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Immune adjuvant efficacy of CpG oligonucleotide in cancer treatment is founded specifically upon TLR9 function in plasmacytoid dendritic cells

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Précis: This study unravels the mechanistic basis for the immune adjuvant effects of CpG oligonucleotide, with implications on how to use it most effectively in combinatorial immunochemotherapy regimens.

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Abstract

The differences in function, location and migratory pattern of conventional dendritic cells (cDCs) and plasmacytoid DCs (pDCs) not only point to specialized roles in immune responses but also signify additive and interdependent relationships required to clear pathogens. We studied the *in vivo* requirement of cross-talk between cDCs and pDCs for eliciting anti-tumor immunity against *in situ* released tumor antigens in the absence or presence of the Toll-like receptor (TLR) 9 agonist CpG. Previous data indicated that CpG boosted tumor-specific T cell responses after *in vivo* tumor destruction and increased survival after tumor re-challenges. The present study shows that cDCs are indispensable for cross-presentation of ablation-released tumor antigens and for the induction of long-term anti-tumor immunity. Depletion of pDCs, or applying this model in type I IFN receptor deficient mice abrogated CpG-mediated responses. CD8α^+ cDCs and the recently identified merocytic cDCs were dependent on pDCs for CpG-induced upregulation of CD80. Moreover, DC-transfer studies revealed that merocytic cDCs and CD8α^+ cDCs were most susceptible to pDC-help and subsequently promoted tumor-free survival in a therapeutic setting. By transferring wild type pDCs into TLR9-deficient mice, we finally showed that TLR9 expression in pDCs is sufficient to benefit from CpG as an adjuvant. These studies indicate that the efficacy of CpG in cancer immunotherapy is dependent on cross-talk between pDCs and specific subsets of cDCs.

Introduction

In the last decades technological advances catalyzed a shift in the treatment of solid tumors from open surgical resection towards less invasive (radiofrequency ablation, cryosurgery, laser ablation) or non-invasive techniques (high-intensity focused ultrasound (1)). These techniques induce direct cell death by protein denaturation and membrane disruption but also induce apoptotic cell death due to cell damage or vascular disruptions. *In situ* tumor destruction hence creates a depot of tumor antigens consisting of dead and dying cells that becomes instantly available for phagocytes, such as dendritic cells (DCs). DCs are well equipped to internalize dying cells and cellular debris and subsequently process tumor antigens for presentation to T cells. The main arm of the adaptive immune system to fight cancer is the activation of tumor-specific cytotoxic CD8 T lymphocytes (CTLs) that recognize and kill tumor cells. *In vivo* generation of CTLs is dependent on the unique mechanism of cross-presentation by DCs; presentation of exogenous antigens on MHC class I. Exploiting the CTL priming capacity of DCs is of major interest for cancer immunotherapy, in particular to enhance anti-tumor immunity after applying *in situ* tumor destruction techniques.

We previously developed a murine model in which a normally lethal melanoma tumor is destructed *in situ* by cryoablation (2). The released tumor antigens preferentially end up in $CD11c^+$ DCs in the draining lymph node (3, 4). Although cryoablated mice remain tumor-free, only ~20-50% of the mice survive a re-challenge with melanoma cells, indicating that a minority of the subjects developed efficient immunological memory against the tumor. These findings emphasized the need to boost anti-tumor immunity by combining tumor destructive treatment with adjuvant immunotherapy. Indeed, administration of the Toll-like receptor (TLR)9 agonist, CpG oligodeoxynucleotides, immediately after the ablation elevate the numbers of CTLs in the lymph node and promoted survival rate upon re-challenge up to 90-100% (4-6). We aimed to elucidate the significance of different DC subsets in the induction of CpG-mediated anti-tumor responses after *in situ* tumor ablation and the importance of TLR9 in the different DC subsets herein.

The unique ability to cross-present antigens has traditionally been attributed to the $CD8\alpha^+$ subset (7), but recent reports showed that $CD8\alpha^-CD11b^-$ cDCs also possess the capacity to present

exogenous antigens on MHC class I (8, 9). These cells were named merocytic DCs after their acquisition of small particles form dying cells through a 'nibbling' process instead of engulfment. In contrast to these cDCs, pDC show a poor functional ability to stimulate naïve T cells in mouse (10) and man (11). The cross-priming capacity of pDCs is still under debate but seems relatively meager when compared to cDCs and may be restricted to specific circumstances (12, 13). Yet, pDCs are able to effectively stimulate pre-activated or memory-type T cells and deliver differentiation (10, 11) and activation (14) signals (particularly type I IFN) for cDCs. The differences in function, location and migratory pattern of cDCs and pDCs may thus not only point to specialized roles in the elicitation of T cell responses but also may signify additive and interdependent relationships resulting in synergistic anti-tumor immunity.

In the present study, we elucidated the specific capacities and interactions of pDCs and different subsets of cDCs in (CpG-stimulated) immune responses against tumor antigens released by *in situ* tumor destruction that requires internalization, processing and presentation by the DCs *in vivo*. Furthermore, we studied whether the exclusive expression of TLR9 in either cDCs or pDCs would be sufficient for the induction of tumor-specific CTLs after combined treatment of ablation with CpG administration.

Materials and Methods

Mice, cell lines and peptides. C57Bl/6J, B6.C-H2.^{bm1}\ByJ (K^{bm1}) and B6.SJL.P*tpcr*^{a} (B6.CD45.1) were obtained from The Jackson Laboratory. TLR9$^{-/-}$ mice were obtained from S. Akira (Department of Host Defense, Osaka University, Osaka, Japan). Act-mOVA transgenic mice were a gift from Dr. M. Jenkins (University of Minnesota Medical School, Minneapolis, NM) and were bred onto a B6.C-*H2*^{bm1}ByJ background (ActmOVA K^{bm1}). OT-I mice were bred on a CD45.1 background and crossed with K^{bm1} mice. IFNα/βR (Ifnar$^{-/-}$) knockout mice were kindly provided by Dr J. Sprent (The Scripps Research Institute Vivarium, La Jolla, CA). Mice were maintained at the animal facility of the La Jolla Institute for Allergy and Immunology (CA, USA) or at the animal laboratory of the NCMLS (Nijmegen, the Netherlands) under specific pathogen-free conditions and were used at 6-12 weeks of age. All experiments were in accordance with the guidelines by the Association for Assessment and Accreditation of Laboratory Animal Care International.

OVA-transfected murine melanoma cell line B16F10 (B16OVA, clone MO5) was kindly provided by Dr. K. Rock (Dept. of Pathology, University of Massachusetts Medical Center, Worchester, MA (15)). Cells were frozen and thawed according to ATCC guidelines for B16F10 and cultured as described before (4). No full authentication was performed, but the expression of OVA, TRP-2 antigens, TLRs and MHC-I and molecules was tested before the experiments by RT-PCR or flow cytometry. In addition the growth characteristics *in vitro* and *in vivo* are closely monitored and compared with previous growth curves. Peptides for the MHC class I-restricted $OVA_{257-264}$ (SIINFEKL) were obtained from A&A laboratories (San Diego, CA). B16-FLT3L cells were cultured in 5% IMDM supplemented with 20 U/mL penicillin, 10 g/mL streptomycin and $5x10^6$ cells were injected to establish FLT3-L-producing tumors in C57BL/6 mice.

***In situ* release of tumor-antigens by cryoablation.** Mice were subcutaneously (s.c.) injected with $5*10^5$ B16OVA cells in PBS and Matrigel. Nine to 11 days after injection, tumors (7-10 mm) were treated in 2 cycles with cryosurgery using a CRY-AC® device armed with a 6 mm probe (Brymill Cryogenic Systems, Ellington, CT, USA). To test long-term tumor protection, the mice were

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challenged with 25-50*10^3 B16OVA cells 40 days after the ablation of the primary tumor and monitored for tumor growth. CpG-ODN 1668 (5'-TCCATGACGTTCCTGATGCT-3') with phosphorothioated backbone was obtained from IDT (San Diego, CA, USA) and injected peri-tumorally (50 g/mouse in 2x15 L) within 15 minutes after ablation. CpG-ODN 1668 is a type B CpG, similar to the clinical-grade available CpG-ODNs used in clinical trials.

Functional ablation of cross-presenting DCs and pDCs. We used a method that was recently described to functionally ablate cross-presentation *in vivo* (16). Tumor-bearing mice were injected with horse *cyto c* (5 mg/mouse; Sigma) on 2 consecutive days before ablation. At this time point the number of CD8α DCs was reduced 2-3 fold. Other DC subsets ($CD11c^+CD11b^+CD172^+$ and $CD11c^{int}B220^+$) were not affected. Plasmacytoid DCs were depleted using the 120g8 antibody administered one and two days prior to ablation (0.5 mg/mouse). Rat IgG was used as a control. We did not observe differences in the number of other lymph node cells. To analyze DCs from the tumor-draining inguinal LN, excised LNs were torn apart using needles in medium containing collagenase D and DNase and incubated at room temperature for 15 minutes. Suspensions were dislodged by re-suspending and EDTA was added before filtration.

Tetramer and intracellular cytokine staining.

Seven to 10 days after ablation, blood, spleen and LN cells were stained with allophycocyanin (APC)- or phycoerythrin (PE)-labeled iTag^{TM} OVA-K^b tetramers (Beckman Coulter), CD8α (clone 53-6.7) and CD44 (clone IM7; eBioscience or BD Pharmingen). Cytokine profiles were assessed after re-stimulation with 0.1 g/ml SIINFEKL peptide. Subsequently, cells were stained with mAbs specific for CD8 TCRβ, CD44 and CD62L, fixed and permeabilized according to Cytofix/Cytoperm kit instructions (BD Pharmingen), and intracellular cytokine staining was performed for IFN-γ (clone XMG1.2) and/or TNFα (clone MP6-XT22; all Abs were purchased from either BD Pharmingen or eBioscience). Samples were collected on a LSRII flow cytometer with Diva software (BD Pharmingen) and data were analyzed with FlowJo software (Tree Star).

Adoptive transfer of OT-I cells and *in vivo* cytotoxicity

OT-I SJL splenocytes were enriched for $CD8^+$ T cells by negative selection according to the manufacturer's instructions (CD8 T cell isolation kit, Miltenyi Biotec). Purity of $V\alpha2^+$ $V\beta5^+$ was typically >98%. Cells were labeled with 1 M CFSE and $5*10^5$ cells were intravenously transferred to mice that were cryoablated. As a control, the same number of CFSE-labeled splenocytes from wild type C57Bl/6J mice were transferred to the same mice. Four days later, spleens were harvested and CFSE dilution was analyzed in $CD8^+CD45.1^+$ (OT-I) and $CD8^+CD45.2^+$ (controls) cells using a LSRII flow cytometer. To address the killing capacity of endogenously generated CTLs splenocytes from $CD45.1^+$ mice were loaded (1 hour incubation at 37°C) with 1 g/ml SIINFEKL peptide or an irrelevant peptide ($E1B_{192–200}$ peptide; VNIRNCCYI) and labeled with CFSE in a concentration of 0.1 M (SIINFEKL) or 2 M ($E1B_{192–200}$). Five $x10^6$ cells of each population was injected into mice of which the B16OVA tumor had been treated with cryoablation +/- CpG 8 days before. Eighteen hours later, spleens were harvested and the differently labeled $CD45.1^+$ populations were analyzed using flow cytometry. The percentage killing was calculated using the formula: 100-((sample-$CFSE^{OVA}$/sample-$CFSE^{control}$)/(naïve-$CFSE^{OVA}$/naïve-$CFSE^{control}$)*100).

Splenic DC isolation and transfer of antigen-loaded DCs

Spleens were isolated from C57Bl/6 mice 10-12 days after injection with $5x10^6$ B16-FLT3-L cells. Spleens were injected with Collagenase/DNase medium and left for 5-10 minutes before they were cut in small pieces. After occasional resuspending during 20 minutes at room temperature the preparation was filtered and washed. The single cell suspension was loaded onto a two-fraction OptiPrep gradient (Sigma) and spun for 20 minutes at room temperature. The low-density cells were washed and generally contained >90% $CD11c^{int/+}$ that contained 10-15% pDCs ($CD11c^{int}BST\text{-}2^+$). Total DC fractions were divided in two suspensions of which the first (cDCs with pDCs) was co-cultured overnight with irradiated (1500 rad) ActmOVA K^{bm1} cells (ratio DC:dying cells = 1:3). The second fraction was depleted of pDCs by magnetic bead sorting and incubated in a similar way (cDCs without pDCs). Cells were harvested and facs-sorted based on the expression of CD11c, B220, BST-

2, CD24, CD8α, CD172 and CD11b. $1x10^5$ DCs were injected into each mouse, which is approximately $4.5x10^6$ per kg. (This is a relatively low amount of cells compared to what is generally used in mouse studies, especially in a therapeutic setting. However, human DC vaccinations are generally performed with multiple injections (2-3) of in average $15x10^6$ cells, which is $0.20\text{-}0.25x10^5$ per kg.)

Statistical analyses. Data were analyzed using Prism software (GraphPad Software, Inc.). Comparisons of one-variable data were performed using a two-tailed unpaired Student's *t* test. Statistical analyses of the survival curves were performed using a log-rank test. Bar graphs represent mean levels $\pm$ standard error of the mean (s.e.m.). $P < 0.05$ was considered statistically significant.

Results

Conventional DCs cross-present tumor antigens that are released by in situ *tumor ablation*

C57Bl/6 mice were inoculated s.c. with $5*10^5$ OVA-transfected B16-melanoma cells. When tumors measured 7-10 mm in diameter (generally 9-11 days after injection) the tumor was destructed *in situ* by cryoablation (4-6). Mice were additionally treated with 2 injections of *cytochrome* (cyto) *c*, an initiator of the mitochondrial apoptosis pathway in cells capable of endosome-to-cytosol transport, such as cDCs (16). In agreement with *Lin et al.* (16), *cyto c* selectively reduced the numbers of splenic $CD11c^+CD11b^-$ cDCs (generally 2-3-fold), whereas other cDC populations ($CD11c^+CD11b^+$) and pDCs ($CD11c^{int}B220^+SiglecH^+$) were unaffected. After cryoablation alone OVA-specific $CD8^+$ T cells were present in blood (Figure 1A), spleen and draining lymph nodes. After *in vitro* re-stimulation with MHC class I-restricted SIINFEKL peptide, $CD8^+$ T cells were expressed the activation marker $CD44^+$ and produced IFN-γ (Figure 1A). Additional treatment with CpG significantly increased the numbers of CTLs *in vivo*. Selective suicide of cross-presenting cDCs by *cyto c* prevented the increment of CTLs in both the absence and the presence of CpG. This indicates that the process of DC cross-presentation is crucial for the induction of immune responses against *in situ* released tumor antigens.

Next, we tested whether pDCs are involved in the generation of tumor-specific CTLs. Both murine cDCs and pDCs express TLR9 mRNA and are responsive to CpG, but only cDCs are able to cross-present cell-associated antigens to $CD8^+$ T cells *in vitro* (data not). Plasmacytoid DCs were depleted in B16OVA-bearing mice using 120g8 monoclonal antibodies before cryoablation +/- CpG treatment. Compared to control Ig-treated animals, CpG-treated but pDC-depleted mice showed reduced numbers of OVA-specific $CD8^+CD44^+$ T cells in blood and IFN-γ^+CD8^+ ($CD44^+$) T cells in both spleen and lymph nodes (Figure 1B). In the absence of pDCs, OVA-specific T cells were still generated after cryosurgery alone (no CpG), suggesting that CTL priming in this model is a unique feature of cDCs but not pDCs. The injection of CpG also drastically increased the cytolytic capacity to kill SIINFEKL-pulsed CFSE-labeled $CD45.1^+$ target cells, which was shown to be dependent on the presence pDCs during priming (Figure 1C)). It was additionally tested whether the presence of

pDCs during the priming phase of the immune response was required to induce CpG-mediated long-term immunity. Mice that remained tumor-free for 40 days after cryosurgery received a re-challenge with a lethal dose of B16OVA tumor cells. As reported previously, only ~50% of the cryoablation-only mice survive the re-challenge, whereas all animals treated with cryoablation and CpG survive (4-6). In contrast, pDC-depleted mice did not show the protective effect of CpG (Figure 1D). These results indicate that CpG-mediated protective immune responses to *in situ* released tumor antigens are highly regulated by pDCs.

CTL priming after cryoablation is dependent on type I interferon receptor signaling

One of the hallmarks of pDCs is the production of massive amounts of type I IFN in response to CpG *in vitro* and *in vivo*. It was tested whether cryoablation with or without CpG treatment could induce anti-tumor responses in mice deficient for the IFN type I receptor (IFNα/βR$^{-/-}$). As expected, the efficacy of CpG to increase OVA-specific (Figure 2A) and IFN-γ-producing (Figure 2B) CD8^{+} T cells after cryoablation was drastically diminished in IFNα/βR$^{-/-}$ mice. Co-administration of CpG increased the *in vivo* killing of SIINFEKL-loaded target cells in wild type mice (Figure 2C). Also IFNα/βR$^{-/-}$ mice started to show increased killing of target cells indicating that CpG might stimulate the immune response to some extent even in the absence of type I IFN signaling.

To dissect whether the disabilities observed in IFNα/βR$^{-/-}$ mice were intrinsic for antigen-presenting cells or for the CD8 T cells themselves, IFNα/βR$^{-/-}$ mice were injected with CFSE-labeled wild type OT-I cells just after ablation and analyzed for proliferation by CFSE dilution. In all circumstances where antigen was released after ablation, OT-I cells were able to divide (Figure 2D). However, the absolute numbers of dividing OT-I cells showed that less cells accumulated in the IFNα/βR$^{-/-}$ mice than in wild type mice, indicating that the type I IFN feedback loop in antigen-presenting cells is important in the onset of the response. Interestingly, IFNα/βR$^{-/-}$ mice also showed lower numbers of CTL priming after cryoablation alone, suggesting that type I IFN signaling is involved in the stimulation of immune responses against *in situ* released antigens from dead cells. Since pDC-depleted mice were able to mount effective CTL responses after ablation only, these data

suggest that other cells may be the source of type I IFN. Indeed, we previously showed that merocytic cDCs produce type I IFN upon the encounter with dying cells (9, 17).

Plasmacytoid DCs determine cDC maturation and antigen loading in CpG-modulated responses

To get more insight in cDC subsets and their susceptibility to pDC-mediated factors, we addressed the antigen uptake capacity and maturation status of the cDCs directly *ex vivo*. Tumor-draining lymph nodes were isolated from 120g8- or isotype-treated mice 1-2 days after cryoablation +/- CpG treatment. Just prior to the ablation OVA-Alexa647 was injected in the tumor to determine the ability of DCs to internalize soluble antigens after ablation +/- CpG. CpG increased the internalization of OVA-Alexa647 by $CD11c^{+}B220^{-}$ cDCs after ablation, while 120g8 did not seem to have an effect on antigen uptake after ablation alone (Figure 3A). Generally, lower uptake of OVA-A647 was observed in DCs from 120g8-treated mice injected with CpG. In all circumstances, ~80% of the $CD11c^{+}$ cells that had internalized OVA-Alexa647 expressed $CD11b^{+}$. These CD11b-expressing DCs were present in the draining LN after CpG-treatment, irrespective of 120g8 treatment (Figure 3B). Also the numbers of antigen-loaded $CD8\alpha^{+}$ DCs and merocytic DCs increased after CpG treatment although to a lesser extent. In addition, the expression of co-stimulatory markers on cDCs was very much increased following CpG treatment, in particular in $CD8\alpha^{+}$ DCs and merocytic DCs (Figure 3C). In pDC-depleted mice, this CpG-mediated up-regulation of CD80 expression was much less pronounced, suggesting that differences in CTL priming may be due to impairment of activation of cross-presenting cDCs.

pDCs stimulate the intrinsic cross-presenting ability of cDCs

In Figure 3, we showed that the *in vivo* maturation of especially $CD8\alpha^{+}$ cDCs and $CD8\alpha^{-}CD11b^{-}$ merocytic DCs after combinational therapy of ablation and CpG is dependent on pDCs. We next studied whether the cross-priming function of resting cDC subsets was differentially regulated by pDC-help. A total number of $1x10^{5}$ sorted DC subsets, loaded with dying ActmOVA K^{bm1} cells (to prevent direct presentation), were transferred into donor mice bearing an established B16OVA tumor

(~4x4 mm). Therapeutic vaccinations with CD8α$^+$ cDC were not successful. However, when CD8α$^+$ cDCs were allowed to interact with pDCs during incubation with irradiated cells almost 40% of the mice were able to clear the established B16OVA tumor after cDC transfers (Figure 4). In line with our previous observations (17), vaccinations with CD8α$^-$CD11b$^-$ alone already extended the period of tumor-free survival. These CD8α$^-$CD11b$^-$ cDCs were also highly responsive to 'pDC-help' as ~50% of the mice survived the outgrowth of a lethal melanoma tumor. DC-vaccination with CD11b$^+$ cDCs cultured in either the absence or presence of pDCs failed to increase survival. This study suggests that CD8α$^+$ cDCs and CD8α$^-$CD11b$^-$ cDCs develop a better intrinsic capacity for immune stimulation when they are permitted to interact with pDCs.

Exclusive TLR9 expression in pDCs suffices to benefit from CpG

The expression of TLR9 may be differently regulated in mice and man with the main discrepancy being the low expression or absence in human cDCs. To study the significance of TLR9 expression in cDCs versus pDCs, B16OVA melanomas in TLR9-deficient mice were cryoablated in the absence or presence of CpG. Cryoablation in TLR9-/- mice resulted in the generation of endogenous OVA-Kb$^+$ CD8 T cells in blood (Figure 5A) and IFN-γ/TNF-α producing CD8 T cells (Figure 5B) in LN. Additional treatment with CpG failed to modulate these responses. Interestingly, the efficacy of CpG was restored when TLR9-deficient mice received a transfer with wild type pDCs. Transfer of pDCs, but not cDCs, after combined treatment of cryoablation and CpG increased the percentages of IFN-γ/TNF-α producing CD8 T cells in the lymph node and OVA-specific T cells in blood when compared to cryoablation alone. To exclude possible effects of CpG directly on the T cells, we also transferred CFSE-labeled OT-I T cells (similar as figure 2D) and confirmed that exclusive expression of TLR9 in pDCs stimulates antigen-specific T cell proliferation (Figure 5C). In addition, pDC-transfer, but not cDC-transfer enhanced the *in vivo* capacity of endogenous T cells to kill SIINFEKL-labeled target cells (Figure 5D). In all, these data suggest that the restricted expression of TLR9 in pDCs, but not in cDCs, suffices for CpG to further stimulate anti-tumor immune responses.

Discussion

Accumulating evidence shows that adaptive immunity is shaped by co-operation between functionally distinct DC subsets. Cross-talk may include the transfer of antigenic material between migratory and LN-resident DCs (18), or cDCs and pDCs (19) that may also synergize to reach optimal activation (20) and physically interact to stimulate CTL-mediated eradication of viruses (14). This is the first study to show the *in vivo* importance of cDC-pDC cross-talk in generating productive CTL responses to *in situ*-released tumor antigens. In cancer therapies where a tumor is instantly destructed *in situ* (*e.g.* radiofrequency ablation, cryosurgery, irradiation) tumor antigens become readily available for phagocytic antigen-presenting cells and thus for presentation to T cells. We have previously shown that CpG synergizes with tumor ablation to induce long-term protection against tumor re-challenges through activation of tumor-specific CTLs. The same conclusions were drawn when using B16F10 parental cells instead of OVA-transfected cells (4-6), indicating that responses to classical tumor antigens follow similar principles. Here we conclude that the clinical efficacy of CpG in mice is critically dependent on the presence of both cDCs and pDCs and the cross-talk between TLR9-expressing pDCs and specific subsets of cDCs.

The present data suggest that cDCs are required for the induction of tumor-specific immunity after ablation. *Cyto c* did not affect MHC class II presentation (16) but selectively decreased the number of cDCs without affecting the numbers of pDCs. This finding supports the notion that the ability of pDCs to phagocytose dead cells and cross-present exogenous antigens *in vivo* appears less efficient when compared to that of cDCs (discussed in (21)). Moreover, tumor-specific CTLs were still present in pDC-depleted animals after ablation emphasizing the leading role of cDCs and not pDCs for antigen presentation and cross-priming in this model. CpG-licensed pDCs were reported to cross-prime OT-I cells after isolation from OVA-protein challenged mice (9 mg/mouse) (22). Compared to these levels, the amount of antigens released after ablation may be too low to be able to detect cross-presentation by pDCs. Functional pDCs were however required to optimally benefit from CpG-mediated immunotherapy evidenced by significantly lower numbers of tumor-specific CTLs and lower survival rates upon secondary challenge in pDC-depleted mice. In the ablation setting *in vivo*,

the generation of both endogenous and transferred IFNα/βR$^{+/+}$ OT-I CD8 T cells was dependent on type I IFN signaling, indicating that type I IFN signaling was important at the level of antigen presentation and not an intrinsic defect of the IFNα/βR$^{-/-}$ CTLs.

The promoter elements of CD80 encoding genes includes several IFN regulatory factor 2 sites, which suggests that the up-regulation of CD80 may be a direct result of type I IFN. CD8α^{+} DCs and merocytic DCs failed to up-regulate the co-stimulatory molecule CD80 in response to CpG *in vivo* when pDCs were depleted before treatment, suggesting that the lack of co-stimulatory signals may have prevented efficient CTL priming. Although our data strongly implicate a role for type I IFN production by pDCs, administration of recombinant IFN-α after cryoablation only slightly increased the influx of DCs in the draining LN and the expression of CD80 on cDCs (not shown). It is well possible that pDCs influence cDCs through other mechanisms as was previously reported in responses to *Listeria monocytogenes.* Kuwajima *et al.* showed that CpG-induced protection from *Listeria* was dependent on CD40-CD40L interactions between cDCs and pDCs and subsequent IL-12 production by cDCs (23). Although adaptive immune responses against antigens from a destructed tumor develop following different mechanisms than those at work in an acute infection model, these data suggest that the cross-talk between pDCs and cDCs after cryoablation+CpG may also be co-stimulation dependent. It was previously shown *in vitro* that cross-talk between pDCs and cDCs requires physical interactions (24). The use of peptides in an *in vitro* co-culture system as used in that study (24) does however overcome the antigen uptake and processing machinery of DCs and may therefore not be directly translated to our pre-clinical model where antigens are derived form dead and dying cells *in situ*.

Type I IFN signaling was shown to be essential to generate anti-tumor responses after ablation both in the presence and absence of CpG. In the latter situation the data suggest that non-pDCs are the source of type I IFN since pDC-depleted mice showed a trend towards increased anti-tumor responses. Indeed, we previously showed that merocytic DCs are a source of type I IFN upon an encounter with dying cells (9, 17). The activation pathways through which immunity is established against *in situ* released tumor antigens in such sterile inflammatory conditions are subject

of intensive research. Previous studies indicated that CTL priming against antigens derived from dying cells injected into the host was independent of MyD88 but dependent on the type I IFN receptor (9). Similar results were found in the cryoablation model. In addition, type I IFN was found essential in local radio-therapy mediated tumor control (25). The way in which these tumor cells die *in vivo* after ablation can thus be considered slightly immunogenic but the independency of MyD88/TRIF suggests that the type I IFN pathway might be an alternative immune stimulatory pathway in addition to those previously proposed (26).

In situ tumor destruction techniques are increasingly applied for the treatment of solid tumors. The *ad hoc* availability of tumor antigens for immune cells makes these treatments particular good candidates for combinations with immunotherapy. The use of CpG in clinical trials so far resulted in variable success (27-30). A common argument on the efficacy of CpG in immunotherapy is the differential expression of TLR9 between mouse and man DCs. Others challenged this argument by showing TLR9 expression in both human pDCs and cDCs (31), and responsiveness to CpG by monocyte-derived DCs (32, 33) and FLT3-L-induced blood DCs (34). An alternative explanation could be suboptimal timing (4) and routing (6) conditions of CpG administration in patients. We here conclude that the exclusive expression of TLR9 in murine pDCs suffices to benefit from the clinical efficacy of CpG in this murine model. The transfer of wild type pDCs, but not cDCs, into TLR9-deficient animals restored the ability to cross-prime antigen-specific and functional CTLs. These data show that although murine cDCs are able to respond to CpG, the exclusive reactivity of pDCs on CpG suffices to induce protective immunity. It will be interesting to determine CpG efficacy in clinical trials that take into consideration the optimal timing (4) and routing (6) prerequisites of CpG administration.

References

1. Kennedy JE. Innovation: High-intensity focused ultrasound in the treatment of solid tumours. Nat Rev Cancer. 2005;5:321-7.
2. den Brok MH, Sutmuller RP, van der Voort R, Bennink EJ, Figdor CG, Ruers TJ, et al. In situ tumor ablation creates an antigen source for the generation of antitumor immunity. Cancer Res. 2004;64:4024-9.
3. den Brok MH, Sutmuller RP, Nierkens S, Bennink EJ, Frielink C, Toonen LW, et al. Efficient loading of dendritic cells following cryo and radiofrequency ablation in combination with immune modulation induces anti-tumour immunity. Br J Cancer. 2006;95:896-905.
4. Nierkens S, den Brok MH, Sutmuller RP, Grauer OM, Bennink E, Morgan ME, et al. In vivo colocalization of antigen and CpG within dendritic cells is associated with the efficacy of cancer immunotherapy. Cancer Res. 2008;68:5390-6.
5. den Brok MH, Sutmuller RP, Nierkens S, Bennink EJ, Toonen LW, Figdor CG, et al. Synergy between in situ cryoablation and TLR9 stimulation results in a highly effective in vivo dendritic cell vaccine. Cancer Res. 2006;66:7285-92.
6. Nierkens S, den Brok MH, Roelofsen T, Wagenaars JA, Figdor CG, Ruers TJ, et al. Route of administration of the TLR9 agonist CpG critically determines the efficacy of cancer immunotherapy in mice. PLoS One. 2009;4:e8368.
7. den Haan JM, Lehar SM, Bevan MJ. CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo. J Exp Med. 2000;192:1685-96.
8. Bedoui S, Prato S, Mintern J, Gebhardt T, Zhan Y, Lew AM, et al. Characterization of an immediate splenic precursor of CD8+ dendritic cells capable of inducing antiviral T cell responses. J Immunol. 2009;182:4200-7.
9. Janssen E, Tabeta K, Barnes MJ, Rutschmann S, McBride S, Bahjat KS, et al. Efficient T cell activation via a Toll-Interleukin 1 Receptor-independent pathway. Immunity. 2006;24:787-99.
10. Krug A, Veeraswamy R, Pekosz A, Kanagawa O, Unanue ER, Colonna M, et al. Interferon-producing cells fail to induce proliferation of naive T cells but can promote expansion and T

helper 1 differentiation of antigen-experienced unpolarized T cells. J Exp Med. 2003;197:899-906.

11. Naranjo-Gomez M, Fernandez MA, Bofill M, Singh R, Navarrete CV, Pujol-Borrell R, et al. Primary alloproliferative TH1 response induced by immature plasmacytoid dendritic cells in collaboration with myeloid DCs. Am J Transplant. 2005;5:2838-48.
12. Kasturi SP, Pulendran B. Cross-presentation: avoiding trafficking chaos? Nat Immunol. 2008;9:461-3.
13. Colonna M, Cella M. Crosspresentation: plasmacytoid dendritic cells are in the business. Immunity. 2007;27:419-21.
14. Yoneyama H, Matsuno K, Toda E, Nishiwaki T, Matsuo N, Nakano A, et al. Plasmacytoid DCs help lymph node DCs to induce anti-HSV CTLs. J Exp Med. 2005;202:425-35.
15. Falo LD, Jr., Kovacsovics-Bankowski M, Thompson K, Rock KL. Targeting antigen into the phagocytic pathway in vivo induces protective tumour immunity. Nat Med. 1995;1:649-53.
16. Lin ML, Zhan Y, Proietto AI, Prato S, Wu L, Heath WR, et al. Selective suicide of cross-presenting CD8+ dendritic cells by cytochrome c injection shows functional heterogeneity within this subset. Proc Natl Acad Sci U S A. 2008;105:3029-34.
17. Reboulet RA, Hennies CM, Garcia Z, Nierkens S, Janssen EM. Prolonged antigen storage endows merocytic dendritic cells with enhanced capacity to prime anti-tumor responses in tumor-bearing mice. J Immunol. 2010;185:3337-47.
18. Allan RS, Waithman J, Bedoui S, Jones CM, Villadangos JA, Zhan Y, et al. Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. Immunity. 2006;25:153-62.
19. Megjugorac NJ, Jacobs ES, Izaguirre AG, George TC, Gupta G, Fitzgerald-Bocarsly P. Image-based study of interferongenic interactions between plasmacytoid dendritic cells and HSV-infected monocyte-derived dendritic cells. Immunol Invest. 2007;36:739-61.

20. Piccioli D, Sammicheli C, Tavarini S, Nuti S, Frigimelica E, Manetti AG, et al. Human plasmacytoid dendritic cells are unresponsive to bacterial stimulation and require a novel type of cooperation with myeloid dendritic cells for maturation. Blood. 2009;113:4232-9.
21. Villadangos JA, Young L. Antigen-presentation properties of plasmacytoid dendritic cells. Immunity. 2008;29:352-61.
22. Mouries J, Moron G, Schlecht G, Escriou N, Dadaglio G, Leclerc C. Plasmacytoid dendritic cells efficiently cross-prime naive T cells in vivo after TLR activation. Blood. 2008;112:3713-22.
23. Kuwajima S, Sato T, Ishida K, Tada H, Tezuka H, Ohteki T. Interleukin 15-dependent crosstalk between conventional and plasmacytoid dendritic cells is essential for CpG-induced immune activation. Nat Immunol. 2006;7:740-6.
24. Lou Y, Liu C, Kim GJ, Liu YJ, Hwu P, Wang G. Plasmacytoid dendritic cells synergize with myeloid dendritic cells in the induction of antigen-specific antitumor immune responses. J Immunol. 2007;178:1534-41.
25. Burnette BC, Liang H, Lee Y, Chlewicki L, Khodarev NN, Weichselbaum RR, et al. The efficacy of radiotherapy relies upon induction of type i interferon-dependent innate and adaptive immunity. Cancer Res. 2011;71:2488-96.
26. Apetoh L, Ghiringhelli F, Tesniere A, Obeid M, Ortiz C, Criollo A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med. 2007;13:1050-9.
27. Manegold C, Gravenor D, Woytowitz D, Mezger J, Hirsh V, Albert G, et al. Randomized phase II trial of a toll-like receptor 9 agonist oligodeoxynucleotide, PF-3512676, in combination with first-line taxane plus platinum chemotherapy for advanced-stage non-small-cell lung cancer. J Clin Oncol. 2008;26:3979-86.
28. Weber J, Thompson JA, Hamid O, Minor D, Amin A, Ron I, et al. A randomized, double-blind, placebo-controlled, phase II study comparing the tolerability and efficacy of ipilimumab administered with or without prophylactic budesonide in patients with unresectable stage III or IV melanoma. Clin Cancer Res. 2009;15:5591-8.

29. Leonard JP, Link BK, Emmanouilides C, Gregory SA, Weisdorf D, Andrey J, et al. Phase I trial of toll-like receptor 9 agonist PF-3512676 with and following rituximab in patients with recurrent indolent and aggressive non Hodgkin's lymphoma. Clin Cancer Res. 2007;13:6168-74.
30. Link BK, Ballas ZK, Weisdorf D, Wooldridge JE, Bossler AD, Shannon M, et al. Oligodeoxynucleotide CpG 7909 delivered as intravenous infusion demonstrates immunologic modulation in patients with previously treated non-Hodgkin lymphoma. J Immunother. 2006;29:558-68.
31. Hoene V, Peiser M, Wanner R. Human monocyte-derived dendritic cells express TLR9 and react directly to the CpG-A oligonucleotide D19. Journal of leukocyte biology. 2006;80:1328-36.
32. Hellman P, Eriksson H. Early activation markers of human peripheral dendritic cells. Hum Immunol. 2007;68:324-33.
33. Mao TK, Lian ZX, Selmi C, Ichiki Y, Ashwood P, Ansari AA, et al. Altered monocyte responses to defined TLR ligands in patients with primary biliary cirrhosis. Hepatology. 2005;42:802-8.
34. Chen W, Chan AS, Dawson AJ, Liang X, Blazar BR, Miller JS. FLT3 ligand administration after hematopoietic cell transplantation increases circulating dendritic cell precursors that can be activated by CpG oligodeoxynucleotides to enhance T-cell and natural killer cell function. Biol Blood Marrow Transplant. 2005;11:23-34.

Figure legends

Figure 1. Both cDCs and pDCs are required to benefit from immune potentiating effects of CpG *in vivo*. C57Bl/6 mice were injected with $5x10^4$ B16OVA cells. When the tumors measured 8-11 mm tumor antigens were released by cryoablation of the tumor *in situ*. Some groups received *cytochrome c* (cyto c; 5 mg/mouse i.p.) one day before and on the day of the ablation and/or CpG (50 g/mouse peri-tumorally) after the ablation. Eight to 10 days after ablation, blood cells were stained for OVA-Kb tetramers. Cells from spleen and draining LN were re-stimulated with SIINFEKL peptide and TCR-β^+CD8α^+ cells were analyzed for the expression of CD44$^+$ and IFN-γ^+ (A). Similar experiments were performed to study the involvement of pDCs. pDCs were depleted using 120g8 monoclonal antibodies injected one and two days before the ablation (B). The killing capacity of the endogenously induced CTLs was assessed by an *in vivo* cytotoxicity assay; splenocytes from CD45.1$^+$ mice were differentially CFSE-labeled and coated with SIINFEKL peptide (CFSE^{low}) or E1B$_{192–200}$ (CFSE^{high}) and injected into mice that had been treated with cryoablation 8 days before. Cytolytic capacity was analyzed 18 hours later by determining the presence of CFSE-labeled cells in the spleen (C). Mean levels +/- standard error of the mean (s.e.m) are shown. Each group consisted of 4-6 mice and these data are representative of 2 similar experiments. Naïve mice and mice that were tumor-free for 40 days after ablation were re-challenged with $50x10^3$ B16OVA cells to determine long-term tumor protection. Tumors measuring 1500 mm^3 were killed according to the animal welfare guidelines. Groups consisted of 8-12 mice (D). The survival curves of naïve, Cryo+CpG and Cryo+CpG+120g8 differed significantly ($p<0.05$) from that of cryo only. 120g8 significantly reduced the survival ratio in the cryo+CpG group.

Figure 2. Priming of endogenous CD8$^+$ T cells and transferred OT-I T cells is abrogated in IFNα/β-R$^{-/-}$ mice. B16OVA-bearing wild type and IFNα/β-R$^{-/-}$ mice were treated with cryoablation. After the ablation, indicated groups were additionally treated with CpG. Eight to 10 days after ablation, blood, spleen and draining LNs were isolated and stained with OVA-Kb tetramers (A).

Spleen and draining LN were re-stimulated with SIINFEKL peptide for 5 hours and analyzed for IFN-γ production (B). Six-8 days after ablation mice received $5x10^6$ SIINFEKL-coated CFSE-labeled (low dose) splenocytes from C57Bl/6 SJL (CD45.1) mice and a similar a number of CFSE-labeled (high dose) splenocytes coated with irrelevant peptide $E1B_{192–200}$. After 18 hours, spleens were harvested and analyzed for the presence of CD45.1 CFSE-labeled cells (C). Tumor-bearing mice were subjected to ablation +/- CpG and injected with CFSE-labeled purified $CD8\alpha^+$ OT-I SJL cells or wild type cells as control population. Three or 4 days after transfer, CFSE dilution was analyzed in splenocytes. Histograms show representative data of cells gated on CD8, Vα2, and Vβ5. Bar graphs show mean values +/- s.e.m of 4-6 mice and are representative of 2 similar experiments (D).

Figure 3. Plasmacytoid DCs determine the function of cDCs. OVA-Alexa-647 (20 g/20 L) was injected into the tumor just prior to ablation +/- CpG. Two days after ablation, LN cells were analyzed for the uptake of Alexa-647 in $CD11c^+$ cells (A). $CD11c^+$ cells that were present in the draining lymph node were sub-fractionated into $CD8\alpha^+$ DCs ($CD11c^+B220^-CD24^+CD172^{lo}CD11b^-$), $CD11b^+$ DCs ($CD11c^+B220^-CD24^{lo}CD8\alpha^-$), and merocytic DCs ($CD11c^+B220^-CD172^{lo}CD8\alpha^-CD11b^{lo}$). Bar graphs show mean values +/- s.e.m of 4-6 mice (B). The relative increase in CD80 expression was determined in each subset (C).

Figure 4. pDCs stimulate the intrinsic cross-priming ability of cDCs against cell-associated antigens. Spleens of B16-FLT3-L-bearing C57Bl/6 mice were harvested. Dendritic cells were enriched using a density gradient and divided in two volumes. One volume was depleted of pDCs using magnetic beads and both volumes were co-cultured with irradiated ActmOVA K^{bml} splenocytes (ratio 1 DC : 3 dying cells). After 18 hours of co-culture and maturation with CpG (0.1 g/ml), the cells were sorted and $1x10^5$ cells were injected into tumor-bearing recipients (tumor size: 4 mm in diameter). Graphs show survival curves of groups consisting of 6-8 mice.

Figure 5. Selective expression of TLR9 in pDCs suffices to benefit from CpG as an adjuvant. B16OVA tumors (7-9 mm in diameter) in TLR9-deficient mice were subjected to cryoablation +/- CpG. Indicated groups s.c. received wild type, FLT3-ligand-grown bone marrow-derived pDCs just after ablation. The percentages of Kb-OVA expressing CD8 T cells in blood and IFN-γ/TNF-α producing CD8 T cells in the draining lymph node are shown in A and B, respectively. Alternatively, $CD8^+$ OT-I cells were transferred i.v. after ablation and the accumulation was analyzed in the draining lymph node of the TLR9-deficient mice 3-4 days later (C). At day 10 after ablation mice received control and SIINFEKL-pulsed and CFSE-labeled target cells and the killing capacity was determined 18 hours after injection (D). Bar graphs show mean values +/- s.e.m of 4-6 mice. In panel A, B and C * indicates significant differences from all other groups.

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Figure 1

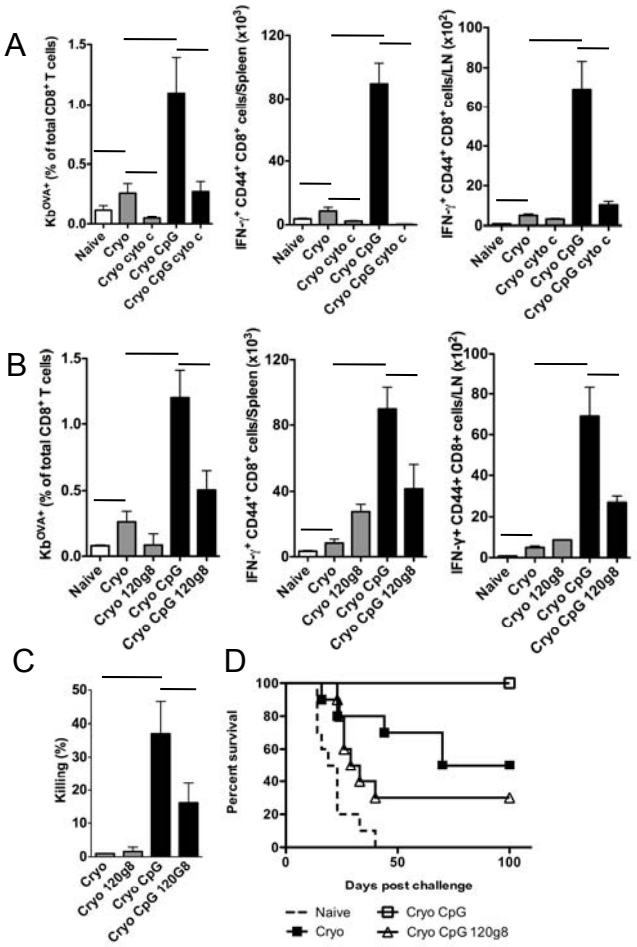

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Figure 2

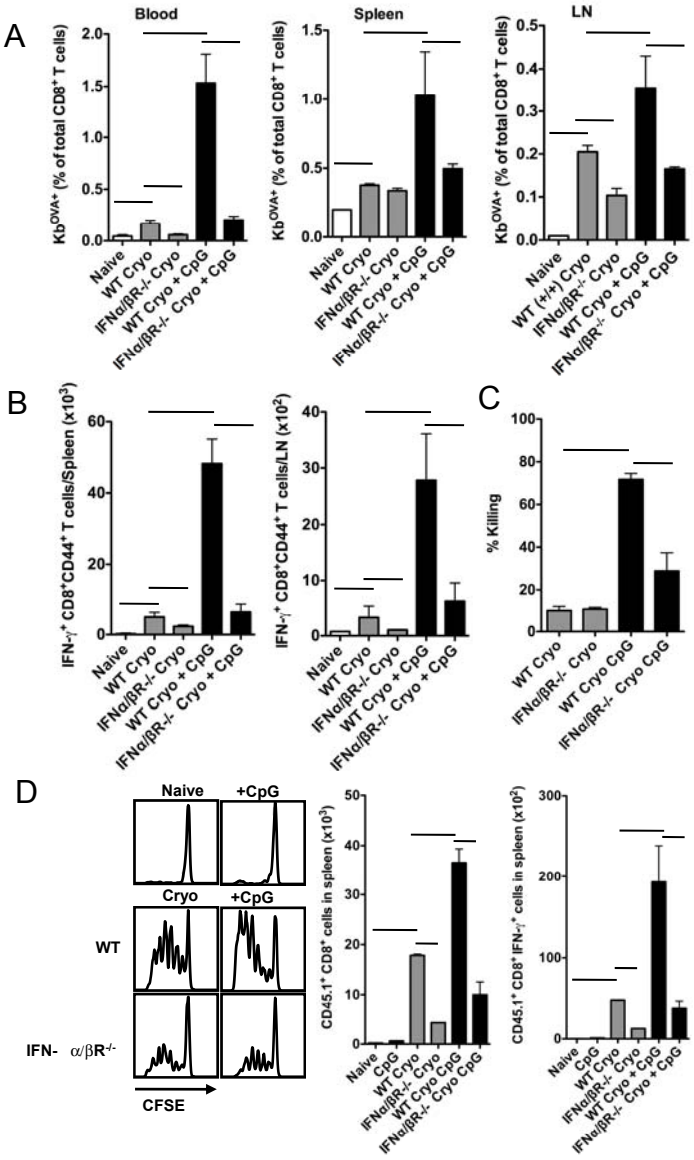

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Figure 3

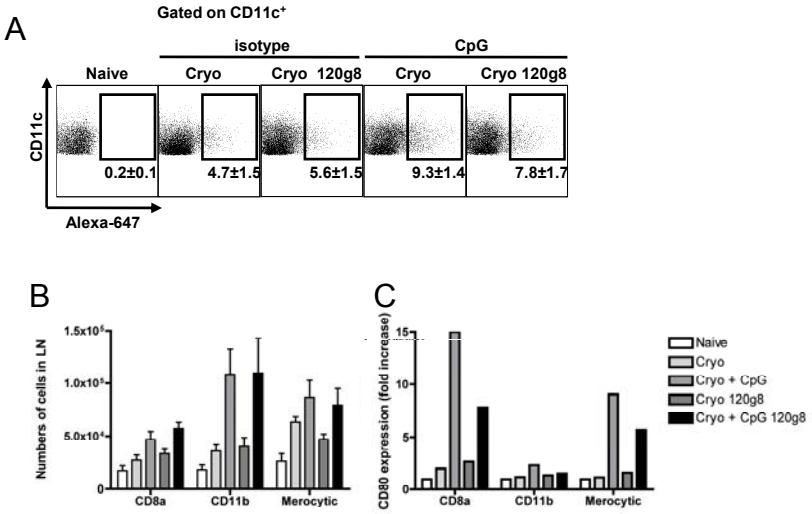

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Figure 4

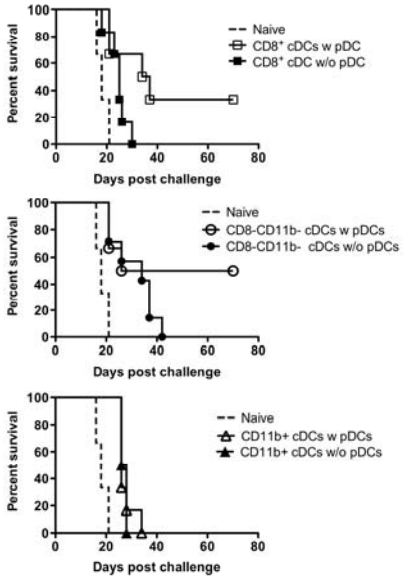

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Figure 5