

Re-engineered CD40 receptor enables potent pharmacological activation of dendritic-cell cancer vaccines *in vivo*

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Modest clinical outcomes of dendritic-cell (DC) vaccine trials call for the refinement of DC vaccine design. Although many potential antigens have been identified, development of methods to enhance antigen presentation by DCs has lagged. We have engineered a potent, drug-inducible CD40 (iCD40) receptor that permits temporally controlled, lymphoid-localized, DC-specific activation. iCD40 is comprised of a membrane-localized cytoplasmic domain of CD40 fused to drug-binding domains. This allows it to respond to a lipid-permeable, high-affinity dimerizer drug while circumventing ectodomain-dependent negative-feedback mechanisms. These modifications permit prolonged activation of iCD40-expressing DCs *in vivo*, resulting in more potent CD8⁺ T-cell effector responses, including the eradication of previously established solid tumors, relative to activation of DCs *ex vivo* ($P < 0.01$), typical of most clinical DC protocols. In addition, iCD40-mediated DC activation exceeded that achieved by stimulating the full-length, endogenous CD40 receptor both *in vitro* and *in vivo*. Because iCD40 is insulated from the extracellular environment and can be activated within the context of an immunological synapse, iCD40-expressing DCs have a prolonged lifespan and should lead to more potent vaccines, perhaps even in immune-compromised patients.

Dendritic cells orchestrate several crucial steps of the adaptive immune response¹. Upon detection of ‘danger signals,’ DCs physiologically adapt to their microenvironment by undergoing a genetic maturation program². The unique machinery of mature DCs allows them not only to induce the activation of naive T cells, but also to regulate their subsequent phenotype and function³. These impressive attributes make DCs ideal natural adjuvants for cancer vaccines⁴. But the limited success of recent clinical trials involving DCs indicates that current strategies need further refinement if DC-based immunotherapy is to be included in the treatment arsenal alongside more conventional modalities of anticancer therapy⁵.

The immunostimulatory properties of DC-based vaccines are limited by their short lifespan and transient activation state within lymphoid tissues. Less than 24 h after exposure to lipopolysaccharide (LPS), DCs terminate synthesis of the T-helper type 1 (T_H1)-polarizing cytokine IL-12 and become refractory to further stimuli⁶, limiting their ability to activate cytotoxic T lymphocytes (CTLs). Other studies indicate that the survival of antigen-pulsed DCs within the draining lymph node is limited to only 48 h after their delivery⁷. These findings underscore the need for improved vaccination strategies capable of either prolonging the activation state and lifespan of DCs, or temporally coordinating the DC activation window with engagement of cognate T cells within lymph nodes.

CD40, a receptor of the tumor necrosis factor (TNF) family, is a particularly good target for manipulation. Unlike the proinflammatory

cytokines or pathogen-associated molecules that DCs encounter throughout the periphery, the DC-expressed CD40 receptor is engaged by CD4⁺ T-helper cells within the lymph node paracortex through its cognate ligand, CD40L^{8–10}. This signal enhances the expression of antigen-presenting and costimulatory molecules, soluble cytokines and several antiapoptotic molecules, ultimately enabling DCs to activate CTLs¹¹. Recent studies have also shown that CD40 stimulation enables DCs to cross-present antigen¹² and overcome peripheral T-cell tolerance¹³. These observations have prompted therapeutic studies of CD40 stimulation. Strategies include systemic delivery of CD40-specific monoclonal antibodies or trimerized CD40L¹⁴, use of CD40-stimulated, antigen-loaded DC-based vaccines¹⁵ and administration of genetically modified CD40L-expressing DCs¹⁶.

Despite its great potential, several properties of CD40 limit its therapeutic development. CD40 is ubiquitously expressed by a variety of other cell types, including B cells, macrophages and endothelial cells¹¹, increasing the likelihood for side effects resulting from systemic administration of CD40 stimuli. In fact, the CD40-CD40L dyad has been shown to promote cardiovascular disease in mice^{17,18} and humans¹⁹. Other studies have shown that agonistic CD40-specific monoclonal antibodies paradoxically suppress inflammatory reactions²⁰. In addition, nontargeted CD40-dependent activation of antigen-presenting cells could trigger polyclonal T-cell expansion,

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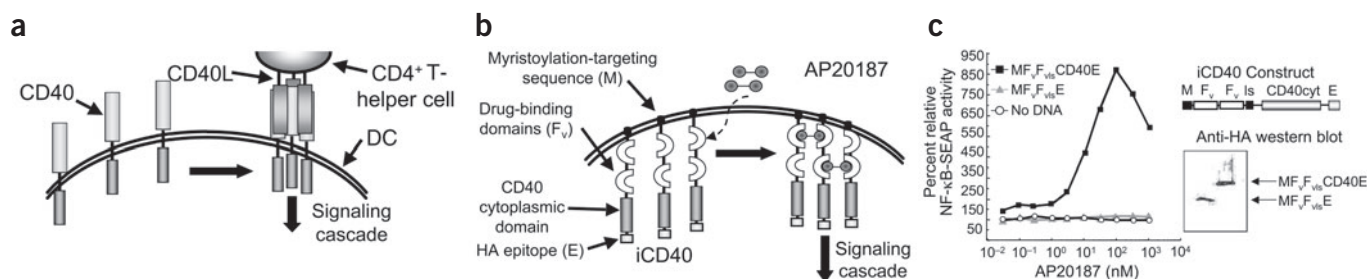


Figure 1 Engineering an insulated, CID-inducible CD40 receptor. **(a)** CD40L-mediated activation of endogenous CD40. **(b)** CID-mediated activation of iCD40. HA, hemagglutinin. **(c)** Activation of iCD40 stimulates NF- κ B transcriptional activity. Jurkat TAg cells were transiently transfected with iCD40 or control vector, MF_vF_{vis}E, along with a NF- κ B-SEAP reporter construct. AP20187 was administered and cell supernatants were assayed for SEAP (secreted alkaline phosphatase) expression. Is, short peptide linker. Hemagglutinin-specific western blot showed comparable expression of iCD40 and vector control. Representative of three independent experiments.

potentially limiting the induction of crucial effector T-cell populations through homeostatic regulation.

Several mechanisms regulate the surface expression of CD40 by targeting its extracellular domain. These include CD40L-induced cleavage by matrix metalloproteinase enzymes²¹, negative feedback degradation by an alternatively spliced CD40 isoform²², and CD40L-mediated endocytosis of CD40 (B.A.H., J.J. and D.M.S., unpublished data). These mechanisms are likely to limit the extent to which CD40-dependent signals can sustain DC activation.

We therefore developed a CD40-based activation system that would extend the prostimulatory state of DCs within lymphoid tissues by providing DC-targeted functionality, temporal control and resistance to CD40-regulatory mechanisms. Several lines of evidence suggest that CD40 trimerization is required for initiating a downstream signaling cascade^{23,24}, making CD40 readily amenable to chemically induced dimerization (CID)²⁵. We engineered a recombinant receptor in which the

cytoplasmic domain of CD40 was fused to ligand-binding domains and a membrane-targeting sequence. Activation of CD40-dependent signaling cascades by this recombinant receptor could be regulated with a lipid-permeable, dimerizing drug. This insulated, inducible CD40 (iCD40) system improved coordination between the initiation of DC activation and the engagement of cognate T cells *in situ* and enhanced DC functionality by circumventing ectodomain-targeted downregulatory pathways.

RESULTS

Engineering the iCD40 receptor

Remote activation of DCs engaged in an immunological synapse may augment a broad spectrum of vaccination strategies. To test this hypothesis, we developed an iCD40 receptor based on CID technology and patterned after the physiological mechanism of endogenous CD40 activation (Fig. 1a). The mouse CD40 cytoplasmic signaling domain fused to a hemagglutinin epitope (E), was subcloned downstream of two tandem

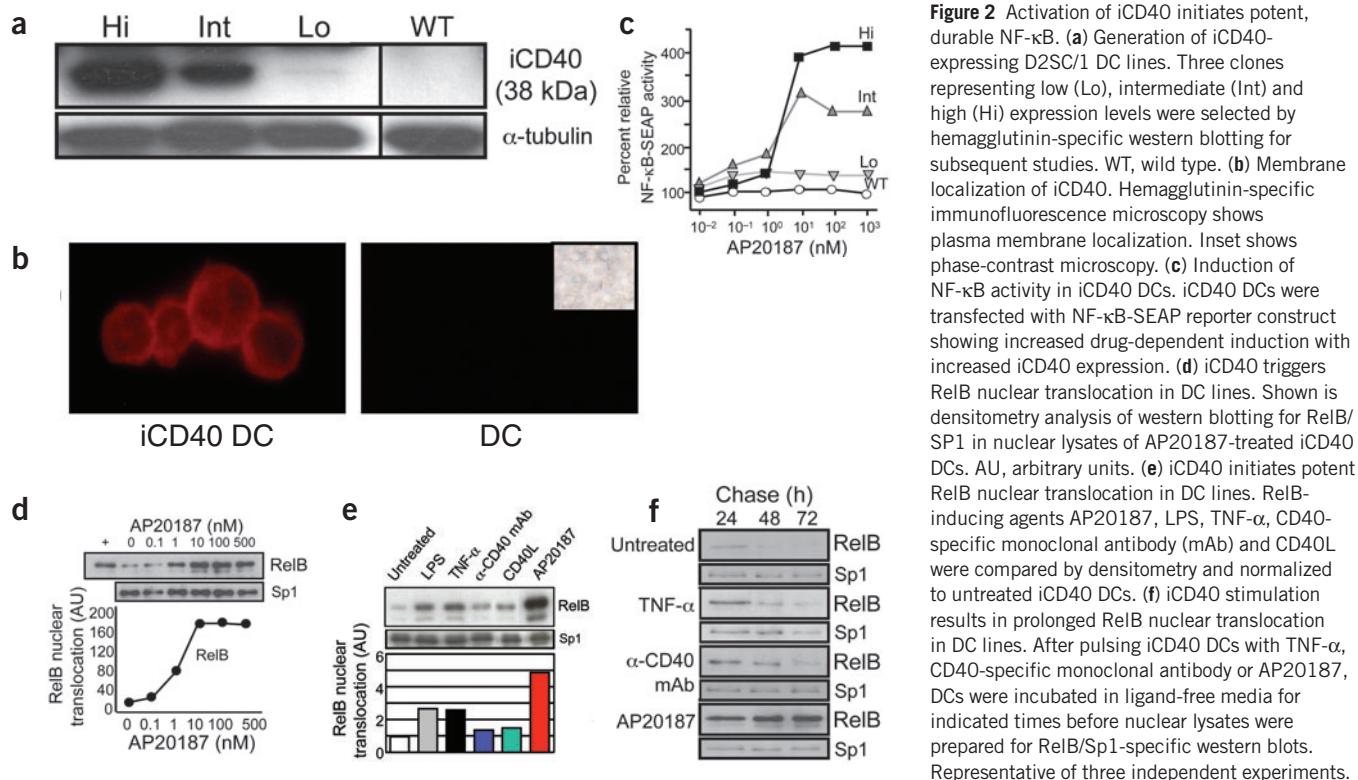


Figure 2 Activation of iCD40 initiates potent, durable NF- κ B. **(a)** Generation of iCD40-expressing D2SC/1 DC lines. Three clones representing low (Lo), intermediate (Int) and high (Hi) expression levels were selected by hemagglutinin-specific western blotting for subsequent studies. WT, wild type. **(b)** Membrane localization of iCD40. Hemagglutinin-specific immunofluorescence microscopy shows plasma membrane localization. Inset shows phase-contrast microscopy. **(c)** Induction of NF- κ B activity in iCD40 DCs. iCD40 DCs were transfected with NF- κ B-SEAP reporter construct showing increased drug-dependent induction with increased iCD40 expression. **(d)** iCD40 triggers RelB nuclear translocation in DC lines. Shown is densitometry analysis of western blotting for RelB/Sp1 in nuclear lysates of AP20187-treated iCD40 DCs. AU, arbitrary units. **(e)** iCD40 initiates potent RelB nuclear translocation in DC lines. RelB-inducing agents AP20187, LPS, TNF- α , CD40-specific monoclonal antibody (mAb) and CD40L were compared by densitometry and normalized to untreated iCD40 DCs. **(f)** iCD40 stimulation results in prolonged RelB nuclear translocation in DC lines. After pulsing iCD40 DCs with TNF- α , CD40-specific monoclonal antibody or AP20187, DCs were incubated in ligand-free media for indicated times before nuclear lysates were prepared for RelB/Sp1-specific western blots. Representative of three independent experiments.

myristoylated (M) domains (from human FKBP12(V36), designated as F_v) that bind the dimerizing drug AP20187 (ref. 26). The resulting chimeric protein, M-F_vF_vCD40-E, is referred to here as iCD40 (Fig. 1b).

Previous signaling studies have indicated that CD40 activates the transcription factor NF- κ B²⁷. We therefore tested the effect of our construct in transiently transfected human Jurkat cells using NF- κ B reporter assays in the presence of titrated AP20187 (Fig. 1c). Increasing amounts of AP20187 resulted in considerable upregulation of NF- κ B transcriptional activity.

iCD40 initiates a potent NF- κ B signal in DCs

To investigate iCD40-mediated DC activation, we studied iCD40 signaling in the immature DC line D2SC/1 (ref. 28). We generated clonal D2SC/1 DC lines stably expressing various amounts of the iCD40 transgene (iCD40 DCs; Fig. 2a). These cells were subjected to immunofluorescence staining to confirm the localization of iCD40 in their membranes (Fig. 2b). Reporter assays were carried out in these stable lines to determine whether iCD40 can also induce NF- κ B activation in DCs. The dose-response curves for the three clones (*i.e.*, Hi, Int and Lo) tested showed increasing induction of NF- κ B with increasing AP20187; however, the two higher-expressing lines showed much more potent response to drug (Fig. 2c). Therefore, the iCD40 DC (Int) line was chosen for subsequent experiments.

Several recent studies have determined that the RelB subunit of NF- κ B is a crucial mediator of DC maturation^{29,30}. Western blot analysis showed that iCD40 triggered RelB nuclear translocation in these DC lines in a drug-dependent manner (Fig. 2d). We then compared the DC activation potency of iCD40 with other traditional maturation stimuli, including LPS, TNF- α , CD40-specific monoclonal antibody and CD40

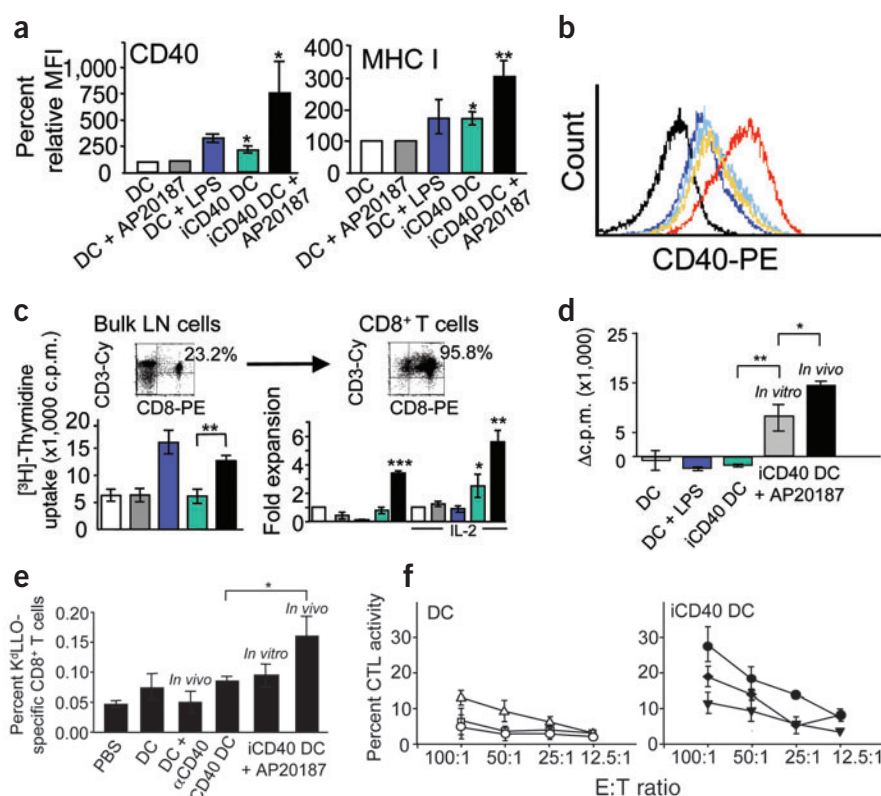
ligand (CD40L; Fig. 2e). To achieve a more rigorous comparison, each agent was administered at the predetermined optimal concentration for RelB induction in iCD40 DCs (data not shown). Among the reagents tested, AP20187-dependent activation of iCD40 DCs generated the most potent RelB signal. Further pulse-chase experiments showed that AP20187 also stimulated a more durable RelB signal than TNF- α or CD40-specific monoclonal antibody (Fig. 2f), suggesting that drug-mediated stimulation of iCD40 DCs maintains a prolonged activation state of at least 3 d.

iCD40 induces DC activation

A key component of DC maturation is the upregulation of several molecules that have crucial roles in the process of T-cell stimulation. We treated iCD40 DCs with AP20187 and analyzed the surface expression of several maturation markers, including ICAM-1/CD54, B7.1/CD80, MHC class I K^d, MHC class II I-A^d and endogenous CD40. Treatment with AP20187 resulted in increased expression of each of these immunostimulatory proteins compared with untreated iCD40 DCs and parental D2SC/1 cells. This increase was comparable to that achieved with LPS stimulation of DCs (Fig. 3a and Supplementary Fig. 1 online). Exposure of iCD40 DCs to excess monomeric drug completely abolished iCD40-stimulated upregulation of DC surface maturation markers (Fig. 3b), confirming that AP20187-mediated physical aggregation of CD40 cytoplasmic domains is required for the activation of these DC lines.

Functional changes accompany DC maturation, including a reduced capacity for endocytosis and enhanced potential for T-cell activation. We investigated CID-induced changes in antigen uptake capacity by measuring the endocytosis of FITC-tagged dextran. Consistent with the maturation marker studies above, drug-mediated activation of iCD40 DCs

Figure 3 iCD40-mediated activation of DC lines *in vitro* and *in vivo*. (a) iCD40 upregulates costimulatory and antigen-presenting molecules in DC lines. Treatment of D2SC/1 DCs with either LPS or AP20187 shows significant induction of CD40 and MHC class I by flow cytometry analysis. Data were normalized to parental D2SC/1 mean fluorescence intensity (MFI). (b) Effect of oligomerization of iCD40 on upregulation of endogenous CD40. In some cultures, excess monomeric AP21995 was added to iCD40 DCs before AP20187 addition. PE, phycoerythrin. Left to right: ■, isotype control; ■, iCD40 DC; ■, iCD40 DC + monomer; ■, iCD40 DC + dimer; ■, iCD40 DC + monomer + dimer. (c) iCD40 DCs promote T-cell expansion in syngeneic mixed lymphocyte reactions (MLRs) in the presence (left) and absence (right) of CD4⁺ T helper cells. Bulk lymph node (LN) cells or purified CD8⁺ T cells were used as responding cell populations, with or without IL-2. Bar colors represent same conditions as a. (d) *In vivo* activation of iCD40 DCs significantly enhances their T-cell immunostimulatory properties. Antigen-specific T-cell proliferation assays were performed after vaccination with treated D2SC/1 DCs. (e) *In vivo*-activated iCD40 DCs expand antigen-specific T cells. Above procedure was repeated with intraperitoneal injections of CD40-specific monoclonal antibody. Spleens were analyzed with PE-labeled K^dLLO₉₁₋₉₉ tetramer. (f) *In vivo* activation of iCD40 DCs generates enhanced CTL-mediated lysis. Procedure as above except that β -gal protein was used as antigen and P.13.1 β -gal-expressing tumor cell line was used as target line. E:T, effector:target ratio. □, PBS; △, DC line; ○, DC line + LPS; ▲, iCD40 DC line; ◆, iCD40 DC line + AP20187 *in vitro*; ●, iCD40 DC line + AP20187 *in vivo*. All data representative three independent experiments. Error bars represent s.d. ****P* < 0.0005; ***P* < 0.005; **P* < 0.05.



resulted in reduced uptake of FITC-dextran similar to that seen in LPS-treated DCs (**Supplementary Fig. 1** online). Our initial *in vitro* approach to determining the T-cell stimulation capacity of iCD40 DCs involved coinubation of mitomycin C-treated D2SC/1 DCs with syngeneic lymph node-derived cells (**Fig. 3c**). The AP20187-treated iCD40 DCs induced T-cell proliferation to levels similar to those in LPS-treated DCs. To further investigate whether this T-cell activation was dependent on CD4⁺ T cells, we repeated the proliferation assays with enriched syngeneic CD8⁺ T cells in the presence and absence of interleukin (IL)-2 (**Fig. 3c**). In contrast to LPS-treated DCs that are dependent on CD4⁺ T-cell-derived helper signals, these assays showed that iCD40 DCs can greatly expand CD8⁺ T cells in culture in a CD4⁺-independent manner.

iCD40 enhances antigen-specific T-cell response *in vivo*

We next investigated the importance of activation timing on the

ability of iCD40 DCs to induce an antigen-specific T-cell response *in vivo*. In preliminary experiments, labeled D2SC/1 DCs migrated to draining lymph nodes upon injection (data not shown). We therefore pulsed parental and iCD40-expressing D2SC/1 DCs with H-2K^d-restricted peptide LLO₉₁₋₉₉ (derived from *Listeria monocytogenes*, listeriolysin O), treated them with LPS or AP20187, and injected them intraperitoneally into syngeneic BALB/c mice. Distinct cohorts were then injected intraperitoneally with AP20187 or agonistic CD40-specific monoclonal antibody 20 h after the initial vaccination. These T-cell proliferation assays showed that *in vivo* activation of iCD40 DCs by AP20187 injection significantly enhances the resulting T-cell response relative to *in vitro* iCD40 activation before DC vaccine delivery (**Fig. 3d**). Using an H-2K^d/LLO₉₁₋₉₉-specific tetramer, we also determined that AP20187-mediated activation of iCD40 DCs *in vivo*, as opposed to *in vitro*, resulted in significantly ($P < 0.5$) enhanced antigen-specific CD8⁺ T-cell expansion over that induced by administration of a CD40-specific monoclonal antibody (**Fig. 3e**). These data are consistent with the unusually potent and prolonged activation state of AP20187-treated iCD40 DCs.

To investigate the antitumor activity of iCD40 DC-stimulated T cells, we conducted CTL assays after vaccinating BALB/c mice with β -galactosidase (β -gal)-loaded DCs using the β -gal-expressing syngeneic tumor line P13.1. Delivery of AP20187 after DC injection resulted in markedly improved tumor-cell killing *ex vivo* relative to DCs stimulated with LPS or prestimulated with AP20187 (**Fig. 3f**).

Ad-iCD40-mediated activation of primary DC vaccines

To characterize iCD40 under more physiological conditions, we developed an iCD40-expressing adenovirus and investigated its activity in primary bone marrow-derived DCs (BMDCs). This vector, Ad-iCD40-EGFP, directs expression of both iCD40 and enhanced green fluorescent protein (EGFP) in transduced BMDCs as verified by western blot and flow cytometry, respectively (**Fig. 4a**). Using quantitative RT-PCR and intracellular cytokine staining (ICCS), we investigated the ability of iCD40 signaling to induce IL-12 cytokine expression in Ad-iCD40-EGFP-transduced BMDCs (iCD40 BMDCs). RT-PCR analysis revealed a nearly eightyfold enhancement of IL-12p35 transcript levels (**Fig. 4b**); this is twofold greater than the synergistic combination of LPS and CD40L and considerably greater than CD40L stimulation alone. AP20187-stimulated iCD40 BMDCs also synthesized considerable levels of IL-12p40, a subunit previously considered to be primarily regulated by danger signals such as LPS (**Fig. 4b**)³¹. This finding paralleled intracellular cytokine staining assays in

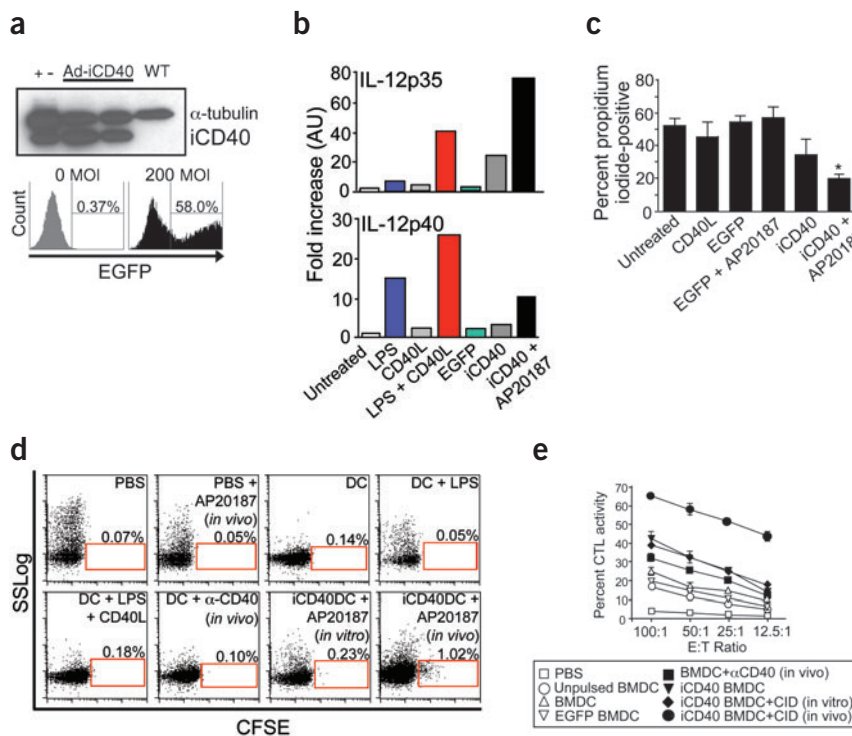


Figure 4 Drug-dependent activation of iCD40-expressing primary BMDCs *in vitro* and *in vivo*. (a) Ad-iCD40-EGFP delivers iCD40 and EGFP transgenes into BMDCs. Top, hemagglutinin-specific western blot analysis of purified BMDCs transduced or mock-infected with virus. Bottom, EGFP expression was confirmed by flow cytometry. (b) CID-induced iCD40 activation potently upregulates IL-12 in primary BMDCs. BMDCs were transduced with Ad-iCD40-EGFP or Ad-EGFP control vector, with or without AP20187. Nontransduced BMDCs were incubated with LPS or CD40L or both. Transcripts of p35 and p40 subunits were analyzed by RT-PCR amplification. (c) iCD40 activation increases BMDC longevity *in vitro*. BMDCs were left untreated or transduced with Ad-iCD40-EGFP or Ad-EGFP, with or without CD40L or AP20187 in serum-free conditions. Day 4 data are shown. (d) *In vivo* activation of iCD40 enhances BMDC longevity *in vivo*. BMDCs were either transduced with Ad-iCD40-EGFP or treated with LPS, CD40L or LPS + CD40L. All BMDCs were peptide-pulsed, stained with CFSE and injected into hind legs of syngeneic mice. Some mice received AP20187 or CD40-specific monoclonal antibody intraperitoneally 20 h after delivery. Draining popliteal lymph nodes were collected 42 h after DC injection, and propidium iodide-negative populations were analyzed by flow cytometry. (e) *In vivo* activation of iCD40-expressing BMDCs enhances CTL killing. Purified BMDCs were mock-transduced or transduced with Ad-EGFP or Ad-iCD40-EGFP, pulsed with LLO₉₁₋₉₉ (unless specified), washed, injected intraperitoneally into BALB/c mice. One group was treated with AP20187 (CID) *in vitro* and other groups were stimulated intraperitoneally with CID or CD40-specific monoclonal antibody, as indicated. A ⁵¹Cr-release assay was conducted using labeled A20-LLO2HygEGFP or A20-HygEGFP tumor lines. All data represent two (b), at least two (d,e), or three (c) independent experiments. Error bars represent s.d. of triplicate measurements.

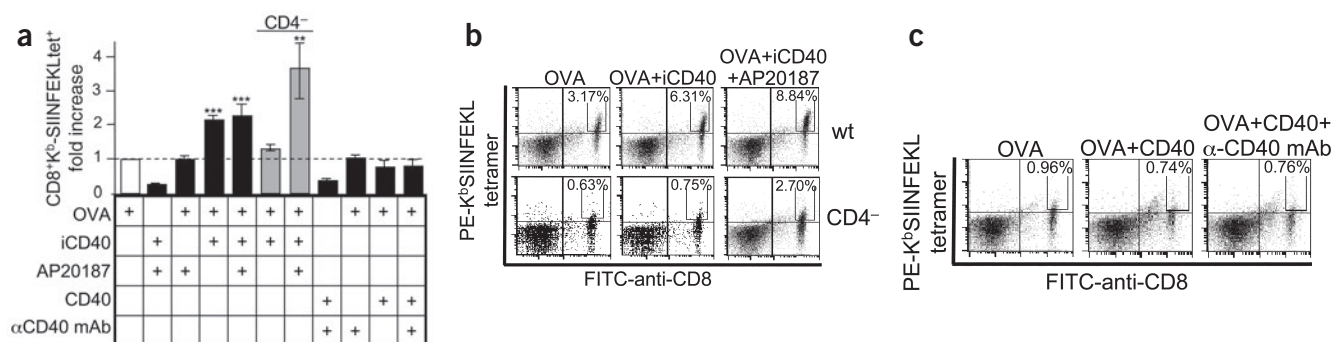


Figure 5 iCD40, but not full-length CD40, augments the immunogenicity of DNA-based vaccines independent of CD4⁺ T cells. (a) Transdermal transfection of both OVA_{257–264}⁻ and iCD40-expressing plasmids shows significant expansion of antigen-specific CD8⁺ T cells relative to OVA alone. Some groups received intraperitoneal injections of AP20187 20 h after vaccination. In some subgroups, a full-length CD40-expressing plasmid (CD40) was substituted for the iCD40 vector used above, and an agonistic CD40-specific monoclonal antibody was delivered intraperitoneally instead of AP20187. *n* = 5 mice per group. ***, *P* < 0.0005; **, *P* < 0.005; *, *P* < 0.05. Splenocytes were collected and analyzed by PE-K^bOVA_{257–264} tetramer/FITC-anti-CD8 flow analysis and data were normalized to OVA alone. SIINFEKL, OVA_{257–264}.

which AP20187-treated iCD40 BMDCs enhanced the production of soluble IL-12p40/p70 by twelvefold (data not shown). These findings indicate that unlike the endogenous CD40 receptor, CID-regulated iCD40 stimulation autonomously induces expression of the bioactive IL-12 heterodimer in a manner that is independent of a secondary microbial priming signal³¹.

In addition to DC activation, DC longevity is another crucial variable that influences T-cell-dependent immunity³². We compared the effects of iCD40 and CD40L on DC survival in an *in vitro* serum-starvation assay (Fig. 4c). We analyzed DC viability by propidium iodide staining and found that drug-activated iCD40 BMDCs survive longer than CD40L-treated DCs. Thus, exposure of iCD40 BMDCs to AP20187 can enhance the longevity of DCs in culture. To investigate iCD40-dependent prosurvival signals *in vivo*, we delivered peptide-pulsed, CFSE-stained untreated or prestimulated BMDCs and iCD40 BMDCs subcutaneously to syngeneic mice. The cohort of mice that were administered untreated BMDCs received an injection of CD40-specific monoclonal antibody, whereas the cohort that was administered iCD40 BMDCs received AP20187 20 h later. Draining popliteal lymph nodes were collected and analyzed by flow cytometry 42 h after vaccination. Consistent with earlier data, postactivation of iCD40 BMDCs by intraperitoneal AP20187 delivery resulted in a substantial enhancement in the number of CFSE⁺ DCs relative to BMDCs prestimulated with LPS, CD40L, LPS/CD40L or AP20187, or post-activated with CD40-specific monoclonal antibody (Fig. 4d). This data strongly suggests that postdelivery activation of iCD40 BMDC vaccines also promotes the longevity of peptide-pulsed primary DCs *in situ*.

The improved IL-12 production and survival of iCD40 BMDCs led us to hypothesize that they should also induce enhanced CTL responses *in vivo*. BMDCs were transduced with the Ad-iCD40-EGFP adenoviral vector, pulsed with LLO_{91–99} and injected intraperitoneally into syngeneic BALB/c mice. Confirming earlier findings, postvaccination AP20187 delivery resulted in substantially improved CTL-dependent killing of LLO_{91–99}-expressing A20 tumor cells relative to iCD40 BMDCs treated with AP20187 before vaccination or to BMDCs stimulated with agonistic CD40-specific monoclonal antibody after vaccination (Fig. 4e and Supplementary Fig. 2 online). Despite modest BMDC transduction efficiencies, these data indicate the efficacy of iCD40 in primary DC-based vaccines.

iCD40 is a potent adjuvant for DNA-based vaccines

Previous studies have shown that Langerhans cells have a crucial role in processing DNA-based vaccines³³. To further analyze the effects of iCD40 on primary DC functionality *in vivo*, we incorporated iCD40-expressing plasmids into a transdermal DNA vaccination protocol³⁴. Gold microparticles were coated with an ovalbumin peptide (OVA_{257–264}) minigene plasmid in the presence or absence of a vector expressing iCD40. Analysis of vaccinated-wild type C57BL/6 mice showed expansion of OVA_{257–264}-specific CD8⁺ T cells. Cotransfection of the iCD40-expression vector increased this OVA_{257–264}-specific T-cell population by approximately twofold after a single vaccination (Fig. 5a,b).

Because iCD40 would predictably obviate the requirement for CD40L provided by CD4⁺ helper T cells, we investigated the ability of the iCD40 receptor to circumvent CD4⁺ T-cell help. We repeated the vaccination procedure in CD4-deficient mice and found that when iCD40 was combined with OVA, there was a reduced overall proportion of OVA_{257–264}-specific CD8⁺ T cells (Fig. 5b), but the stimulatory effect of the iCD40 activation switch was highly drug inducible (greater than threefold; Fig. 5a,b). Therefore, although there was a partial requirement for helper T cell-derived cytokines for maximum efficacy, drug-dependent expansion of antigen-specific CD8⁺ T cells can occur in a manner independent of CD4⁺ helper T cells.

In an additional gene-gun experiment, we examined whether the full-length endogenous CD40 receptor, subcloned into the same vector backbone, could induce comparable stimulatory effects. Despite verification that the intact CD40 molecule was expressed by this plasmid (data not shown), K^b-OVA_{257–264} tetramer analysis showed that full-length CD40 could not enhance antigen-specific CD8⁺ T-cell responses (Fig. 5a,c). In addition, postvaccination injection of CD40-specific monoclonal antibody only moderately improved the immune response to OVA_{257–264} antigen. These data further indicate that the insulated, drug-regulated iCD40 receptor is capable of transducing a more potent activation signal than the full-length CD40 molecule.

iCD40 enhances antitumor immune responses

We next investigated the *in vivo* antitumor efficacy of iCD40 DC-based vaccines and the *in situ* role of iCD40-expressing DCs in tumor immunosurveillance. To establish a therapeutic tumor model, C57BL/6

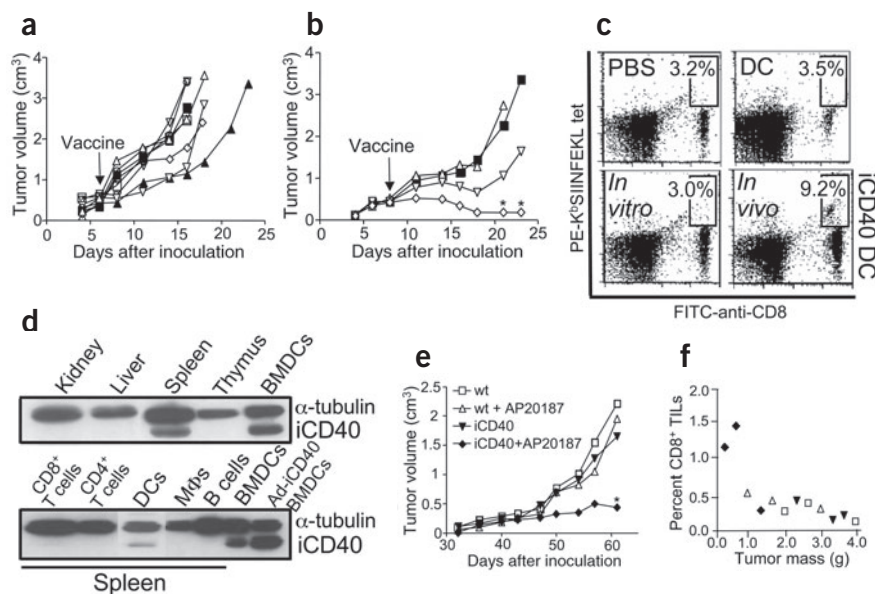


Figure 6 iCD40 enhances the efficacy of DC-based tumor vaccines and the potency of DC-mediated tumor immunosurveillance. **(a)** Activation of SIINFEKL-pulsed BMDCs with LPS or CD40L or both *in vitro*, or with CD40-specific monoclonal antibody *in vivo*, show no efficacy toward large (> 0.5 cm³) EG.7-OVA tumors. □, PBS.exp.1; ▲, PBS.exp.2; ▽, DCs; ◇, DC + LPS; ○, DC + LPS/CD40L; ■, DC + rat IgG; △, DC + CD40-specific monoclonal antibody *in vivo*. **(b)** *In vivo* drug-mediated activation of iCD40-expressing DCs eliminates established EG.7-OVA tumors after a single vaccination. ■, PBS; △, iCD40 DC; ▽, iCD40 DC + AP20187 *in vitro*; ◇, iCD40 DC + AP20187 *in vivo*. *n* = 3–5 mice per group. Representative of two independent experiments. *, *P* < 0.05. **(c)** *In vivo* activation of iCD40 BMDCs elicits significant increase in tumor antigen-specific CD8⁺ T cells in peripheral blood of tumor-bearing mice. Dual plots represent at least two mice per group. **(d)** iCD40-transgenic mice show DC-specific expression of iCD40 receptor. Hemagglutinin-specific western blots were performed on whole tissue lysate or lysate prepared from microbead-purified cells. Mφs, macrophages. **(e)** iCD40-transgenic mice administered AP20187 are more resistant to growth of a prostate cancer cell line. Male iCD40 transgenic and nontransgenic littermates were inoculated with TRAMP-C2 prostate cancer cell line. Some cohorts received intraperitoneal injections of AP20187 every 3–4 d. WT, wild type. *n* = 5 mice per group. Data represent two independent experiments. **P* < 0.05. **(f)** iCD40 activation of DCs in tumor-bearing hosts increases CD8⁺ tumor-infiltrating lymphocytes (TILs) in tumor tissue. Flow analysis from single-cell suspension of TRAMP tumors resected on day 62 after inoculation. *n* = 3 mice per group.

mice were inoculated subcutaneously with the EG.7-OVA thymoma tumor line and allowed to progress until tumor volumes reached approximately 0.5 cm³. These tumor-bearing mice were vaccinated with either SIINFEKL-pulsed wild-type or iCD40 BMDCs. Vaccination with wild-type BMDCs, either untreated or stimulated in culture with LPS and CD40L or *in vivo* with CD40-specific monoclonal antibody, did not slow the overall tumor growth rate (Fig. 6a). In contrast, *in vivo* drug-mediated iCD40 activation of BMDC vaccines resulted in sustained decreases in tumor size (Fig. 6b). In addition, the response rate to *in vivo*-activated iCD40-expressing BMDC vaccines was significantly (*P* < 0.05) higher than the response rates to wild-type BMDCs under all other vaccination conditions (70% compared to 30%). To confirm the elicitation of tumor antigen-specific T-cell responses in tumor-bearing mice, we performed H-2K^b OVA_{257–264} tetramer analysis on peripheral blood CD8⁺ T cells. This analysis verified the presence of an expanded population of K^bOVA_{257–264}-specific CD8⁺ T cells exclusively in mice vaccinated with *in vivo*-activated iCD40 BMDCs (Fig. 6c and data not shown).

To further investigate the capacity of iCD40 to enhance DC-dependent regulation of tumor growth *in situ*, we engineered a DC-targeted, iCD40-expressing transgenic mouse. Tissue expression analysis by western blot showed restricted iCD40 expression in splenic and BMDCs,

thus confirming CD11c promoter specificity (Fig. 6d). Vaccination of wild-type C57BL/6 mice with AP20187-activated, SIINFEKL-pulsed iCD40 BMDCs induced greater *in vivo* proliferation of adoptively transferred CFSE-labeled OT-I (from K^b-SIINFEKL-specific TCR transgenic mice) CD8⁺ T cells than either nonactivated transgenic BMDCs or wild-type BMDCs stimulated with AP20187 (data not shown). These results prompted us to test the ability of the iCD40 DC activation system to suppress the growth of a highly tumorigenic prostate cancer cell line *in situ*. We subcutaneously inoculated the flanks of iCD40-transgenic male mice and their nontransgenic littermates with syngeneic TRAMP-C2 prostate cancer cells. A cohort of both iCD40-transgenic and wild-type mice received biweekly intraperitoneal injections of AP20187. AP20187-treated iCD40-transgenic mice were highly resistant to TRAMP prostate tumor growth (Fig. 6e). An immunological mechanism for the suppression of TRAMP tumor growth was supported by flow cytometry analysis, which revealed increased numbers of infiltrating CD8⁺ T cells within the resected tumor tissue of AP20187-treated iCD40-expressing mice (Fig. 6f). These findings indicate a crucial role for DCs in tumor immunosurveillance and suggest that *in vivo* iCD40 stimulation of DC-based vaccines can greatly enhance the potency of their antitumor activity.

DISCUSSION

The iCD40 receptor endows DC-based vaccines with enhanced immunostimulatory properties, including the ability to temporally manipulate DC activation. Other investigators have found that transient production of IL-12 blunts the T-cell activation capacity of ‘exhausted’ DCs^{6,35}. These findings imply that prolonging the interaction between activated DCs and T cells should confer a more robust cellular immune response. Indeed, recent work has shown that persistent coadministration of Toll-like receptor (TLR) ligands enhances DC-based vaccines³⁶. We have chosen to use a nontoxic CID-based system to modulate the initiation of DC activation so that the period of optimal DC-dependent antigen presentation is likely to occur within the lymphoid microenvironment.

Restricted expression of iCD40 by antigen-pulsed DCs permits amplification of targeted T-cell populations. Because the engineered DC vaccine expresses a drug-dependent, tissue-specific receptor, this approach should enable the selective activation of DCs capable of presenting the antigen(s) of interest. Unlike the administration of systemic DC stimuli, this specificity should avoid the nonspecific polyclonal expansion of T cells that could compete for space (or DC access) in the T-cell compartment of lymphoid tissues and ultimately limit the proliferation of antitumor T cells³⁷.

This work shows that iCD40 provides a more potent and extended activation of DCs relative to full-length endogenous CD40. Even though we used an identical backbone vector to ensure similar expression levels, iCD40 induced a significantly more robust K^bOVA_{257–264}-specific T cell response than CD40, involving up to 80% of all splenic CTLs after

a single transdermal vaccination. We propose that this effect is caused, at least in part, by the ability of iCD40 to evade several ectodomain-targeted inhibitory mechanisms. In B cells, ligand engagement induces the cleavage of the CD40 extracellular domain by the matrix metalloproteinase TNF- α -converting enzyme²¹. In maturing DCs, an additional isoform of CD40 has been described that promotes the degradation of full-length CD40. We have confirmed these findings in the D2SC/1 DC line and have further found that iCD40 is resistant to this negative feedback pathway (**Supplementary Fig. 3** online). We have also found the endogenous CD40 receptor to undergo ligand-induced downregulation (data not shown). These regulatory pathways could attenuate the signaling capacity of the full-length CD40 receptor and, based on recent findings, promote CD40-induction of the immunosuppressive IL-10 cytokine³⁸.

Some investigators have shown that CD40 agonistic monotherapy is sufficient for the induction of an effective immune response¹³, but more recent evidence indicates that microbial secondary signals are necessary for optimum DC activation³¹. This is illustrated by the synergistic effect of LPS (or other TLR ligands) and CD40L on DCs for IL-12 upregulation (**Fig. 4**). Our study shows that iCD40 can circumvent the requirement for these nonspecific TLR ligands. In fact, our results seem to far surpass with those of a recent study in which synergism between a CD40-specific monoclonal antibody and a TLR-7 agonist generated a K^bOVA_{257–264}-specific CD8⁺ T-cell response that peaked at only ~9% of splenic CD8⁺ T cells³⁹.

Finally, a CID-dependent, DC-specific activation system for CD40 promises to avoid the unwanted side effects that are likely to occur over time after systemic administration of soluble CD40L or CD40-specific monoclonal antibody. This potential toxicity primarily involves the CD40-dependent stimulation of endothelial cells. Studies have shown that CD40 stimulation promotes the stabilization of arterial thrombi by activating the vascular endothelium^{17,40}. Consistent with these findings, substantial elevations of surface CD40L on platelets and plasma levels of soluble CD40L have been detected in patients with acute cerebral ischemia¹⁹. Several reports have also shown that CD40 stimulation promotes the development of atherosclerotic plaques, implicating the CD40 receptor as a proangiogenic stimulus^{18,41,42}.

The numerous advantages provided by iCD40 suggest that a DC vaccine expressing such a drug-regulated 'switch' has clinical potential. In an effort to translate these findings to the bedside, phase 1 toxicity studies with an AP20187 analog, AP1903, have shown that the dimerizing agent reached effective serum concentrations without generating adverse side effects⁴³. In addition, our group has engineered a human iCD40 receptor that induces drug-dependent NF- κ B activity. All available data suggests that each of the negative regulatory mechanisms previously ascribed to mouse CD40 also exist in the human system, supporting the contention that an insulated iCD40 receptor could greatly potentiate DC vaccine efficacy. In practice, the implementation of such a strategy is feasible because the *ex vivo* preparation of DCs provides the ideal opportunity for their transduction by iCD40-expressing viral vectors. Finally, this work illustrates how our knowledge of basic DC biology can dictate the design of new vaccination strategies.

METHODS

Mice. Six- to eight-week-old C57BL/6 mice (H-2^b) were obtained from The Center for Comparative Medicine (Baylor College of Medicine). BALB/c (H-2^d) and CD4-deficient B6.129S2-Cd4^{tm1Mak}/J mice (H-2^b) were purchased from Harlan Labs and The Jackson Laboratory, respectively.

Plasmid, adenoviral and transgene constructions. See **Supplementary Methods** online.

Cell lines. Jurkat TAg cells were maintained in complete DMEM. The D2SC/1 DC line (H-2^d; gift from Sang-Mo Kang, University of California, San Francisco) was cultured in IMDM containing 10% fetal bovine serum, 20 μ M β -mercaptoethanol and antibiotics. iCD40-expressing D2SC/1 DC lines were derived by electroporation of parental D2SC/1 cells (324 mV, 950 μ F; Gene Pulser II, Bio-Rad) with 20 μ g of piCD40-IRES-neo plasmid, followed by G418 selection (0.6 mg/ml) and limiting dilution.

DC purification. BMDCs were harvested and cultured as described previously⁴⁴. Briefly, bone marrow cells were flushed from tibias and femurs of mice and cultured in RPMI containing 10% fetal bovine serum, 50 μ M β -mercaptoethanol, antibiotics, 20 ng/ml mouse granulocyte-macrophage colony-stimulating factor (BioSource) and 10 ng/ml mouse IL-4 (RDI). Cell suspensions were cocultured with CD11c-coated microbeads (Miltenyi Biotec) and applied to two consecutive magnetic columns to achieve >95% purity.

SEAP (secreted alkaline phosphatase) reporter assays and fluorescence microscopy. See **Supplementary Methods** online.

Western blots. D2SC/1 DC lines were treated with *Escherichia coli* LPS (0.5 μ g/ml; Sigma-Aldrich), mouse TNF- α (1,000 U/ml; BioSource), mouse CD40L (1 μ g/ml; R&D Systems), mouse CD40-specific monoclonal antibody 3/23 (10 μ g/ml; BD PharMingen) or AP20187 (100 nM; Ariad Pharmaceuticals). Nuclear lysates were prepared using the Transfactor Extraction Kit (Clontech Laboratories) and probed with mouse RelB- and mouse Sp1-specific monoclonal antibodies (Santa Cruz Biotechnology).

Real-time RT-PCR. BMDCs were transduced with the Ad-iCD40-EGFP and Ad-EGFP viral vectors and treated with AP20187, soluble mouse CD40L alone, or CD40L with LPS (1 μ g/ml). RNA was extracted from BMDCs using the RNeasy Protect Mini Kit (Qiagen), treated with DNase I and reverse-transcribed with the High Capacity cDNA Archive Kit (Applied Biosystems). Amplification of IL-12p35 and IL-12p40 was achieved using TaqMan Assays-On-Demand primer sets with TaqMan MGB probes (Applied Biosystems) and quantified using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems).

T-cell proliferation assays. Mitomycin C-treated D2SC/1 cells were cocultured with bulk lymph node cells or CD8⁺ T cells from BALB/c mice for 5 d and pulsed with [³H]-thymidine before analysis with a liquid scintillation counter. D2SC/1 DCs were also pulsed with 50 μ g/ml LLO_{91–99} in the presence or absence of LPS or AP20187 and injected intraperitoneally into BALB/c mice. In some cases, this was followed 18–20 h later by intraperitoneal injection of 50 μ g AP20187 or 100 μ g agonistic CD40-specific monoclonal antibody FGK-45 (gift from Rene E. M. Toes, Leiden University, Netherlands). FGK-45 was purified using an ImmunoPure Protein G IgG Purification Kit (Pierce), tested for purity and functionally verified. Collected splenocytes were cocultured with LLO_{91–99}-pulsed or nonpulsed D2SC/1 cells for 5 d in the presence of 2 ng/ml mouse IL-2 (R&D Systems). The change in counts per minute (c.p.m.) was calculated by subtracting the c.p.m. in the absence of LLO_{91–99} from the c.p.m. in the presence of LLO_{91–99}.

In vivo BMDC survival assays. Purified BMDCs were transduced with Ad-iCD40-EGFP, pulsed with SIINFEKL peptide, stained with intracellular CFSE dye (Molecular Probes)³² and injected subcutaneously into the hind legs of syngeneic mice. iCD40 BMDCs were either exposed to AP20187 in culture or activated postadministration by intraperitoneal injection of AP20187. Untransduced DCs were treated with LPS, CD40L or both in culture, or activated by postdelivery injection of CD40-specific monoclonal antibody. Draining popliteal lymph nodes were collected ~42 h later, and propidium iodide-negative cells were analyzed by flow cytometry.

⁵¹Cr-release assays. BMDCs were transduced with the Ad-iCD40-EGFP vector, pulsed with LLO_{91–99} with or without AP20187, and injected intraperitoneally into BALB/c mice. In some cases, BMDC injection was followed by intraperitoneal injection of AP20187 or agonistic CD40-specific monoclonal antibody. Splenocytes were pulsed with LLO_{91–99} peptide in the presence of mouse IL-2 for 5 d. CTL assays were performed with ⁵¹Cr-labeled A20-LLO2-HygEGFP and A20-HygEGFP stable tumor target cell lines.

Gene-gun DNA vaccination. Wild-type C57BL/6 and CD4-deficient mice were immunized with plasmids pCMV-OVA_{257–264}, pCD40-IRES-hrGFP or pCD40-IRES-hrGFP by gene-gun transfection with the Helios Biolistic Device (Bio-Rad)³⁴. In some cases, this was followed by intraperitoneal injection of AP20187 or CD40-specific monoclonal antibody.

In vivo tumor vaccination studies. EG.7-OVA cells (American Type Culture Collection) were expanded in culture and injected subcutaneously into C57BL/6 mice. Tumor size was allowed to increase to ~0.5 cm³ before DC vaccines were administered. BMDCs were isolated, purified, transduced and pulsed with the SIINFEKL peptide as described above. The vaccine contained either nonactivated, *in vitro*-activated or *in vivo*-activated BMDCs. Two orthogonal tumor measurements were recorded every 3 d. Tumor volume was calculated as $m_1^2 \times m_2 \times 0.5236$.

Statistical analysis. Statistical significance was determined based on the Student *t*-test. All data are shown as mean \pm s.d.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We thank M. Brenner, C. Rooney and D. Lewis for reviewing drafts of this manuscript; K. Freeman for discussions; and E. Nikitina and T.-A. Nguyen for technical assistance. This work was supported by a Robert C. and Janice McNair MD/PhD Training Fellowship at Baylor College of Medicine (to B.A.H.) and Department of Defense grant PC010463 (to D.M.S., K.M.S. and B.A.H.) and a Prostate Cancer Research Initiative grant (to D.M.S. and J.J.).

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 27 August; accepted 22 December 2004

Published online at <http://www.nature.com/naturemedicine/>

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