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Research Article **Vaccines**

The in vivo therapeutic efficacy of DC-based cancer vaccines is limited by suboptimal DC maturation protocols. Although delivery of TLR adjuvants systemically boosts DC-based cancer vaccine efficacy, it could also increase toxicity. Here, we have engineered a drug-inducible, composite activation receptor for DCs (referred to herein as DC-CAR) comprising the TLR adaptor MyD88, the CD40 cytoplasmic region, and 2 ligand-binding FKBP12 domains. Administration of a lipid-permeant dimerizing ligand (AP1903) induced oligomerization and activation of this fusion protein, which we termed iMyD88/CD40. AP1903 administration to vaccinated mice enabled prolonged and targeted activation of iMyD88/CD40-modified DCs. Compared with conventionally matured DCs, AP1903-activated iMyD88/CD40-DCs had increased activation of proinflammatory MAPKs. AP1903-activated iMyD88/CD40-transduced human or mouse DCs also produced higher levels of Th1 cytokines, showed improved migration in vivo, and enhanced both antigen-specific CD8^{+} T cell responses and innate NK cell responses. Furthermore, treatment with AP1903 in vaccinated mice led to robust antitumor immunity against preestablished E.G7-OVA lymphomas and aggressive B16.F10 tumors. Thus, the iMyD88/CD40 unified "switch" effectively and safely replaced exogenous adjuvant cocktails, allowing remote and sustained DC activation in vivo. DC "licensing" through iMyD88/CD40 may represent a mechanism by which to exploit the natural synergy between the TLR and CD40 signaling pathways in DCs using a single small molecule drug and could augment the efficacy of antitumor DC-based vaccines.

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A composite MyD88/CD40 switch synergistically activates mouse and human dendritic cells for enhanced antitumor efficacy

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The in vivo therapeutic efficacy of DC-based cancer vaccines is limited by suboptimal DC maturation protocols. Although delivery of TLR adjuvants systemically boosts DC-based cancer vaccine efficacy, it could also increase toxicity. Here, we have engineered a drug-inducible, composite activation receptor for DCs (referred to herein as DC-CAR) comprising the TLR adaptor MyD88, the CD40 cytoplasmic region, and 2 ligand-binding FKBP12 domains. Administration of a lipid-permeant dimerizing ligand (AP1903) induced oligomerization and activation of this fusion protein, which we termed iMyD88/CD40. AP1903 administration to vaccinated mice enabled prolonged and targeted activation of iMyD88/CD40-modified DCs. Compared with conventionally matured DCs, AP1903-activated iMyD88/CD40-DCs had increased activation of proinflammatory MAPKs. AP1903-activated iMyD88/CD40-transduced human or mouse DCs also produced higher levels of T_H1 cytokines, showed improved migration in vivo, and enhanced both antigen-specific CD8+ T cell responses and innate NK cell responses. Furthermore, treatment with AP1903 in vaccinated mice led to robust antitumor immunity against preestablished E.G7-OVA lymphomas and aggressive B16.F10 tumors. Thus, the iMyD88/CD40 unified "switch" effectively and safely replaced exogenous adjuvant cocktails, allowing remote and sustained DC activation in vivo. DC "licensing" through iMyD88/CD40 may represent a mechanism by which to exploit the natural synergy between the TLR and CD40 signaling pathways in DCs using a single small molecule drug and could augment the efficacy of antitumor DC-based vaccines.

Introduction

By virtue of their ability to process and present antigens, DCs are highly efficient at priming and expanding antigen-specific T cells. Tumor antigen–pulsed DCs have generated great interest as immunotherapeutic vaccines for cancer. However, in numerous clinical trials, ex vivo–differentiated and matured DCs have garnered modest clinical responses (1, 2). One possible explanation for this disparity is that most ex vivo–matured DCs have suboptimal activation due to weak, nonsynchronized, and noncontextual maturation, as evidenced by low expression of maturation markers and T_H1 cytokines (e.g., IL-12), poor migratory ability, and diminished survival (3). A number of proinflammatory cytokine cocktails have been utilized to mature and activate DCs, including the "gold standard," consisting of IL-1β, IL-6, TNF-α, and PGE$_2$. In this maturation cocktail (MC), PGE$_2$ enhances DC migration via CCR7 upregulation. However, PGE$_2$ can also increase the IL-10/IL-12 ratio in DCs, leading to expansion of T_H2 cells or Tregs (4). Furthermore, DC activation by inflammatory cytokines alone can result in poor CD4+ T effector function, potentially leading to tolerogenic outcomes (5).

Modest clinical outcomes of DC vaccines have thus driven the development of improved pro-T_H1 and pro-CTL DC maturation protocols. Several protocols use exogenous CD40 ligand (CD40L), which can partially mimic lymph node CD4+ T cell–mediated activa-

tion of CD40 receptor on the DC surface. However, CD40L-induced negative feedback mechanisms can still constrain prolonged CD40 signaling and DC activation (6). Systemic CD40L administration can also have deleterious effects due to the widespread expression of CD40 on several cell types, including endothelial cells and platelets. To overcome this drawback, we previously reengineered CD40 using an inducible FKBP system. FK506-binding protein 12 (FKBP12) belongs to the immunophilin family of highly conserved proteins that bind immunosuppressive drugs such as FK506, rapamycin, and cyclosporin A (7). Endogenous human FKBP12 has, however, been adapted to bind with high specificity to a lipid-permeable dimerizing drug (e.g., AP1903) or chemical inducer of dimerization (CID) by introducing a point mutation (FKBP12v36) in the FK506-binding domain that abolishes binding to its immunosuppressive counterparts (8). Binding of FKBP12v36 domains from separate proteins to CID leads to ligand-induced dimerization/oligomerization of FKBP-containing proteins. According to this strategy, we fused the cytoplasmic signaling domain of CD40 to tandem copies of FKBP12v36. The reengineered CD40 was localized to membrane lipid rafts using a myristoylation-targeting sequence, enabling receptor activation in a physiological context. The CID system enabled inducible CD40 activation in DCs, independent of its cognate CD40L, either in culture or in vivo without detectable adverse events. In vivo drug administration induced significant antitumor responses by tumor antigen–pulsed, inducible CD40-expressing DCs (iCD40-DCs) (9).

Nevertheless, studies by our laboratory and others have now shown that along with CD40 costimulation, potent activation and

licensing of human DCs requires additional immunological adjuvants, including the potent TLR4 ligand LPS (10, 11). Combined stimulation by LPS and CD40L can promote a second wave of IL-12 production by DCs and exponential expansion of CD8+ CTLs (12–14). Combining CD40 and TLR stimulation in vivo has also been shown to transform tumor-infiltrating DCs from an immunosuppressive to immunostimulatory phenotype, thereby overcoming tolerance to self antigens in a tumor microenvironment (15). However, the many known adverse effects of LPS in vivo, including "toxic shock" in humans, have limited the use of LPS in late-stage clinical studies (16). An LPS analog, monophosphoryl lipid-A (MPL-A), has been shown to have lower toxicity, but can also induce IL-10 and dampen proinflammatory responses, making MPL-A a less effective adjuvant (17). Furthermore, MPL-A induces TLR4 signaling dominantly through TRIF, rather than MyD88, resulting in the upregulation of type I IFNs that, in turn, can curb DC survival (18, 19). To overcome the limitations of LPS-associated toxicity and proapoptotic signaling of some clinical-grade LPS analogs, we created a dimerizable TLR4 "switch" fused to iCD40 to enhance DC activation.

TLRs signal through homo/heterodimerization of their cytoplasmic Toll/IL-1 receptor (*TIR*) domains, leading to recruitment and activation of associated adaptor molecules (20). Of the known mammalian TLRs, all but TLR3 signal through the adaptor protein MyD88 via a TIR-TIR homotypic interaction. Aggregation of MyD88 leads to the recruitment and activation of IRAK family kinases via DD-DD (wherein DD indicates *death domain*) interactions, triggering a signaling cascade that culminates in p38, JNK, and NF-κB activation (21). Although TLR4 also binds to the TIR-containing adaptor, TRIF, MyD88 is the critical mediator for generating T_H1 responses (22). Therefore, we have engineered a CID-inducible MyD88 (iMyD88) containing tandem FKBP12v36 domains along with a myristoylation-targeting sequence for membrane localization.

We show that iMyD88 induces higher NF-κB activation and requires several logs lower CID concentrations in vitro for NF-κB induction relative to iCD40-expressing cells. To achieve simultaneous activation of both CD40- and MyD88-dependent signaling pathways, we further fused the MyD88 and CD40 signaling domains with 2 CID-binding domains to generate a composite, inducible activation switch (iMyD88/CD40). iMyD88/CD40 signaling in DCs leads to enhanced CID-dependent DC activation, cytokine production, and migration in vivo. iMyD88/CD40 significantly (*P* < 0.01) enhances DC-mediated tumor antigen–specific T cell responses in mouse models of melanoma and lymphoma. Finally, iMyD88/CD40-modified human DCs, in addition to their improved survival, markedly enhance T cell responses to human tumor antigens upon CID treatment. This is the first report of a composite activation receptor for DCs (DC-CAR) that unifies 2 separate signaling pathways with the use of a single drug. CID-mediated DC licensing represents what we believe is a novel strategy for exploiting the natural synergy between CD40 and TLR signaling that can markedly improve on MCs in augmenting the therapeutic efficacy of antitumor DC-based vaccines.

Results

Development of a composite DC activation receptor, iMyD88/CD40, that enables enhanced NF-κB activation. One established method for achieving high-level DC activation ex vivo is through combined stimulation of CD40 and TLRs. We previously developed a CD40

activation switch that enables prolonged CD40 signaling in DCs in the absence of exogenous CD40L (9). To replace potentially toxic TLR ligands, we subcloned the cytoplasmic TIR domains of several human TLRs, including TLR3, TLR4, TLR7, TLR8, and TLR9, 5′ or 3′ of tandem human FKBP12v36 (F_v) domains, membrane targeted using a myristoylation sequence. Since TLR signaling induces activation of transcription factor NF-κB, we used an NF-κB–responsive SEAP reporter assay to screen these chimeric constructs following transient transfection in HEK293 cells and treatment with dimerizing drug AP1903 or nonclinical analog AP20187 (23) (designated CID). Although split-GFP reconstitution assays and molecular modeling of TLRs support a model for dimerization-induced activation (24, 25), we observed only modest NF-κB induction by any of these FKBP12-fused chimeric TLR constructs (data not shown), suggesting that more subtle allosteric changes or intracellular targeting are additional requirements for "out-of-context" TIR activation. Therefore, we redirected our focus downstream of TLR signaling to the TIR domain–containing adaptor molecule, MyD88, which appears to also signal through an aggregation mechanism (21).

In addition to full-length MyD88, as part of our initial characterization, we evaluated truncated alleles lacking either the C-terminal TIR domain alone (designated MyD88ΔTIR) or lacking both the C-terminal TIR and intermediate domains (MyD88ΔC). SEAP reporter assays showed that both the DD and previously described intermediate domain of MyD88 (26) were required for inducible MyD88–mediated (iMyD88-mediated) NF-κB activation when fused in frame (either 5′ or 3′) of 2 myristoylated F_v drug-binding domains (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI44327DS1). In contrast, the TIR domain was not required for downstream signaling. The iMyD88ΔTIR (henceforth referred to as iMyD88) was selected for fusion to CD40. We inserted the CD40 cytoplasmic domain (CD40c) into the iMyD88 variants and screened all 6 possible permutations (not shown) to generate the optimum composite receptor, referred to as iMyD88/CD40 (Figure 1A). Compared with previously generated iCD40 (9) and an established constitutive CD4-TLR4 (27), iMyD88 and iMyD88/CD40 induced around 5-fold and 10-fold higher NF-κB reporter activation, respectively, even at approximately 2 logs lower CID concentration. Similarly, with increasing concentrations of CID (>1 nM), both iMyD88 and iMyD88/CD40 induced several-fold higher NF-κB reporter activation compared with iCD40. Thus, iMyD88/CD40 had a superior dose response and higher magnitude of NF-κB induction relative to iMyD88 alone or iCD40 alone (Figure 1B). Protein levels of all chimeric receptors were comparable, as determined by Western blot (Figure 1C), and could not account for the dramatic differences seen.

To help understand the synergistic NF-κB induction observed, we evaluated the activation of individual NF-κB subunits. LPS stimulation of TLR4 is known to induce nuclear translocation of NF-κB subunits RelA and c-Rel (28), whereas CD40 stimulation primarily induces RelB (29). To determine which NF-κB subunits were activated by iMyD88 and iMyD88/CD40, we analyzed nuclear translocation of RelA, RelB, and c-Rel subunits in the human monocytic cell line THP-1. Cells were transduced with adenoviral vectors (Ad) expressing iCD40, iMyD88, iMyD88/CD40, or control protein, LUC, stimulated with or without CID and LPS (as indicated), and nuclear extracts analyzed. Consistent with previous observations, LPS and iMyD88 activated RelA and c-Rel, while iCD40 activated only RelB. In contrast, iMyD88/CD40 induced the simultaneous nuclear local-
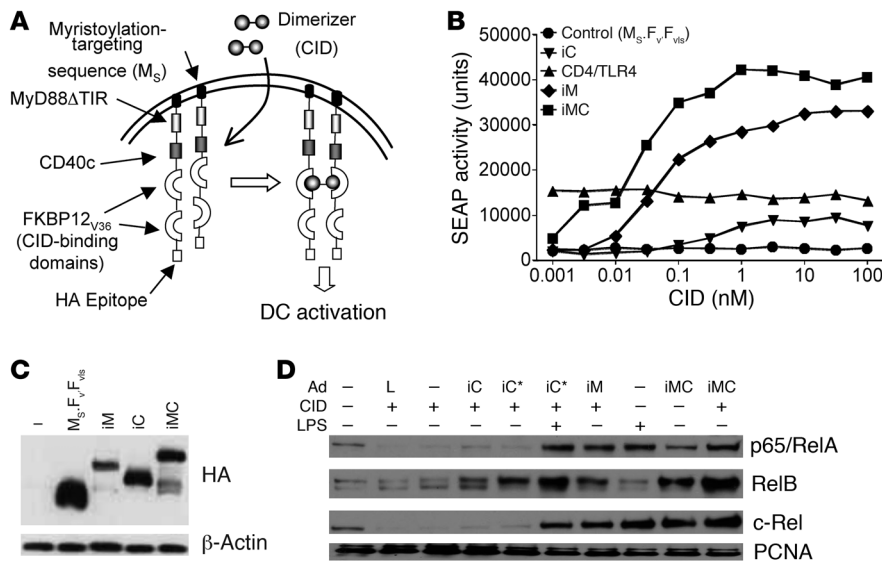
Figure 1

Designing a CID-based, composite activation receptor for DCs triggering robust NF-κB activation. (**A**) CID-mediated activation of iMyD88/CD40 composite receptor. HA, hemagglutinin tag; MyD88ΔTIR, amino-terminal region of MyD88 lacking the TIR domain; CD40c, cytoplasmic domain of CD40. (**B**) iMyD88/CD40 activates NF-κB transcriptional activity in a CID-dependent manner. 293 cells were transiently transfected with constructs expressing iMyD88/CD40 (iMC), iMyD88 (iM), iCD40 (iC), constitutive TLR4 (CD4/TLR4) as a positive control, or control backbone vector, Ms.Fv′Fvls.E (ls, short glycine-serine linker peptide), along with an NF-κB–SEAP reporter. After overnight CID treatment, cell supernatants were analyzed for SEAP activity. (**C**) Full-length expression of iMyD88/CD40, iMyD88, and iCD40 confirmed by Western blot with anti-HA mAb. (**D**) iMyD88/CD40 triggers simultaneous nuclear translocation of all 3 NF-κB Rel family subunits, RelA, RelB, and c-Rel at 60 minutes following treatment with CID. 48 hours after Ad5 transduction (100 MOI) with Ad5-iCD40 (iC), Ad5F35-iCD40 (iC*), Ad5-iMyD88 (iM), Ad5-iMyD88/CD40 (iMC), or Ad5-LUC (L), THP-1 cells were stimulated with 100 nM CID or LPS (1 μg/ml) for 60 minutes. Nuclear lysates were prepared and analyzed by Western blotting. All data are representative of at least 3 independent experiments.

ization of all 3 Rel subunits, and addition of CID enhanced this translocation (Figure 1D). Thus, the composite receptor, iMyD88/CD40, increases NF-κB activation in a quantitative and qualitative manner consistent with previous observations on the effects of combining CD40 and MyD88 signaling pathways.

iMyD88/CD40 improves activation of primary mouse BM-derived DCs. Since iMyD88/CD40 enhanced NF-κB activation by several-fold, we next investigated whether NF-κB induction would translate to the maturation status of mouse BM-derived DCs (BMDCs), as measured by CD40, B7.1/CD80, B7.2/CD86, and MHC-II (I-A[b]) surface expression. Treatment of Ad-iMyD88/CD40–transduced DCs with CID resulted in drug-dependent increases in cell-surface expression of CD40, CD86, and MHC-II compared with Ad-iCD40–transduced DCs (iCD40-DCs) or Ad-iMyD88–transduced DCs (iMyD88-DCs). This effect was comparable to that achieved by high-dose (1 μg/ml) LPS treatment or a combination of LPS and CD40L (Figure 2A). In contrast, BMDCs expressed high levels of CD80 that were not changed by receptor transduction or CID treatment (data not shown). These results show that iMyD88/CD40 activation can upregulate a functionally diverse array of immunostimulatory molecules on BMDCs.

In addition to costimulatory molecule expression, functional changes indicative of DC maturity include reduced phagocytic capacity. We tested the ability of DCs to internalize FITC-dextran.

Activation by Ad-iMyD88/CD40 (20,000 viral particles [vp]/cell) significantly decreased the phagocytic capacity of DCs, relative to control Ad-LUC–transduced cells. Transduction of DCs with 4-fold lower Ad-iMyD88/CD40 (5000 vp/cell) revealed a significant reduction in phagocytosis in a CID-dependent manner (Supplemental Figure 2). Therefore, following injection, CID-treated iMyD88/CD40-DCs are incapacitated for additional antigen uptake in vivo. In contrast, nonactivated iMyD88/CD40-DCs (exposed to moderate [5000 vp/cell] Ad) retain their phagocytic potential, similar to immature DCs.

These results also help explain the low level of ligand-independent activation mediated by iMyD88/CD40 in some assays. Overexpression of proteins can promote spontaneous oligomerization. Predictably, lowering iMyD88/CD40 expression eliminates basal signaling in the absence of CID (Supplemental Figures 3 and 4). Thus, CID-independent signaling by iMyD88/CD40 seen in some experiments is an expected by-product of overexpression that can be titrated away when basal signaling is undesirable. In other circumstances, low-level basal signaling could "jump start" maturation.

One of the hallmarks of activated DCs is the enhanced secretion of proinflammatory cytokines. The mode of DC activation determines the array of cytokines produced during an inflammatory response (30). Previous studies have demonstrated that DC activation through simultaneous CD40 and TLR stimulation induces synergistic production of the T$_H$1 cytokine IL-12 (11, 31, 32). While CD40 stimulation induces IL-6, TNF-α, IL-15, and IL-12p40, adjuvants such as LPS are needed for BMDCs to efficiently upregulate IL-12p35 as well as IL-1α and IL-1β (33). In addition to IL-12, pro-T$_H$1 cytokine IFN-γ plays an important role in generating antitumor immunity in response to DC vaccines (34). Therefore, we tested the ability of iMyD88/CD40 to induce proinflammatory cytokines and chemokines in BMDCs. Upon CID addition, we observed up to a 5- to 10-fold increase in IFN-γ, IL-12, IL-1α, and IL-1β production by iMyD88/CD40-DCs (Figure 2B and Supplemental Figure 5). This increase was 2- to 4-fold higher than the combined effects of CD40L and LPS through endogenous receptors. Thus, CID-dependent iMyD88/CD40 signaling in BMDCs results in increased surface expression of maturation markers and a significant increase in bioactive IL-12p70 ($P < 0.0005$) and IFN-γ ($P < 0.05$) production compared with treatment with iMyD88, iCD40, or a combination of iCD40 and LPS.

iMyD88/CD40 enhances downstream proinflammatory signaling. To further investigate the molecular basis for iMyD88/CD40-mediated DC activation and IL-12 production, we tested the phosphorylation of p38, JNK, and IKK associated with IL-12p70 secretion in DCs (35). Low-level basal signaling by iMyD88/CD40 induced the phosphorylation of both IKKα/β and JNK, which was further
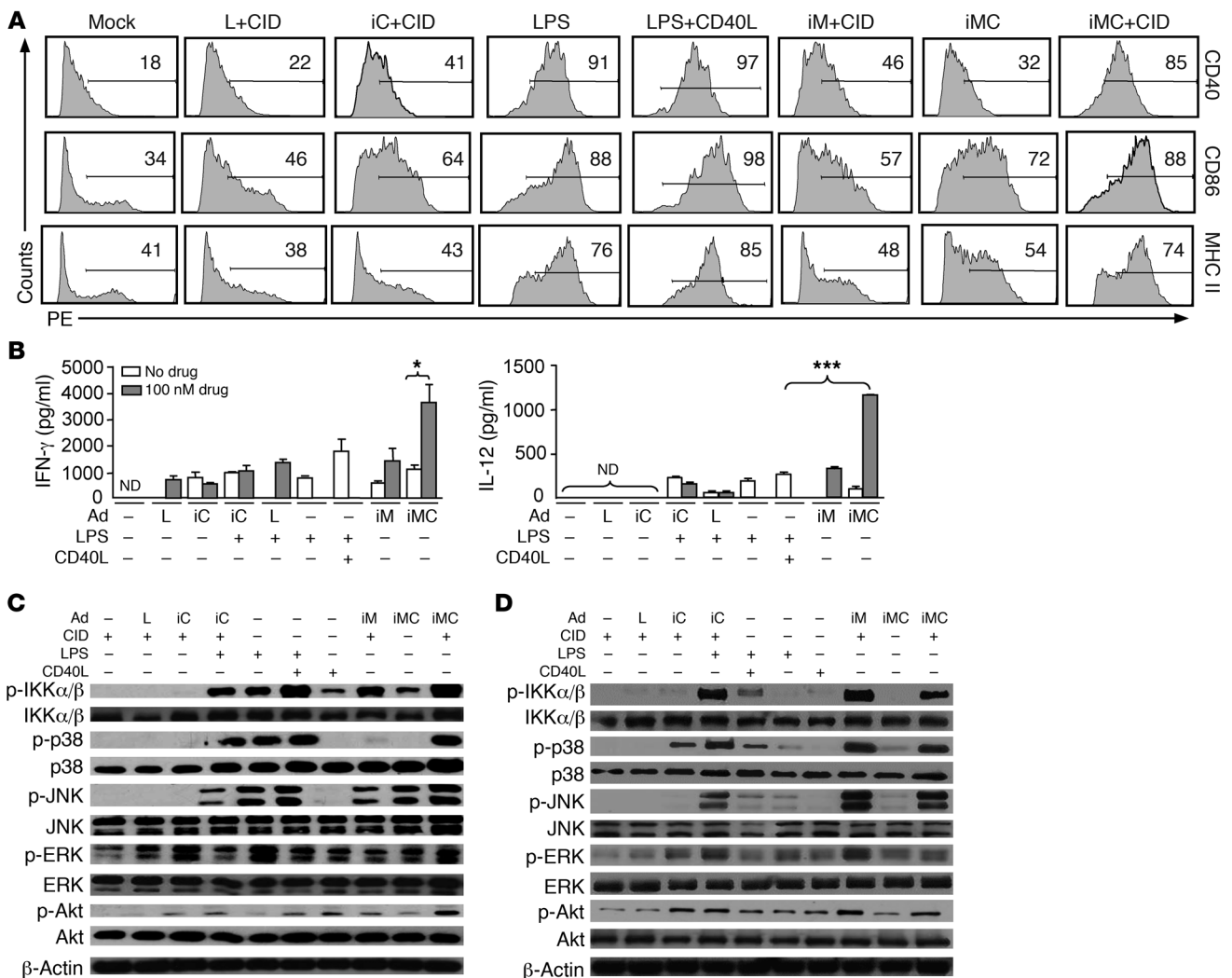
Figure 2
iMyD88/CD40 induces potent activation of primary BMDCs. (**A**) Upon CID treatment, iMyD88/CD40 upregulates immunostimulatory molecules on BMDCs. Immature BMDCs were transduced with 100 MOI Ad and stimulated with CID (100 nM), LPS (1 μg/ml), or soluble CD40L (1 μg/ml) for 48 hours, followed by flow cytometry to measure surface expression of CD40, CD86, and MHC II. Histogram numbers represent percentages of CD40+, CD86+, or MHC II+ cells within the CD11c+ gate. (**B**) CID-induced iMyD88/CD40 activation induces robust proinflammatory cytokine production by BMDCs. Supernatants from Ad-transduced BMDCs, stimulated with CID, LPS, or recombinant mouse CD40 ligand (5 μg/ml) for 48 hours, were analyzed in duplicate in a multiplex cytokine bead assay. *$P < 0.05$ (1-tailed); ***$P < 0.0005$ (2-tailed). Similar results were obtained for mouse soluble (PeproTech Inc.) and multimeric (Axxora, LLC) CD40L. (**C**) iMyD88/CD40 activates proinflammatory signaling molecules in BMDCs. 48 hours after Ad treatment, BMDCs were stimulated with CID, LPS, or recombinant CD40L for 30 minutes. Cell lysates were harvested and analyzed using antibodies to phosphorylated and unphosphorylated IKKα/β, p38, JNK, ERK, and Akt. (**D**) iMyD88/CD40 induces synergistic activation of signaling molecules in human THP-1 cells. THP-1 cells were treated with Ad as listed in part **C** and activated and analyzed as above. Data are representative of at least 3 (**A**, **B**, and **D**) or 2 (**C**) independent experiments.

enhanced by CID addition in iMyD88/CD40-BMDCs (Figure 2C). Potent CID-dependent activation of IL-12p70–promoting p38 and prosurvival Akt (36) were also observed. However, stimulation with LPS alone was sufficient to activate some downstream signaling in BMDCs. Therefore, to better reveal cooperative effects of CD40 and LPS signaling, we tested signaling by iMyD88/CD40 in human monocyte-derived DCs (MoDCs) and also a human clonal monocytic cell line, THP-1, chosen to overcome commonly observed donor-dependent variability in MoDCs. Consistent with mouse DCs, iMyD88/CD40 increased the phosphorylation of

IKKα/β, p38, and JNK, as well as Akt, in a CID-dependent manner (Figure 2D and Supplemental Figure 6), suggesting that combinatorial activation of multiple IL-12–promoting factors could contribute to the observed CID-dependent synergistic IL-12 production by iMyD88/CD40-DCs. Thus, iMyD88/CD40 induction can augment DC licensing by boosting the activation state of several key signaling molecules linked to pro-T_H1 cytokine production, serving to amplify acquired immune responses.

iMyD88/CD40 enables activation, survival, and T cell–stimulatory functions of human MoDCs. To demonstrate the physiological relevance
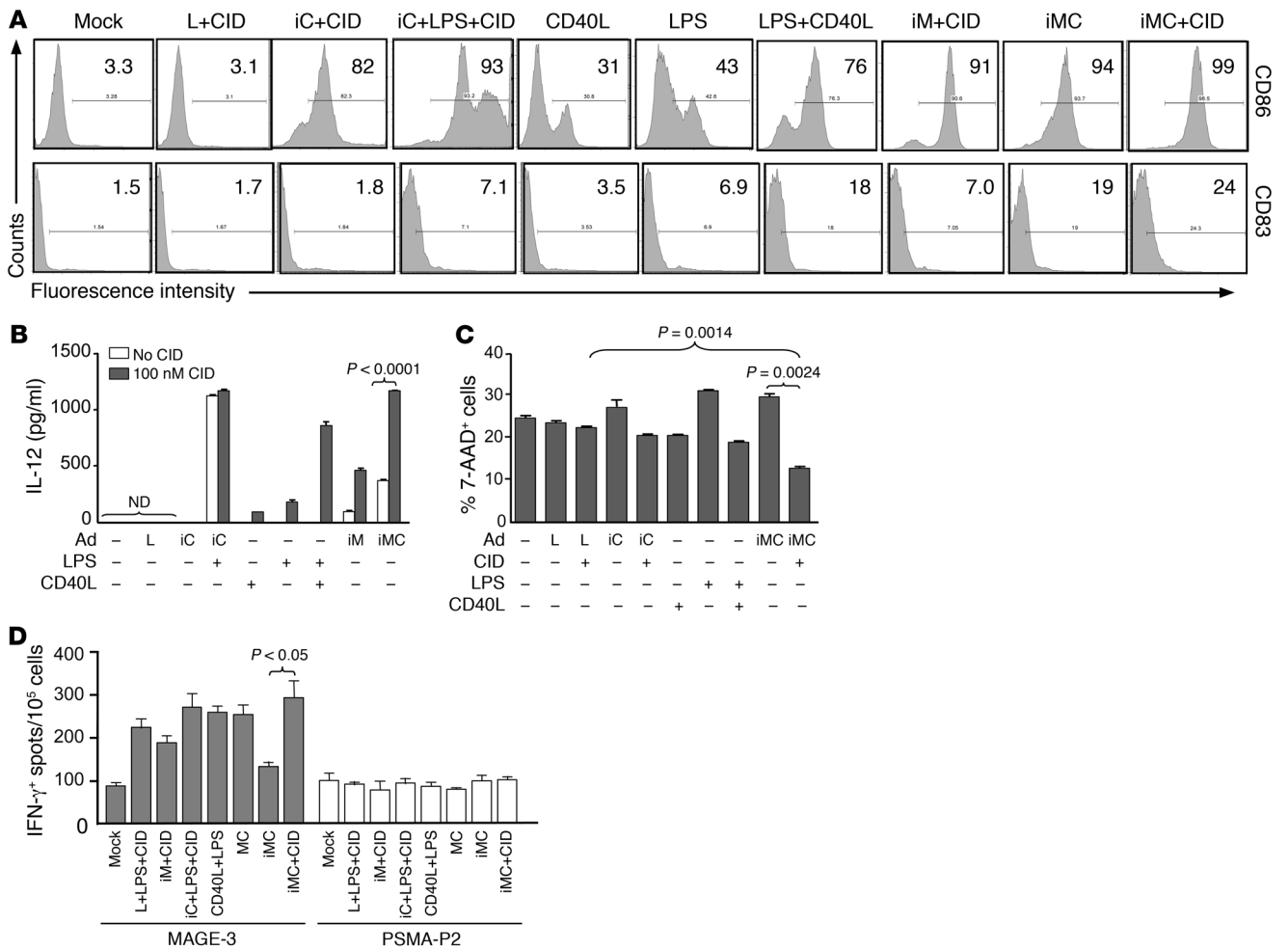
Figure 3

iMyD88/CD40 enhances the activation, survival, and T cell–stimulatory function of human DCs. (**A**) CID-mediated iMyD88/CD40 activation upregulates costimulatory molecules on MoDCs. MoDCs treated with Ad (10,000 vp/cell) and CID or enhanced CD40L (1 μg/ml) and LPS (1 μg/ml) for 48 hours were analyzed by flow cytometry. Histogram numbers represent percentages of CD83+ or CD86+ DCs. (**B**) iMyD88/CD40 augments IL-12p70 production upon CID addition. MoDCs were Ad transduced and activated with CID/LPS/CD40L. 48 hours later, supernatants were analyzed in duplicate using ELISA. (**C**) iMyD88/CD40 improves MoDC survival. MoDCs were transduced with Ad activated for 1 day, washed, and plated in serum-free human DC media. Data indicate percentage of 7-AAD+ (dead) cells on day 9 as analyzed in duplicate by flow cytometry. (**D**) iMyD88/CD40-DCs boost MAGE-3–specific CTL responses in a CID-dependent manner. MAGE-3–pulsed MoDCs, Ad-transduced and activated with CID or combinations of LPS+CD40L or MC, were used to stimulate autologous CD8+ T cells. Following 2 rounds of stimulation with DCs, T cells were assayed in triplicate by IFN-γ ELISpot assay. PSMA-P2, irrelevant HLA-A2–restricted PSMA peptide. All data represent mean ± SEM, *P* values (2-tailed), representative results out of 3 (different donors) presented for all assays.

of iMyD88/CD40 as a DC activation receptor, we tested the activation status of iMyD88/CD40-treated human MoDCs and their ability to prime autologous T cell responses to a melanoma antigen, MAGE-3. Similar to mouse BMDCs, we found that iMyD88/CD40 crosslinking in vitro consistently activates MoDCs, inducing CID-dependent upregulation of costimulatory molecules CD83 and CD86 on MoDCs (Figure 3A and Supplemental Figure 7). Upon CID addition, iMyD88/CD40-DCs also produced significantly higher levels of bioactive IL-12p70 than they did without CID (*P* < 0.0001), comparable to or higher than iCD40+LPS+CID-treated DCs (Figure 3B and Supplemental Figure 8). Moreover, the magnitude of IL-12 induction depended on the levels of iMyD88/CD40 transgene expression. MoDC transduction with increasing MOI of Ad-iMyD88/CD40 led to a linear increase in the magnitude

of IL-12 produced over a 2-day period (Supplemental Figure 9). Additionally, CID-treated iMyD88/CD40-MoDCs had significantly (*P* < 0.005) improved survival in vitro, up to 9 days following activation, compared with MoDCs activated with LPS+CD40L or LUC-modified DCs (Figure 3C and Supplemental Figure 10). These data show that iMyD88/CD40 activation not only enhances human MoDC maturation and IL-12 production, but also prolongs MoDC survival in vitro, consistent with the elevated phosphorylation of antiapoptotic Akt observed in these cells.

To determine whether improved activation status translated to robust DC licensing, we set up a coculture assay to test priming of autologous antigen-specific T cell responses. We tested the generation of IFN-γ+ CTL responses to HLA-A2+–restricted human melanocyte–associated peptide antigen MAGE-3$_{271-279}$. MoDCs (from
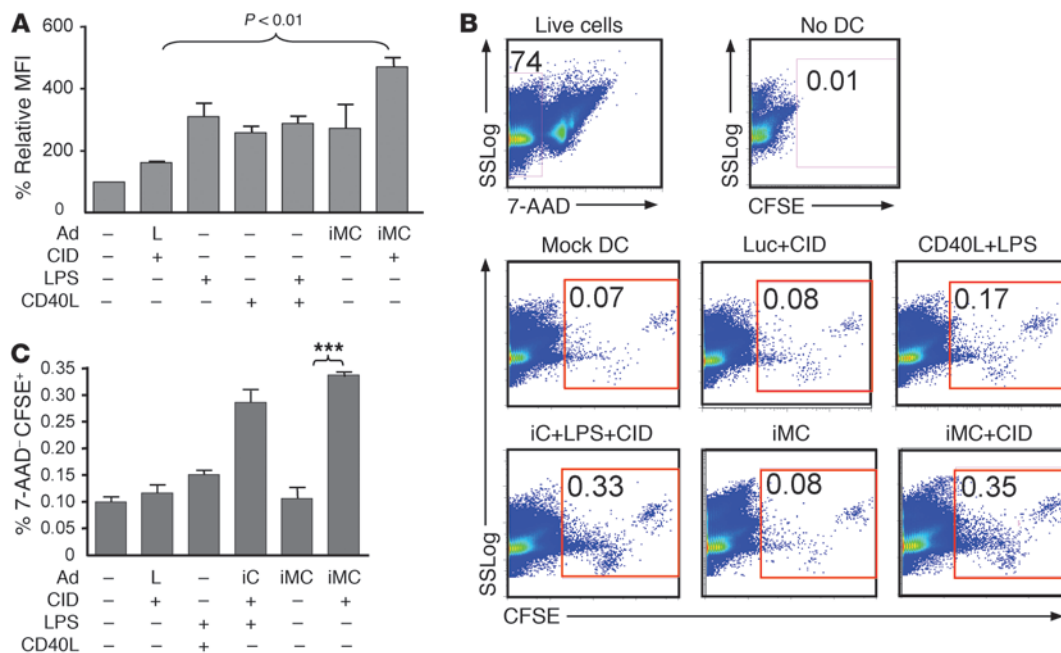
Figure 4
iMyD88/CD40 enhances CCR7 expression and in vivo migration of BMDCs. (**A**) iMyD88/CD40 upregulates CCR7 expression on BMDCs on treatment with CID. Immature BMDCs were Ad transduced (20,000 vp/cell) and stimulated with CID (100 nM) or LPS (250 ng/ml) and CD40L (2 μg/ml) for 24 hours. Percentage of CD11c+ DCs expressing CCR7 was determined by flow cytometry. Data normalized to mock-treated DCs, representative of 3 independent experiments. (**B** and **C**) iMyD88/CD40 enhances migration of BMDCs in vivo in a CID-dependent manner. Purified, CFSE-labeled BMDCs treated with Ads for 24 hours were injected s.c. into the footpads of C57BL/6 mice (n = 5) followed by CID administration (i.p.) the same day. 3 days after injection, cells from draining popliteal lymph nodes were stained with 7-AAD and percentage of viable CFSE+ cells was determined by flow cytometry. (**B**) Representative dot plots with numbers indicative of CFSE+ fraction. (**C**) Data represent means + SEM. ***$P < 0.0001$ (2-tailed), representative of 3 independent experiments.

HLA-A2+ donors) were transduced with adenoviruses expressing iCD40, iMyD88, iMyD88/CD40, or control protein, LUC and stimulated with or without CID and LPS, as indicated. Nontransduced cells were either untreated or matured using standard MC or a combination of CD40L and LPS. Following 2 rounds of stimulation with activated DCs, T cells were analyzed by ELISpot assay. CID-treated iMyD88/CD40-DCs induced a significantly ($P < 0.05$) higher frequency of MAGE-3$_{271-279}$–specific T cells, comparable to that observed with MC, CD40L+LPS, or iCD40+LPS+CID-treated MoDCs (Figure 3D). In addition, the percentage of activated DCs expressing IL-12p70 and effector CTLs expressing IFN-γ and perforin, as determined by intracellular staining, was higher in the iMyD88/CD40+CID treatment group compared with no CID treatment or LUC+CID (Supplemental Figure 11). Additionally, intracellular IL-12 was detected up to 1 week following removal of DC activation stimuli (data not shown), suggesting durable IL-12 production by iMyD88/CD40+CID-treated DCs. These results demonstrate that modification of MoDCs to express iMyD88/CD40 improves their immunogenic potency in a CID-dependent manner.

It is noteworthy that we selected Ads to deliver our transgenes. While Ads combine robust transgene expression with low cytotoxicity, the underlying rationale for their clinical use (37), Ad-modified DCs are also primed to present adenoviral capsid components (38, 39). However, these vector-associated antigens did not preclude antitumor efficacy in our studies. Nevertheless, future iterations of this vaccine will encompass nonviral delivery of the iMyD88/CD40 to circumvent potential off-target effects of Ads.

iMyD88/CD40 induces migration of BMDCs in vivo. In addition to DC activation, migration to regional, draining lymph nodes is critical for priming naive T cells (40–42). LPS induces surface expression of CCR7, required for trafficking to tissue-draining lymph nodes. Activation of iMyD88/CD40 in mouse DCs resulted in augmented CCR7 expression, comparable to CD40L+LPS treatment (Figure 4A). To test the effect of iMyD88/CD40 on DC migration in vivo, we injected CFSE-labeled BMDCs, activated with LPS or iMyD88/CD40+CID in vitro, s.c. into the hind footpads of C57BL/6 mice, followed by i.p. CID administration. Immature DCs or Ad-LUC–transduced DCs served as controls. Approximately 72 hours after footpad DC injection, viable cell populations in the draining popliteal lymph nodes were analyzed. We observed a significantly ($P < 0.05$) higher percentage of CFSE+ cells in the lymph nodes of mice receiving CID-treated iMyD88/CD40-BMDCs compared with mice injected with LUC-BMDCs (Figure 4, B and C). Thus, elevated CCR7 expression by iMyD88/CD40-DCs correlates with improved migration from footpads following in vivo drug delivery.

iMyD88/CD40 enhances the therapeutic efficacy of DC tumor vaccines and antitumor immune responses. To assess the therapeutic efficacy of iMyD88/CD40-DCs, we evaluated these cells in 2 distinct tumor models, B16.F10 melanoma and EG.7-OVA lymphoma (Figure 5). In both models, s.c. tumors were preestablished at the time of DC vaccination. In B16.F10-based experiments, DCs were pulsed with tyrosinase-related protein 2–derived (*TRP2*-derived), immunodominant, H2-K^b–restricted peptide SVYDFFVWL. iMyD88/CD40-DC immunization of mice bearing 7-day preestablished B16.
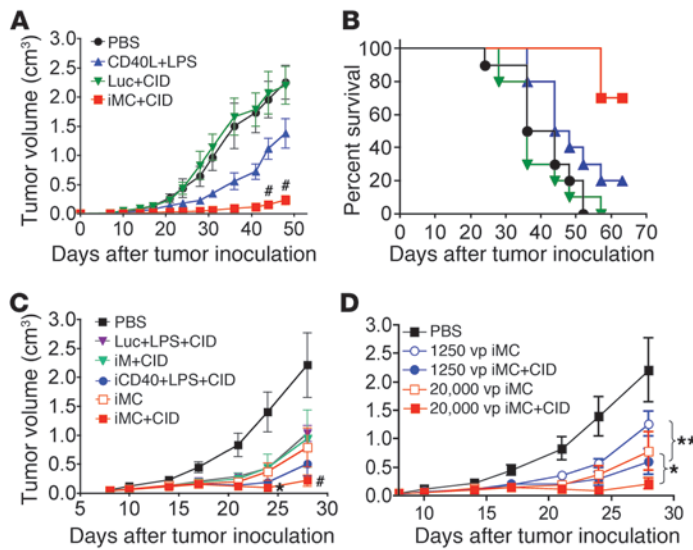
Figure 5
iMyD88/CD40 enhances the therapeutic efficacy of DC-based vaccines against ectopic tumors. (**A**) iMyD88/CD40-DC vaccine effectively controls preestablished aggressive B16 tumors. C57BL/6 mice (*n* = 10) bearing approximately 5-mm B16.F10 tumors were vaccinated s.c. 3 times, 7 days apart, with TRP2$_{180–188}$–pulsed BMDCs transduced with Ad (20,000 vp/cell) followed by in vivo CID administration. Tumor growth was monitored biweekly. #*P* < 0.001, iMyD88/CD40+CID-treated DCs versus CD40L+LPS-DCs (at indicated time points) or LUC+CID-DCs (from day 28, using 2-way ANOVA). (**B**) Enhanced survival of B16.F10-bearing mice treated with iMyD88/CD40-DCs (*n* = 10). *P* = 0.0005, iMyD88/CD40+CID-treated DCs compared with control groups, based on log-rank test. (**C**) Vaccination with 1 dose of iMyD88/CD40-DCs plus in vivo CID eradicates preestablished E.G7-OVA tumors. On day 8 after inoculation of EG.7-OVA tumors, mice (*n* = 6) were vaccinated with OVA-pulsed, Ad-transduced (20,000 vp/cell) BMDCs, activated with 50 nM CID and/or LPS (250 ng/ml) and CD40L (2 μg/ml). *simP* < 0.05, iMyD88/CD40+CID DCs versus LUC+LPS+CID DCs; #*P* < 0.001, iMyD88/CD40+CID DCs versus LUC+LPS+CID DCs or iMyD88/CD40 DCs (no in vivo CID); from 2-way ANOVA. (**D**) Elevated iMyD88/CD40 transgene expression correlates with improved DC vaccine efficacy. E.G7-OVA tumor-bearing mice (*n* = 6) were vaccinated with BMDCs transduced with either 20,000 vp or 1,250 vp/cell of Ad-iMyD88/CD40 and activated in vitro with 50 nM CID, with or without in vivo CID, *simP* < 0.05 (2-tailed), 20,000 vp iMC+CID DCs versus 20,000 vp iMC DCs (no in vivo CID); **simP* < 0.01 (2-tailed), 1,250 vp iMC+CID DCs versus 1,250 vp iMC DCs (no in vivo CID). Data representative of 2 independent experiments. Data are represented as mean ± SEM.

F10 tumors, followed by in vivo administration of CID (AP1903) and 2 booster vaccines, significantly reduced tumor progression (*P* < 0.001, 4/10 mice in the iMyD88/CD40+CID vaccination group were B16 tumor–free on day 57) and enhanced survival compared with PBS and all DC vaccine groups (*P* = 0.0005) (Figure 5, A and B). In E.G7-OVA tumor experiments, only a single vaccine, comprising iMyD88/CD40-DCs pulsed with OVA, was sufficient to inhibit the growth of 7-day preestablished tumors (Figure 5C). To determine whether the level of iMyD88/CD40 transgene expression influenced the efficacy of our DC vaccine, we transduced BMDCs with both low (1,250 vp/cell) and high (20,000 vp/cell) doses of Ad-iMyD88/CD40, achieving approximately 5% and 50% BMDC transduction levels, respectively (Supplemental Figure 12). Although higher transgene expression in DCs correlated with bet-

ter vaccine efficacy, even at relatively low levels of iMyD88/CD40 expression, peptide-pulsed DC vaccines effectively controlled the tumors in a CID-dependent manner (Figure 5D). Thus, iMyD88/CD40-modified DC vaccines can effectively control preexisting tumors at both high and suboptimal levels of transgene expression.

To further evaluate the induction of antigen-specific adaptive immunity, we analyzed the generation of OVA-specific CD8+ T cell responses in E.G7-OVA tumor-bearing mice following vaccination. Analysis of peripheral blood and spleens of vaccinated mice showed that iMyD88/CD40-DCs induced a higher frequency of OVA$_{257–264}$–specific (SIINFEKL-specific), IFN-γ–producing CD8+ T cells compared with controls (i.e., DCs modified with iMyD88 alone or iCD40 alone or DCs activated with LUC+LPS or CD40L+LPS) (Figure 6A and Supplemental Figure 13). Similar to tumor growth, enhanced T cell expansion in vivo was CID- and transgene expression–dependent (Figure 6A).

To test the functional status and cytotoxic potential of the vaccine-elicited CD8+ T effector cells, we performed in vitro and in vivo cytotoxicity assays. Splenocytes from DC-vaccinated mice were used as effectors in the in vitro assay, whereas DC-vaccinated mice were injected with SIINFEKL-pulsed splenocytes from a naive syngeneic mouse to test antigen-specific lysis of targets in vivo. Consistent with elevated CD8+ T cell expansion, splenocytes from iMyD88/CD40-DC–immunized mice induced higher levels of specific lysis of SIINFEKL-pulsed (63% ± 16% for in vivo CTL assay) (Figure 6B) or E.G7-OVA (65%–81% for in vitro assay) target cells (Figure 6C) relative to control DCs. Interestingly, we found that splenocytes from iMyD88/CD40-DC–immunized mice also induced up to 70% lysis of NK target cell YAC-1 (Figure 6C). Overall, these results indicate that iMyD88/CD40-DC–based vaccines can effectively activate both innate NK responses and antigen-specific CD8+ T cell responses, accounting for their enhanced tumoricidal potential compared with DCs matured with an exogenous CD40L+LPS combination.

Finally, we attempted to evaluate the role of various proinflammatory cytokines in vaccine efficacy. In our studies, iMyD88/CD40-BMDCs expressed several-fold enhanced levels of cytokines and chemotactic factors, including IFN-γ, IL-12, and IP-10/CXCL10 (Figure 2B and Supplemental Figure 5C), which could all potentially contribute to the enhanced antitumor immune responses observed (34, 43, 44). Therefore, we tested whether iMyD88/CD40-modified BMDCs from IFN-γ, IP-10, IL-12p35, or IL-12p40–deficient (KO) mice or control WT mice could induce SIINFEKL-specific CD8+ T cells in tumor-naive C57BL/6 WT mice. Notably, peripheral blood analysis 1 week after vaccination revealed that while IL-12p35 and IL-12p40 were absolutely necessary for the initiation of SIINFEKL-specific CD8+ T cells, both IFN-γ and IP-10 were also required for maximum induction of CTL responses in vivo (Figure 6D). Collectively, these results suggest that modification of DC vaccines with iMyD88/CD40 significantly (*P* < 0.0001) increases antigen-specific antitumor responses that in turn require multiple DC-secreted factors, including IFN-γ, IP-10, and especially IL-12 subunits p35 and p40. Our data show that modification of DCs ex vivo with this DC-CAR, which we believe to be novel, enables 2-step DC activation before and after vaccination, perhaps in a more physiological setting, enabling improved immunotherapy against preestablished tumors.
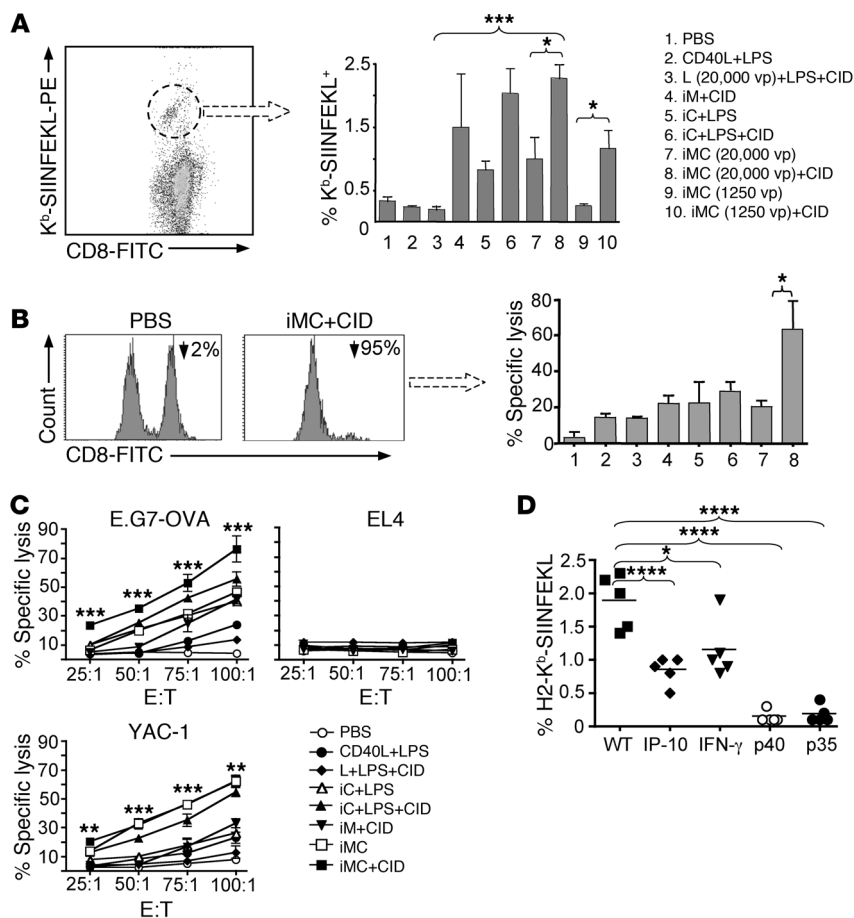
Figure 6

iMyD88/CD40-DC vaccination generates robust antitumor immunosurveillance in vivo. (**A**) iMyD88/CD40-DCs enhance antigen-specific CD8+ T cell responses in vivo. E.G7-OVA tumor-bearing mice (*n* = 3) were immunized as described in Figure 5, C and D. 1 week later, PBMCs were analyzed for CD8+ K^b-SIINFEKL+ cells. Data represent mean + SEM. *P < 0.05; ***P < 0.0005 (2-tailed). (**B**) iMyD88/CD40-DC+CID delivery enhances tumor antigen–specific cytotoxic potential in vivo. Mice were treated as described above (*n* = 3). 2 weeks later, splenocytes from syngeneic C57BL/6 mice were pulsed with OT-I (SIINFELKL) peptide, CFSE-labeled (CFSE^{hi}), and injected i.v. as targets. The next day, CFSE+ splenocytes were quantified. Histogram numbers represent percentages of CFSE^{hi} cells. Data represent mean + SEM. *P < 0.05 (1-tailed). (**C**) Combined activation of innate and adaptive antitumor responses by iMyD88/CD40-DCs. Splenocytes from immunized mice were cocultured with ^{51}Cr-labeled E.G7-OVA or control EL-4 (OVA−) or YAC-1 (NK targets) cells at different effector to target (E:T) ratios. Data represent mean ± SEM (*n* = 3). **P < 0.01; ***P < 0.001, iMyD88/CD40+CID versus all groups; 2-way ANOVA. (**D**) DC-secreted factors IP-10, IFN-γ, and IL-12 mediate antitumor effects of iMyD88/CD40-DC vaccine. SIINFEKL-pulsed, iMyD88/CD40-modified BMDCs from WT or IP-10, IFN-γ, IL-12p40, or IL-12p35 KO mice were activated with CID in vitro and in vivo following footpad injection. PBMCs were analyzed as discussed in **A**. Each data point corresponds to an individual mouse. Horizontal bars denote the mean for each group. Data represent mean + SEM (*n* = 5). *P < 0.05; ****P < 0.0001 (1-way ANOVA). Representative of 2 independent experiments.

Discussion

A major challenge in the generation of DC vaccines has been the ability to achieve optimal DC activation, which would enable prolonged DC survival, increased migration to lymph nodes, and more potent antigen-specific T cell priming following in vivo delivery. Improvements in all of these characteristics could potentially contribute to a more effective therapeutic cancer vaccine.

Here, building on our previous preclinical and clinical work developing a dimerizer-inducible CD40 receptor, we have designed a chimeric, inducible signaling "switch," iMyD88/CD40, that potentiates strong antitumor immune responses. iMyD88/CD40 replaces adjuvants, such as LPS, used with first-generation iCD40, overcoming technical hurdles and risks of LPS toxicity in clinical applications.

Using the syngeneic CID system, we initially developed inducible MyD88, consisting of MyD88's death and intermediate domains, but devoid of its TIR domain. Removal of the TIR domain should minimize unproductive or confounding upstream interactions of iMyD88 with endogenous TIR-containing proteins, which include the TLR4 complex, other surface-localized and endosomal TLRs, or IL-1 receptor family members (45). Integration of this approach with our previously engineered iCD40 generated the composite, drug-inducible MyD88/CD40 activation switch. Upon addition of CID, iMyD88/CD40 triggering enables temporal control of DC activation both in vitro and in vivo. Moreover, restricting CID administration to narrow windows following DC administration should permit time for DC migration to draining lymph nodes, where proinflammatory cytokine production can be elaborated at not only the "right time" after vaccination, but also at the "right place," namely, within draining lymph nodes where migrated DCs can prime antigen-specific T cells. Additionally, CID removal or decay should return the system to background levels of activation, potentially acting as an additional safety mechanism to further minimize any risk of adverse events.

Other recent strategies to improve DC function have included attempts to inactivate negative feedback pathways or molecules, such as suppressor of cytokine signaling 1 (SOCS1). The uncontrolled "removal of brakes" resulted in breaking of tolerance to self antigen, tyrosinase-related protein 2 (TRP-2), promoting autoimmune vitiligo and hair loss in mice bearing B16 melanoma tumors (34). In contrast, administering TRP2-pulsed, iMyD88/CD40-enhanced DCs coupled with in vivo CID delivery did not trigger similar autoimmune pathology in mice bearing B16 melanoma tumors when followed for up to 9 months. Still, the iMyD88/CD40-DC vaccination strategy effectively induced regression and stasis of 7-day preestablished tumors, highlighting the utility of a regulatory switch for efficient DC immunotherapy. While we did observe expected basal signaling in some in vitro assays, this can be titrated away and does not seem to diminish antitumor efficacy.

Appropriately, iMyD88 activation does not appear to entirely phenocopy TLR4 signaling, as it lacks MyD88-independent, TRIF-dependent signaling. However, in addition to its antiviral effects,

TRIF can promote endotoxin tolerance, DC apoptosis, and skewing toward a T_H2 phenotype (22, 46, 47). TRIF also activates the production of type I IFNs. Although IFN-α/β can promote the survival and maintenance of activated T cells (48), type I IFNs can also negatively regulate TLR4-mediated DC survival that would be disadvantageous in the context of ex vivo DC vaccine development (19). Finally, a recent study reported that between the 2 main TLR4 signaling pathways, TRIF was more critical for macrophage activation while MyD88 was necessary and sufficient to potently activate DCs in response to LPS (49). In summation, TRIF induction of negative regulatory pathways may counterbalance its positive role as an adjuvant in DC activation. Our studies have shown that iMyD88 alone can effectively replace LPS-mediated signaling and proinflammatory responses in DCs, which are further enhanced several-fold by the DC-CAR iMyD88/CD40. Therefore, we propose that, in addition to positive regulatory effects, circumventing the TRIF pathway with the use of iMyD88/CD40 may overcome proapoptotic signals, resulting in prolonged DC survival and improved T_H1 responses.

In addition to improving longevity, iMyD88/CD40 triggering also enhances migration and activation of mouse and human DCs. Migration of activated DCs to draining lymph nodes is critical for priming antigen-specific T cells and acts as a chemokine source, which increases lymphocyte recruitment and subsequent proliferation (41, 42). Thus, a collective increase in DC migratory ability, survival, and activation could contribute toward priming a stronger T cell response.

At the molecular level, nuclear localization of Rel subunits in response to CD40 or LPS stimulation is associated with enhanced antigen presentation and an upregulation of maturation markers on DCs (29, 50, 51). Using the human monocytic cell line THP-1, we demonstrated simultaneous activation of all 3 NF-κB Rel subunits by iMyD88/CD40, correlating with increased expression of costimulatory molecules on the surface of mouse and human DCs. This chimeric, inducible, intracellular receptor can enhance signaling without the possible limitations caused by ectodomain cleavage and endotoxin tolerance observed with CD40L and LPS treatment, respectively (6, 52, 53). CID treatment of iMyD88/CD40-DCs also enhanced production of T_H1 cytokines IL-12 and IFN-γ, although we did not rule out that elevated IFN-γ had resulted from autocrine-positive feedback caused by high IL-12p70 levels. Furthermore, we observed elevated phosphorylation of IKK, p38, and JNK, all previously implicated in IL-12 and IFN-γ production, as well as increased activation of the downstream PI3K effector, Akt, which promotes DC survival (36). These results provide a potential mechanistic explanation for combined additive activation of overlapping signaling pathways resulting in the enhanced activity of iMyD88/CD40. Thus, heightened induction of downstream proinflammatory mediators by iMyD88/CD40 can result in high-level DC activation, promoting tumor antigen–specific immune responses in vivo that effectively control preestablished tumors. At the same time, a variety of other parameters could further influence the antitumor efficacy of the iMyD88/CD40-DC vaccine, including transgene levels or vaccine schedule. Additional studies will be needed to evaluate the full mechanisms involved in the induction of durable antitumor responses by iMyD88/CD40-DCs.

Current clinical protocols for tumor immunotherapy have included in vivo administration of TLR agonists, such as TLR9-binding, unmethylated CpG oligodeoxynucleotides or TLR7/8-binding resiquimod/R848. Although TLR agonists can boost immune responses, the systemic use of TLR agonists poses a risk of triggering autoimmunity (54). On the other hand, intratumoral delivery of TLR adjuvants limits their use to easily accessible, local rather than distant metastatic lesions (54). In addition, untargeted TLRs on the surface of tumor cells have been shown to promote their growth and immune escape following ligand administration, confounding antitumor goals (55). These findings further support the need for development of adjuvants, such as iMyD88/CD40, that can be targeted and activated specifically in DCs. While the use of iMyD88/CD40 in ex vivo–differentiated DCs solves a major obstacle in DC vaccine efficacy, the generation and production of clinical-grade autologous DC vaccines remain an expensive and cumbersome process (56). To overcome this caveat, the iMyD88/CD40 gene therapy platform should also be amenable to in vivo DC-targeting strategies currently under consideration. One of several such strategies would involve the ligand-mediated delivery of both iMyD88/CD40 and tumor antigen–encoding DNA elements to DC receptors, such as CD205 or DNGR1/CLEC9A (57, 58), providing the platform of a broadly applicable "off-the-shelf" vaccine.

Methods

Mice, cell lines, peptides, and dimerizer drug. Six- to eight-week-old female C57BL/6 mice were purchased from the Center for Comparative Medicine at Baylor College of Medicine and Harlan Sprague Dawley Laboratory. CD45.1 C57BL/6 mice, B6.129S1-IL12a^{tm1Jm}/J (IL-12p35–deficient), B6.129S1-IL12b^{tm1Jm}/J (IL-12p40–deficient), B6.129S1-Ifnγ^{tm1Ts}/J (IFN-γ–deficient), and B6.129S4-Cxcl10^{tm1Adl}/J (IP-10–deficient) mice were purchased from the Jackson Laboratory and maintained under pathogen-free conditions in the Transgenic Mouse Facility at Baylor College of Medicine. Animal procedures were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine. E.G7-OVA, EL-4, YAC-1, HEK293, B16.F10, and THP-1 cells were purchased from ATCC and cultured as recommended by the vendor. Peptides were produced at 80%–95% purity by Genemed Synthesis Inc. Dimerizer drugs, AP1903, and AP20187 were provided by ARIAD Pharmaceuticals.

Recombinant plasmids and Ads are described in Supplemental Methods.

Preparation and activation of mouse and human DCs. Murine BMDCs were prepared as previously described with modifications (9, 36).

BMDCs were transduced with Ad5 vectors (with 5 μl of GeneJammer transfection reagent [Agilent Technologies]/1 × 10^6 cells for in vivo experiments) and activated using 50–100 nM CID (AP1903, Formatech), 2–5 μg/ml CD40L (recombinant CD40L from Peprotech Inc., or multimeric enhanced CD40L from Axxora LLC) and 10–1000 ng/ml LPS (Sigma-Aldrich), where indicated.

Human MoDCs were generated from the blood of healthy donors as previously described (11). Human MoDCs were activated with Ad5 or Ad5F35 vectors and CID (100 nM) or 1 μg/ml of multimeric enhanced CD40L (Axxora LLC), 10 ng–1 μg/ml of LPS (Sigma-Aldrich), and MC containing 10 ng/ml of TNF-α, 10 ng/ml of IL-1β, 150 ng/ml of IL-6 (R&D Systems), and 1 μg/ml of PGE$_2$ (Cayman Chemicals).

Tumor experiments. Groups (n = 5–10) of C57BL/6 female mice were inoculated s.c. with 50,000 B16.F10 tumor cells or 100,000 E.G7-OVA cells. To prepare DC vaccines, BMDCs were pulsed with TRP2$_{180–188}$ (10 μg/ml) or OVA (10 μg/ml), transduced with 20,000 vp/cell of Ad5 vectors, and activated with 50 nM CID (AP1903), LPS (250 ng/ml), or CD40L (2 μg/ml) overnight. On day 7 (when tumors were measurable or palpable), BMDCs (2 × 10^6 cells/mouse) were injected into the footpads of mice. Booster vaccines were given at 7-day intervals to mice bearing B16.F10 tumors. Mice in CID treatment groups received i.p. injections of CID (AP1903) at 5 mg/kg 24 hours following DC vaccination. Tumors were measured biweekly, and tumor volume was calculated as 0.5236 × length × width^2. Mice with tumors of greater than 10% of body weight or exceeding 1 cm^3 were euthanized and graphed with last recorded tumor volume.

DC survival assay. MoDCs from CD14+ precursors grown in DC medium supplemented with GM-CSF and IL-4 were transduced with Ads (10,000 vp/cell) and/or activated with combinations of 1 μg/ml enhanced CD40L (Axxora LLC), 250 ng/ml LPS, and 100 nM CID (AP1903). 16 hours later, cells were washed and plated in serum-free human DC media without growth factors or activating stimuli. Cell viability was assessed at different time points by flow cytometry, using a 7-aminoactinomycin D (7-AAD) assay (BD Biosciences).

ELISpot and in vitro cytotoxicity assays. Splenocytes from E.G7-OVA–bearing mice were purified and stimulated with $OVA_{257-264}$ (SIINFEKL) H2-K^b–restricted peptide, and irrelevant $TRP2_{180-188}$ (SVYDFFVWL) peptide (negative control). 96-well nitrocellulose-base plates (Millipore) were coated overnight with 10 μg/ml anti-mouse IFN-γ mAb (AN 18; Mabtech Inc.), washed with PBS, and blocked with complete RPMI 1640. Splenocytes were seeded into wells and incubated with 1 μg/ml of peptides for 20 hours at 37°C in 5% CO_2. Cells were washed away with PBS (0.5% Tween-20) and further incubated with 1 μg/ml biotinylated anti-mouse IFN-γ antibody (R4-6A2; Mabtech) at 37°C for 2 hours. The avidin/biotinylated enzyme complex (Vector Laboratories) was added for 1 hour followed by detection of IFN-γ–secreting cells using 3-amino-9-ethylcarbazole substrate (Sigma-Aldrich). The results were evaluated by ZellNet Consulting Inc. using KS ELISPOT 4.3 software. Human IFN-γ ELIspot assays were performed with blood from HLA-A2+ healthy volunteers, as previously described (11).

CTL and NK responses were assessed by a standard ^{51}Cr-release assay. In CTL assays, splenocytes pooled from immunized mice were cocultured with OVA-expressing E.G7-OVA cells or EL4 control cells. YAC-1 cells were used as target cells in NK cell assay. All target cell lines were labeled with 0.1 μCi of ^{51}Cr (PerkinElmer Inc.). Different numbers of effector cells were incubated with a constant number of target cells (5×10^4/well) in 96-well U-bottomed plates for 6 hours at 37°C in triplicate. Supernatants were harvested and ^{51}Cr release was measured using a MicroBeta Trilux Counter (PerkinElmer Inc.). Percentage of lysis was calculated as follows: (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100.

In vivo CTL assay. Mice bearing E.G7-OVA tumors were used in this study. Syngeneic splenocytes from naive C57BL/6 mice were used as in vivo target cells. Cells were labeled by incubation for 10 minutes at 37°C with either 6 μM CFSE (CFSE^{hi} cells) or 0.6 μM CFSE in complete RPMI 1640 medium (CFSE^{lo} cells). CFSE^{hi} cells were pulsed with SIINFEKL peptide, and CFSE^{lo} cells were incubated with control peptide *SVYDFFVWL*. A mixture of 4×10^6 CFSE^{hi} and 4×10^6 CFSE^{lo} cells was injected i.v. via tail vein into tumor-bearing hosts. After 16 hours of in vivo incubation, mice were euthanized, splenocytes were collected, and single-cell suspensions were analyzed for detection and quantification of CFSE-labeled cells. Percentage of lysis was calculated as follows: 100 – ([percentage SIINFEKL-pulsed] × [percentage SVYDFFVWL-pulsed]$^{-1}$ × 100).

In vivo DC migration assay. To analyze DC migration in vivo, BMDCs were enriched using the CD11c magnetic microbead separation method (Miltenyi Biotec). Purified BMDCs were transduced with Ad (10,000 vp/cell) and activated with CID (100 nM) or LPS (250 ng/ml). The following day, DCs were labeled with 6 μM CFSE for 30 minutes and injected s.c. into the footpads of C57BL/6 mice (2×10^6 cells per mouse). Mice of LUC+CID, iCD40+LPS+CID, and iMC+CID groups were administered CID (AP1903, 5 mg/kg) i.p. the same day as DC administration. On day 3 after injections, single-cell suspensions of draining popliteal lymph nodes were stained with 7-AAD and percentage of CFSE+ viable cells was analyzed by flow cytometry (600,000 events were acquired).

Flow cytometry and cytokine expression analysis. Following treatment with adenoviruses and activation with CID (100 nM), LPS (1 μg/ml), and CD40L (1 μg/ml), expression of DC markers on the surface of mouse BMDCs and human MoDCs was analyzed by flow cytometry as described in Supplemental Methods. To analyze peripheral blood lymphocytes (PBLs), cells were purified on Histopaque 1083 gradient (Sigma-Aldrich). To analyze intracellular expression of HA-tagged transgenes or cytokines, cells were fixed and permeabilized using BD Cytofix/Cytoperm Kit (BD Biosciences). Data were analyzed using FlowJo software (Tree Star Inc.)

The expression of cytokines in culture supernatants was analyzed according to manufacturers' instructions using ELISA kits for mouse and human IL-12p70 (BD Biosciences) and a Milliplex Mouse Cytokine Bead Assay (Milliplex Co.).

Western blotting. To prepare nuclear extracts, THP-1 cells were treated with either 100 MOI of Ad5 or Ad5F35 vectors and 100 nM CID (AP1903) or LPS (1 μg/ml), and nuclear extract kit (Active Motif) was used according to manufacturer's instructions. To prepare cell lysates, BMDCs and THP-1 cells were activated with 10,000 vp/cell of Ad5 or Ad5F35 vectors and 100 nM CID (AP1903), LPS (10 ng/ml), or recombinant CD40L (2 μg/ml of soluble CD40L for BMDCs, 1 μg/ml of enhanced human CD40L for THP-1 cells) followed by lysis with buffer comprising 2 mM PMSF, 20 μg/ml protease inhibitor cocktail and phosphatase inhibitors, including 2 mM sodium orthovanadate, sodium pyrophosphate, and β-glycerophosphate (Sigma-Aldrich). Proteins were detected using antibodies (Cell Signaling Technology Inc.) to Rel subunits and phosphorylated and total IKKα/β, p38, JNK, ERK1/2, and Akt molecules.

Statistics. Data are represented as mean ± SEM. Data were analyzed using unpaired Student's *t* test to calculate 2-tailed or 1-tailed *P* values to determine statistical significance in differences when comparing 2 treatment groups in all assays. One-way ANOVA followed by Bonferroni's multiple comparison test was used to compare multiple treatment groups. Two-way ANOVA followed by Bonferroni's test was used to assess statistical significance of differences in tumor growth between multiple treatment groups at different time points. Survival was recorded by Kaplan-Meier graphs, with significance determined by the log-rank test. Data were analyzed using GraphPad Prism version 5.0 software (GraphPad).

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