31]. This early transgene expression is also accompanied by upregulation of several genes involved in inflammatory responses and chemotaxis, among others, which can be responsible of promoting maturation and migration of skin DC. Similarly, we observed that intradermal DNA vaccination significantly increased transcript levels of IFN-α

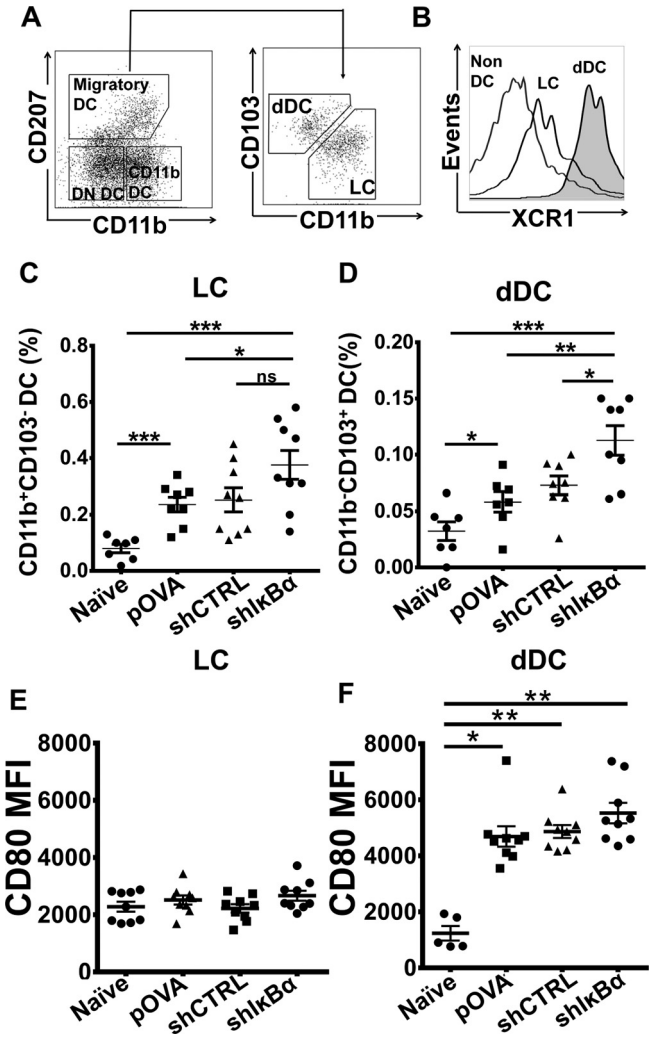

Fig. 3. shIκBα promotes migration of mature dDC to draining lymph nodes one day after DNA vaccination. C57BL/6 mice were intradermally electroporated with pOVA, shCTRL and shIκBα. Naïve mice were used as controls. CD103$^+$ dermal DC (dDC: CD207$^+$CD11b$^-$CD103$^+$XCR1$^+$) and Langerhans cells (LC: CD207$^+$CD11b$^+$-CD103$^-$XCR1$^-$) were analyzed one day after DNA electroporation in inguinal lymph nodes by flow cytometry. **A.** Gating strategy to define LC and dDC in the lymph nodes based on the expression of CD207, CD11b and CD103. **B.** Expression of the cross-presenting DC marker XCR1 in LC versus dDC. **C-D.** Quantification of the percentages of LC (C) and dDC (D) present in draining lymph nodes. **E-F.** Geometric mean fluorescence intensity (MFI) of CD80 in LC (E) and dDC (F). Bars are the mean ± SEM. Results are from two independent experiments, n = 7–9 mice per group. Statistical analysis was performed using Mann-Whitney unpaired *t* test. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; ns: not significant.

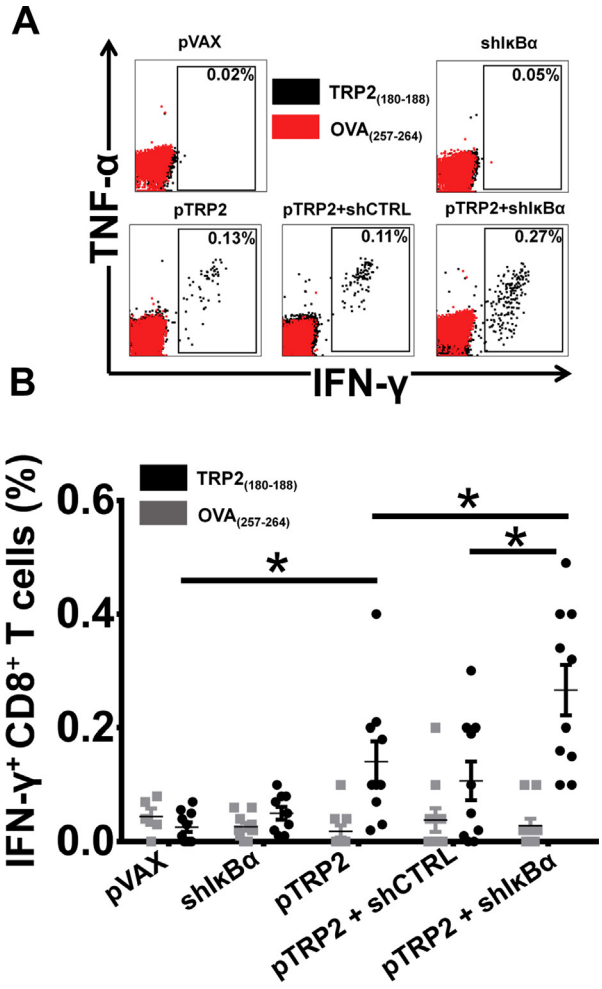

Fig. 4. Detection of TRP2-specific CTL responses induced by DNA vaccination. C57BL/6 mice (n = 10) were intradermally electroporated with pVAX, shIκBα + pVAX, pTRP2 + pVAX, pTRP2 + shCTRL, pTRP2 + shIκBα twice at two-week interval. CD8$^+$ T cell responses was analyzed in blood 12–13 days after last vaccination by peptide stimulation followed by intracellular cytokine staining. **A.** Representative dot-plot of IFN-γ and TNF-α producing CD8$^+$ T cells after *in vitro* stimulation with TRP2$_{(180-188)}$ (showed in black) and control peptide OVA$_{(257-264)}$ (showed in red). **B.** Quantification of IFN-γ-producing CD8$^+$ T cells for each group. Bars are the mean ± SEM. Results are from two independent experiments, n = 10 mice per group. Statistical analysis was performed using Mann-Whitney unpaired *t* test. $^*p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and IL-6, which were further enhanced by shIκBα (Fig. 1) and may be responsible for promoting migration of skin migratory DC to lymph nodes. In particular, dDC have a recognized role in carrying out antigen cross-presentation and activating CD8$^+$ T cells, suggesting that its accumulation in draining lymph nodes could lead to enhanced CTL responses. Indeed, we demonstrated that combining shIκBα with a DNA vaccine encoding the melanoma antigen TRP2 (pTRP2) led to increased CTL responses and tumor protection in melanoma models of lung metastasis and solid tumors.

Different factors determine the ability of DC to mediate T cell immune responses after vaccination, including migration to the lymph nodes, antigen cross-presentation and maturation status [32,33]. These factors are regulated in DC after sensing administrated DNA through endosomal and cytosolic innate immune receptors or by proinflammatory microenvironment produced by resident cells, other than DC, that sense administrated DNA. In addition, innate immune activation may be triggered by the formation of hairpin RNA structures or short RNA species that can be sensed by innate immune receptors, such as TLR3, TLR7, TLR8, PKR and RIG-I, leading to the production of type I IFNs and proinflammatory cytokines via NF-κB [34]. As mentioned before, we observed that intradermal DNA vaccination increased transcript levels of IFN-α and IL-6 (Fig. 1). Moreover, levels of IFN-α were further enhanced by shCTRL and even more by shIκBα. Hence, NF-κB pathway is crucial for controlling the superior ability of DC to initiate T cell responses [33,35,36]. However, whether shIκBα is promoting NF-κB activation directly in transfected DC or other skin-resident cells was not addressed in this study. Relevant work in the field indicates that DNA vaccine-encoded antigens are presented mainly by cross-presentation carried out by DC that migrate to the site of vaccination [37], which implies that DNA vaccine-encoded antigens are preferentially up taken and expressed by cells residing in the skin that are not specialized in activation of CD8$^+$ T cells. Although the uptake and expression of shIκBα by skin

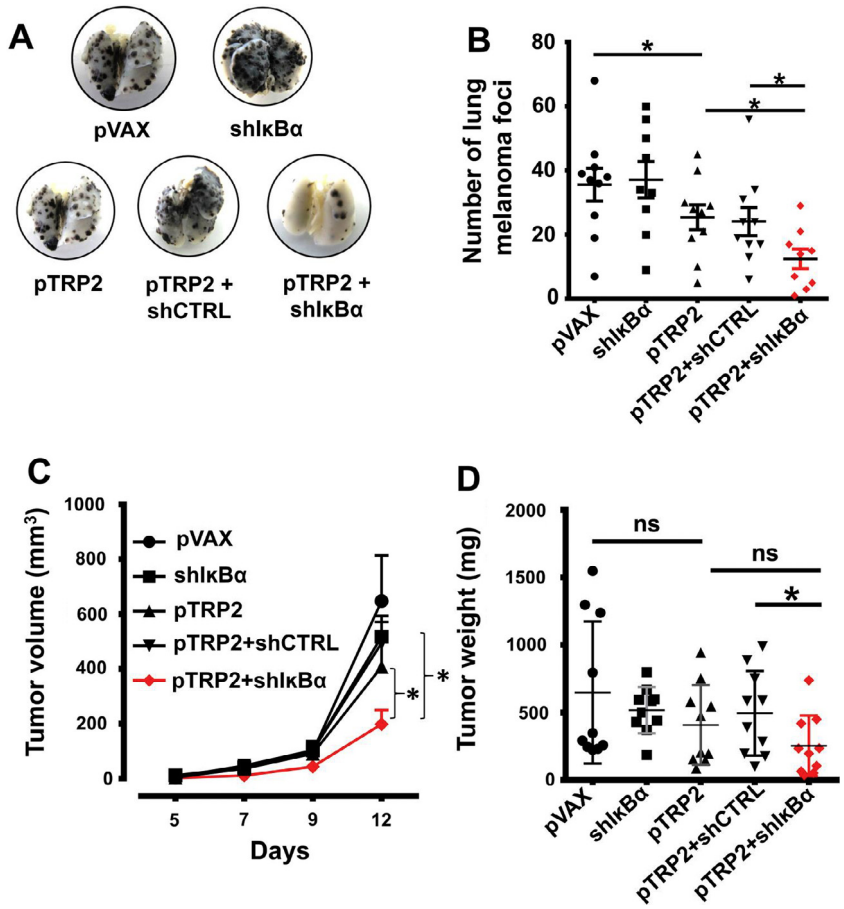

Fig. 5. Suppression of lung metastasis and solid tumor growth induced by DNA vaccination in combination with shIκBα. C57BL/6 mice (n = 10) were intradermally electroporated with shIκBα + pVAX, pTRP2 + pVAX, pTRP2 + shCTRL, pTRP2 + shIκBα. **A-B.** For lung metastasis assay, mice were vaccinated twice at two-week interval and one day after were intravenously challenged with 4×10^5 B16F10 melanoma cells. Lung metastasis foci were analyzed three weeks later. Representative mouse lungs (A) and quantification of lung metastasis foci (B) are shown for each condition. **C-D.** In the case of therapeutic setting, mice were subcutaneously challenged with 2.5×10^5 B16F10 melanoma cells and intradermally vaccinated one day later. Tumor growth was measured every two days (C) and tumor weight was measured after sacrificing the mice (D). Bars are the mean ± SEM. Results are representative from two independent experiments, n = 5 mice per group. Statistical analysis was performed using Mann-Whitney unpaired *t* test. $^*p < 0.05$; ns: not significant.

DC subsets cannot be ruled out, it seems unlikely that shIκBα would be expressed only by relevant cross-presenting DC subsets. This may suggest that the shIκBα-enhanced DC migration observed in this study relies on DC-extrinsic factors, such as the generation of a stronger inflammatory milieu upon vaccination. The role of non-immune cells has been largely studied. Keratinocytes, being part of the first line of defense [38–40] have shown that expression of innate immune receptors enables them to produce proinflammatory mediators, including cytokines, chemokines and antimicrobial peptides in a NF-κB-dependent manner, which contribute to the initiation of adaptive immune responses [41–43]. Regardless of which cells are uptaking the shIκBα, it is fair to conclude that adjuvants that promote NF-κB activation have the potential to enhance the T cell responses elicited by DNA or even protein-based vaccines.

The factors dictating vaccine efficacy remain poorly characterized. The results obtained here indicate that migration of mature dDC to draining lymph nodes is a factor that positively correlates with the magnitude of $CD8^+$ T cell responses and antitumor efficacy. Other studies have also shown that pre-conditioning the vaccination site can significantly improve DC migration to the lymph nodes and efficacy of tumor antigen-loaded DC vaccines through a chemotaxis-dependent mechanism [44,45]. Therefore, adjuvants that promote the migration of cross-presenting DC represent an attractive approach to enhance the potency of vaccines against cancer.

Finally, this study also highlights the versatility of DNA vaccines that facilitates the co-delivery of DNA-encoded adjuvants, also known as genetic adjuvants. In this regard, RNAi-based genetic adjuvant silencing molecules that inhibit the induction of T cell immune responses represent a novel strategy to modulate the magnitude of antitumor CTL immune responses elicited by DNA vaccines [22]. This strategy seems advantageous since it avoids the expression of a second protein with immunomodulatory properties, such as GM-CSF or CXCL10, that can compete for the expression machinery with the antigen or even become targeted by adjuvant-specific adaptive immune responses generated during vaccination [46]. Overall, our study suggests that adjuvants promoting NF-κB activation represent an attractive strategy to promote migration of mature dDC to draining lymph nodes and the generation of antitumor CTL responses elicited by DNA vaccines.

Conflict of interest statement

The authors declare no competing financial interest.

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Appendix A. Supplementary material

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