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# Administration of α-galactosylceramide impairs the survival of dendritic cell subpopulations in vivo

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## ABSTRACT

**In this study, we examine whether recognition of α-GalCer presented on CD1d-expressing DCs and B cells in vivo elicits the cytotoxic activity of iNKT cells and elimination of α-GalCer-presenting cells. We report that i.v. injection of α-GalCer induced a decrease in the percentage and number of splenic $CD8^+$Langerin$^+$ DCs, while $CD8^-$ DCs were not affected. The decline in $CD8^+$ DC numbers was clearly detectable by 15 h after α-GalCer injection, was maximal at 24–48 h, returned to normal by day 7, and was accompanied by a reduced cross-presentation of OVA protein given i.v. to specific $CD8^+$ T cells in vitro. The decrease in the numbers of $CD8^+$ DCs required iNKT cells but was independent of perforin, Fas, or IFN-γ, as it was observed in mice deficient in each of these molecules. In contrast, treatment with a TNF-α-neutralizing antibody was effective at reducing the decline in $CD8^+$ DC numbers and DC activation. Treatment with immunostimulatory CpG ODN also resulted in DC activation and a decreased number of $CD8^+$ DCs; however, the decline in DC number was a result of down-regulation of CD11c and CD8 and did not require iNKT cells or TNF-α. Although $CD8^+$Langerin$^+$ DCs appeared to be selectively affected by α-GalCer treatment, they were not required for early iNKT cell responses, as their prior depletion did not prevent the increase in serum TNF-α and IL-4 observed after α-GalCer treatment. Thus, iNKT cells regulate the survival of $CD8^+$ DCs through a mechanism that does not appear to involve direct cell killing. *J. Leukoc. Biol.* 89: 753–762; 2011.**

## Introduction

Type I NKT, or iNKT, cells are a unique cell type characterized by expression of cell surface receptors associated with NK cells and a TCR composed by an invariant TCR α-chain, Vα14-Jα281, predominantly paired with a β-chain containing Vβ8.2, Vβ7, or Vβ2 [1, 2]. Unlike the TCR of conventional T cells, the TCR of iNKT cells recognizes glycolipid antigens in the context of the nonpolymorphic MHC I-like molecule CD1d [3], expressed on a variety of cell types, including B cells and DCs. Upon TCR engagement, iNKT cells rapidly produce copious amounts of cytokines, including IL-12, IL-4, and IFN-γ [4], thereby supporting the activation of other immune cells such as NK [5] cells and DCs [6, 7] and the development of further immune responses. The type of cytokine produced appears to depend on the iNKT ligand [8, 9]. α-GalCer induces strong IFN-γ and IL-4 responses, and the lower affinity ligand OCH induces predominantly IL-4. iNKT cells have a powerful immunoregulatory role and can influence a wide array of host responses and pathologies, including tumor rejection [10].

The glycolipid antigen α-GalCer is a powerful activator of iNKT cells in vivo [3] and is used to elicit the adjuvant activity of iNKT cells. Cytokine production by iNKT cells is rapidly followed by the up-regulation of the maturation markers CD80 and CD86 on spleen DC [6, 7], IL-12 production, and IFN-γ production by iNKT and NK cells. iNKT cells can also directly activate DCs via a CD40/CD40L interaction [11], thereby enhancing $CD4^+$ and $CD8^+$ T cell immune responses to coadministered soluble antigens [6, 7, 12]. This adjuvant effect requires antigen and α-GalCer to be injected simultaneously and be presented on the same APC in vitro [12] and in vivo [7], further supporting a mechanism whereby iNKT cells "license" DCs via a cognate interaction in vivo.

iNKT cells also express effector molecules that induce cell death, such as perforin [13] and the membrane-bound members of the TNF superfamily FasL and TRAIL [14, 15]. A number of studies have demonstrated that mouse and human iNKT cells can kill tumor cells in vitro [13, 15–17], and human iNKT cells can kill DCs in vitro [18, 19]. Killing by iNKT cells has also been demonstrated in vivo [20] and shown to

Abbreviations: α-GalCer, α-galactosylceramide, CD40L=CD40 ligand, DTR=diphtheria toxin receptor, FasL=Fas ligand, iNKT cell=invariant NK T cell, ODN=oligodeoxynucleotide, PKO=perforin knockout

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correlate with high CD1d expression and high-affinity ligands on target cells, and to be dependent almost exclusively on Fas/FasL interactions [20]. It has been proposed that DC killing is one of the immune regulatory functions of iNKT cells to prevent excessive antigen stimulation [18, 21] and to regulate the Th1/Th2 balance by removing DCs that are a major source of IL-12 [19].

In this study, we investigated the effect of iNKT cell activation on DC survival in vivo. We found that $CD8^+$ DC numbers in the spleen were substantially reduced as early as 15 h after α-GalCer treatment. The loss of $CD8^+$ DCs did not require perforin expression by host cells but was mediated at least in part by TNF-α, which is secreted abundantly and rapidly by activated iNKT cells [22]. DC activation and loss of the $CD8^+$ DC subpopulation appeared to be linked events, as they were induced by the same experimental treatments. Concomitant with the loss of $CD8^+$ DCs, α-GalCer treatment impaired the ability of spleen cells to cross-present antigens injected i.v., a function that is characteristic of $CD8^+$ DCs. Thus, iNKT cells regulate DC survival in vivo through a mechanism that does not appear to involve direct cell killing.

## MATERIALS AND METHODS

### Mice

The C57BL/6 and PKO [23] mice were from The Jackson Laboratory (Bar Harbor, ME, USA); B6.MRL-lpr and B6.IFN-$\gamma^{-/-}$ [24] were from the Walter and Eliza Hall Institute (Melbourne, Australia). Langerin-DTReGFP and Langerin-EGFP knock-in mice, expressing the human DTR and/or EGFP under the control of the Langerin promoter [25], were kindly provided by Bernard Malissen (Centre d'Immunologie de Marseille-Luminy, France). CD1d$^{-/-}$ mice [26] were kindly provided by Chyung-Ru Wang (University of Chicago, IL, USA). Mice from these strains were bred and maintained at the Malaghan Institute of Medical Research Biomedical Research Unit, Victoria University (Wellington, NZ). Experimental procedures were approved by the Victoria University Animal Ethics Committee and conducted in accordance with institutional guidelines.

For in vivo treatments with CpG ODN, C57BL/6 mice were obtained from the Animal Resource Centre (Perth, Australia). B6.Jα18$^{-/-}$ [10], B6.CD1d$^{-/-}$ [27], and B6.TNF-α$^{-/-}$ [28] mice were from the Queensland Institute of Medical Research Animal Facility (Australia). All animal experimentation was performed with the approval of the Animal Ethics and Experimentation Committee of the University of Western Australia (Australia), according to the guidelines of the National Health and Medical Research Council (Australia).

### Flow cytometry

Anti-FcγRII (clone 2.4G2), anti-CD8 (clone 2.43), and anti-CD11c (clone N418) mAb were purified from hybridoma culture supernatants using protein G-Sepharose (Pharmacia Biotech, Sweden), and conjugated to biotin, FITC, and Alexa 647, respectively. Anti-B220 PerCP, anti-CD3 PerCP/Cy5, anti-CD8 PerCP, anti-CD8 allophycocyanin/Cy7, anti-CD19 PerCP/Cy5, anti-CD24 PE, anti-CD11c allophycocyanin, and anti-NK1.1 PE were purchased from BD PharMingen (San Diego, CA, USA), and anti-CD86 PE and anti-CD45.1 PE were from eBioscience (San Diego, CA, USA). All antibody incubations were in FACS buffer containing 10 mM EDTA (Sigma-Aldrich, St Louis, MO, USA), 0.01% $NaN_3$ (Sigma-Aldrich), and 2% FCS (Gibco, Invitrogen, Auckland, NZ) on ice for 10 min. PI (BD PharMingen) was added to samples to exclude dead cells. All data were collected on a FACSCalibur (Becton Dickinson, Mountain View, CA, USA) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

### Phenotyping of DCs from spleen

Spleens were harvested and digested in a solution of 0.41 mg/ml Liberase CI (Roche, Mannheim, Germany) and 0.1 mg/ml DNase I (Roche) for 30 min at 37°C. For the last 5 min, EDTA (Sigma-Aldrich) was added to a final concentration of 10 mM. Spleens were then pressed through a 70-μM cell strainer (BD Biosciences, Bedford, MA, USA) into IMDM (Gibco, Invitrogen). RBCs were lysed in ammonium chloride-Tris buffer, and 1–2 × $10^6$ cells were added to a 96-well round-bottom plate (BD Biosciences) and incubated with anti-FcγRII and the indicated antibodies.

### In vivo treatments

Mice were injected i.v. with 200 ng α-GalCer or OCH (kindly provided by Dr. Gavin Painter, Industrial Research Ltd., Lower Hutt, NZ) or with other doses as indicated. Control mice received an equivalent volume of PBS. To neutralize TNF-α, mice were treated i.p. with 10 mg/kg anti-TNF-α (clone TN3-19.12, BioLegend, San Diego, CA, USA). Twenty hours later, mice were injected i.v. with 200 ng α-GalCer, and after a further 48 h, spleens were harvested and processed as above. Serum samples were taken 3 h after α-GalCer injection to determine residual TNF-α levels. To deplete Langerin$^+$ DC, Langerin-DTReGFP mice were given 1 μg DT (Sigma-Aldrich), injected i.p. 3 days and 1 day before treatment with α-GalCer. Serum was collected for cytokine analysis 3 h after α-GalCer treatment. Mice were treated with 20 nmol CpG ODN 1668 (Geneworks, Adelaide, South Australia, Australia)/mouse or the equivalent amount of PBS by i.p. injection and killed 18 h later.

### Measurement of serum cytokines

Serum levels of IL-4 and TNF-α were determined using a bead multiplex immunoassay (Bio-Plex™ systems, Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. Briefly, serum samples were diluted 1:4 in an assay buffer containing 0.1% BSA (Henderson, Auckland, NZ), 0.05% Tween 20 (Sigma-Aldrich), 0.005% $NaN_3$ (Sigma-Aldrich), and 2.5 mM EDTA, all dissolved in PBS (Gibco, Invitrogen). Samples were transferred to 96-well filter plates (L-Plate, Abacus ALS, Auckland, New Zealand) and incubated at room temperature for 30 min, after which detection antibodies were added and incubation continued for a further 10 min. After washing, samples were resuspended in assay buffer and analyzed on a Bio-Plex™ 200 (Bio-Rad Laboratories). Cytokine concentrations were determined against commercial standards (Bio-Rad Laboratories).

### In vitro T cell proliferation assays

C57BL/6 mice were left untreated or injected i.v. with 2 mg low endotoxin OVA (Profos AG, Regensburg, Germany) and 200 ng α-GalCer at the times indicated in the figures. Forty-two hours after OVA injection, mice were killed, and spleen DCs were enriched by centrifugation on a Nycodenz gradient (Sigma-Aldrich) and magnetic selection using anti-CD11c MACS beads (Miltenyi Biotec, Germany). The resulting populations were 77–95% $CD11c^+$ cell by flow cytometry and were titrated into 96-well plates together with 1 × $10^5$ magnetically purified $CD8^+$ T cells from OT-I mice. Cells were incubated for 96 h at 37°C and labeled with tritiated thymidine over the last 24 h of culture to measure proliferation. Data for 30,000 DCs/well are shown.

### Data analyses

Statistical analyses were conducted using the Prism 4 software (Graphpad Software Inc., San Diego, CA, USA) using the tests indicated in the individual figure legends. Tests for normality were carried out on the numbers of $CD8^+$ and $CD8^-$ DCs in untreated and α-GalCer-treated mice from pooled experiments, and the populations were found to be normally distributed. $P$ values $<0.05$ were considered significant.

## RESULTS

### The number of splenic CD8$^+$ DCs decreases after treatment with α-GalCer

To investigate the effects of cognate interactions between iNKT cells and DCs, we treated C57BL/6 mice with the iNKT cell ligand α-GalCer i.v. and examined spleen DC populations at different times after treatment. At 48 h after injection, the number of CD8$^+$ DCs was reduced dramatically in mice treated with α-GalCer, whereas the number of CD8$^-$ DCs (**Fig. 1A** and **B**), T cells, and B cells did not change (data not shown). Coadministration of OVA protein did not alter or prevent CD8$^+$ DC loss (data not shown). The number of CD8$^+$ DCs was already reduced substantially 15 h after α-GalCer treatment and was lowest at 24 h (Fig. 1C). By 3 days after α-GalCer treatment, the CD8$^+$ DC population started to recover, and CD8$^+$ DC numbers returned to homeostatic levels by Day 7 (Fig. 1C). A similar decrease in the number of CD8$^+$ DCs was observed in LNs at 24 h and 48 h after α-GalCer treatment (data not shown). Thus, treatment with α-GalCer induced a systemic decrease in the number of CD8$^+$ DCs.

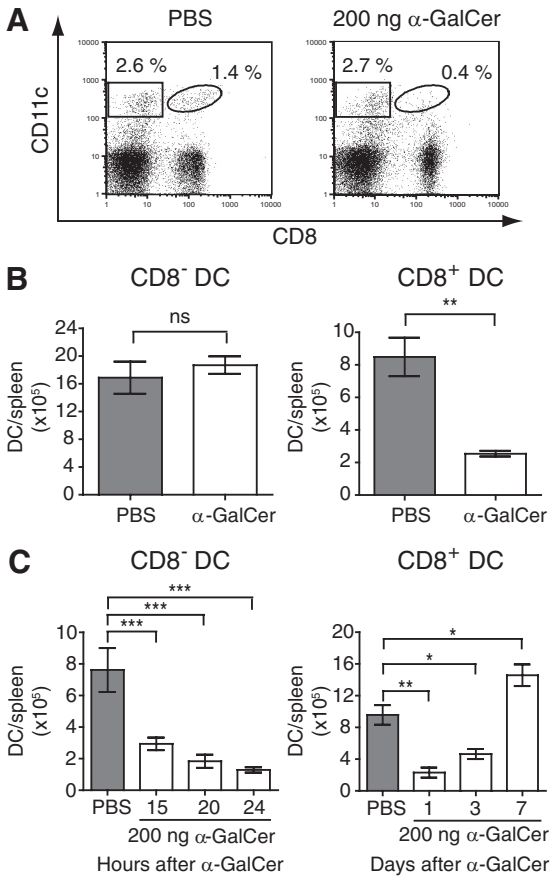


**Figure 1. The number of splenic CD8$^+$ DCs declines after treatment with α-GalCer.** C57BL/6 mice were injected with PBS or 200 ng α-GalCer i.v. and killed at different time-points for flow cytometry analysis. (A) Representative dot plots of CD8 and CD11c expression in spleens of mock-treated mice and mice treated with α-GalCer 48 h earlier. Only PI$^-$B220$^-$ events are shown. (B) Numbers of CD11c$^+$CD8$^-$ (left panel) and CD11c$^+$CD8$^+$ (right panel) spleen cells 48 h after the indicated treatments. (C) Numbers of splenic CD11c$^+$CD8$^+$ DCs at different times after α-GalCer treatment. Bars represent mean ± SEM from four to seven mice/group. ***$P < 0.001$; **$0.001 < P < 0.01$; *$0.01 < P < 0.05$; and ns, not significant by one-way ANOVA with a Tukey post hoc test. Data are representative of two to six independent experiments.

The reduction in numbers of CD8$^+$ DCs could be a result of down-regulation of the CD8 marker or loss of the cells themselves. To address this question, we examined the expression of two additional markers expressed on CD8$^+$ DCs: Langerin and CD24 [29]. Langerin-expressing cells were monitored using Langerin-EGFP mice, which express the EGFP marker in Langerhans cells and also in a proportion of CD8$^+$ DCs in LN and spleen [25]. As shown in **Fig. 2A**, few CD8$^+$EGFP$^+$ cells were found in the spleens of α-GalCer-treated Langerin-EGFP mice (Fig. 2A); these cells were almost exclusively CD11c$^+$ (Fig. 2B). In contrast, the number of Langerin-EGFP$^-$ cells remained unchanged (Fig. 2C). Similarly, the number of CD24$^+$ DCs declined after α-GalCer treatment, and the CD24$^-$ DC population remained stable (Fig. 2D). Together, these data strongly support the notion that α-GalCer does not simply induce down-regulation of the CD8 marker on DCs but leads to the loss of a subpopulation of splenic DCs that expresses the markers CD8, CD24, and Langerin.

### Requirement for iNKT cells and effector molecules in the loss of CD8$^+$ DCs after α-GalCer treatment

We wished to establish whether the loss of CD8$^+$ DCs after α-GalCer treatment required iNKT cells. We used CD1d$^{-/-}$ mice, which lack iNKT as a result of the lack of positive selection in the thymus. As shown in **Table 1**, injection of α-GalCer into CD1d$^{-/-}$ mice did not affect the number of CD8$^+$ DCs, indicating that iNKT cells were necessary for the loss of CD8$^+$ DCs.

iNKT cells possess cytotoxic ability and have been reported to kill DCs presenting α-GalCer in vitro [18, 19]. To investigate whether the observed decrease in CD8$^+$ DCs required expression of cytotoxic mediators, PKO and B6.MRL-lpr mice, which are defective in perforin- and Fas-dependent cytotoxicity, respectively, were treated with α-GalCer i.v., and splenic CD8$^+$ DCs were enumerated 24 h later. As shown in Table 1, injection of α-GalCer in C57BL/6 and PKO mice resulted in a similar loss of CD8$^+$ DCs, while CD8$^-$ DCs were not affected in either strain of mice (not shown). C57BL/6 and PKO mice responded to the injection of α-GalCer with similar increases in the expression of CD86 on all DC populations (data not shown). Injection of α-GalCer in B6.MRL-lpr mice also induced a decrease in CD8$^+$ DCs, although this was less marked than in C57BL/6 controls; interestingly, up-regulation of CD86 on DCs was also less marked in B6.MRL-lpr than in C57BL/6 mice (data not shown). Lastly, CD8$^+$ DC loss was also observed in IFN-γ$^{-/-}$ mice treated with α-GalCer (Table 1). Therefore, the loss of CD8$^+$ DCs after α-GalCer treatment occurs independently of perforin, Fas, or IFN-γ.

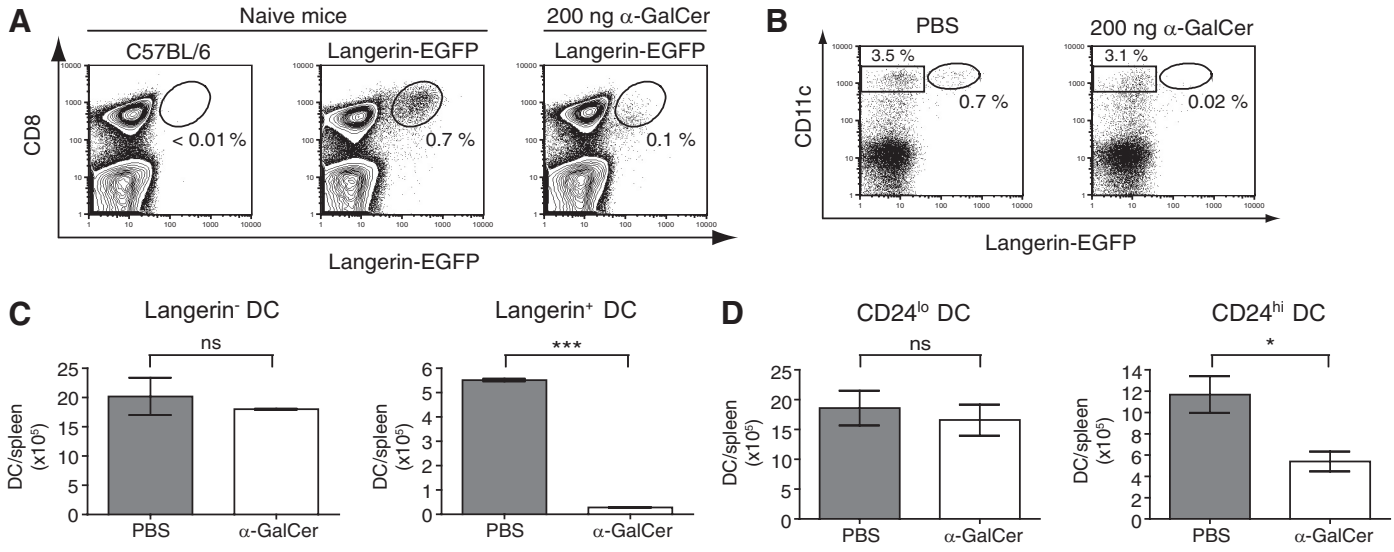


**Figure 2. The loss of CD8$^+$ DCs following α-GalCer treatment is not a result of down-regulation of the CD8 marker**. C57BL/6 and Langerin-EGFP mice were treated with α-GalCer and analyzed as described in Fig. 1. (A) Representative dot plots of CD8 and EGFP expression in spleens from untreated mice and mice treated with α-GalCer 24 h earlier. Only PI$^-$B220$^-$ events are shown. (B) Representative dot plots of CD11c and EGFP expression in spleens from Langerin-EGFP mice 24 h after the indicated treatments. Only PI$^-$B220$^-$ events are shown. (C) Numbers of CD11c$^+$EGFP$^-$ (left) and CD11c$^+$EGFP$^+$ (right) spleen cells in Langerin-EGFP mice 24 h after the indicated treatments. (D) Numbers of CD11c$^+$CD24$^{lo}$ (left) and CD11c$^+$CD24$^{hi}$ (right) spleen cells in C57BL/6 mice treated as indicated 48 h earlier. Bars represent mean ± SEM from three to four mice/group. ***$P < 0.001$; *$0.01 < P < 0.05$; and ns, not significant by an unpaired Student's $t$ test. Data are representative of two independent experiments.

## Treatment with the α-GalCer analog OCH induces a decrease in the number of CD8$^+$ DCs

iNKT cells are known to produce a wide range of cytokines upon in vivo treatment with glycolipid ligands. We wished to determine the effects of the α-GalCer analog OCH in relation to the number of splenic CD8$^+$ DCs in vivo. Compared with α-GalCer, OCH engages the iNKT receptor with lower affinity [30] and induces similar IL-4 production but lower IFN-γ [9]. As shown in **Fig. 3A**, the number of CD8$^+$ DCs was decreased significantly in mice treated with OCH, although somewhat less profoundly than in α-GalCer-treated mice. Comparable doses of α-GalCer and OCH were required for this decrease. DC activation after injection of different doses of α-GalCer was also examined. Interestingly, the decrease in CD8$^+$ DC numbers and the increased cell surface expression of CD86 were observed at similar doses of α-GalCer (Fig. 3A and B), suggesting that DC activation and cell loss may be triggered in the same conditions and might be related events.

## Neutralization of TNF-α prevents the reduction of CD8$^+$ DCs after α-GalCer treatment

The decrease in numbers of CD8$^+$ DCs after α-GalCer treatment might be a result of the effects of cytokines secreted as a

**TABLE 1. The Depletion of CD8$^+$ DCs Following α-GalCer Treatment Requires iNKT Cells but Is Independent of the Expression of Cytotoxic Molecules**

| Strain | $n^a$ | α-GalCer treatment | No. CD8$^+$ DC/spleen ± SD (thousands) | % of untreated | $P^b$ |
|---|---|---|---|---|---|
| C57BL/6 | 22 | – | 702 ± 272 | 26.9% | <0.0001 |
| | 24 | + | 189 ± 107 | | |
| CD1d$^{-/-}$ | 5 | – | 984 ± 232 | 100.1% | 0.993 |
| | 5 | + | 986 ± 291 | | |
| PKO | 7 | – | 857 ± 329 | 29.8% | 0.0002 |
| | 9 | + | 256 ± 116 | | |
| B6.1pr | 5 | – | 850 ± 193 | 49.7% | 0.0020 |
| | 5 | + | 422 ± 88 | | |
| IFN-γ$^{-/-}$ | 7 | – | 830 ± 256 | 30.4% | <0.0001 |
| | 8 | + | 253 ± 81 | | |

Mice were injected i.v. with PBS (–) or 200 ng α-GalCer (+) and sacrificed 18–24 h later. Numbers of CD11c$^+$CD8$^+$ cells/spleen ± SD are shown. [a] $n$, Number of mice/group pooled from eight (C57BL/6), three (IFN-γ$^{-/-}$ and PKO), two (CD1d$^{-/-}$), and one (B6.1pr) independent experiments, respectively. [b] $P$ values were calculated using an unpaired, two-tailed Student's $t$ test.

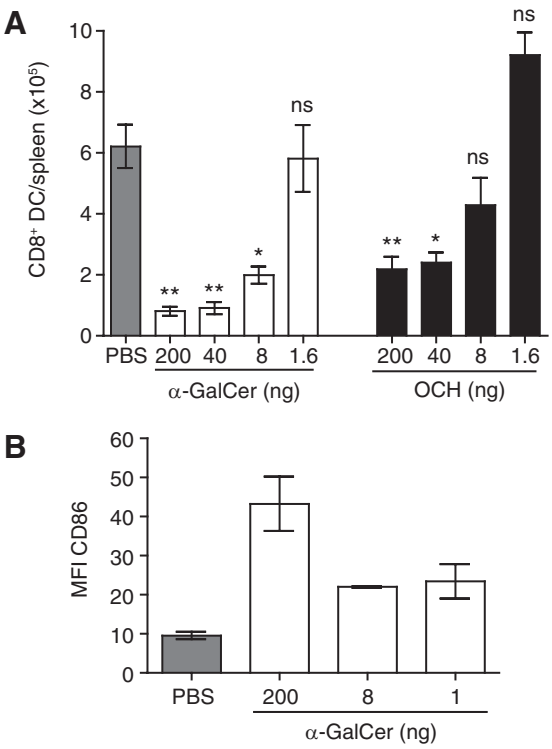


**Figure 3. Treatments with OCH or α-GalCer induce similar decreases in the number of splenic CD8$^+$ DCs.** C57BL/6 mice were treated with α-GalCer or OCH and analyzed as described in Fig. 1. (A) Numbers of spleen CD11c$^+$CD8$^+$ cells 48 h after treatment with the indicated doses of OCH or α-GalCer. ***$P < 0.001$; **$0.001 < P < 0.01$; *$0.01 < P < 0.05$; and ns, not significant by one-way ANOVA with a Tukey post hoc test. $P$ values are for comparisons with the PBS control group. (B) Expression of CD86 on spleen CD11c$^+$CD8$^+$ cells 24 h after treatment with 200 ng α-GalCer. Bars represent mean ± SEM from three to four mice/group. MFI, Mean fluorescence intensity.

consequence of iNKT cell activation. TNF-α, which is produced in α-GalCer-treated mice early after injection [22], is a known inducer of cell death [31]. To establish the possible role of TNF-α in the loss of CD8$^+$ DCs, C57BL/6 mice were treated with 10 mg/kg TNF-α-neutralizing antibody, and 20 h later, they were injected with α-GalCer. Blood was collected from all mice 3 h after α-GalCer treatment to determine residual levels of serum TNF-α. As shown in **Fig. 4A**, mice injected with α-GalCer had high levels of serum TNF-α, and TNF-α was below the level of detection in mice pretreated with TNF-α-neutralizing antibody.

Mice treated with α-GalCer and anti-TNF-α were also examined for numbers of CD8$^+$ DCs in spleen and LNs 48 h after α-GalCer administration. As in previous experiments, the numbers of CD8$^+$ DCs were reduced significantly in mice treated with α-GalCer; this decrease was reversed substantially but not completely by treatment with anti-TNF-α (Fig. 4B and C). No change was observed in the number of CD8$^-$ DCs in any of the experimental groups (Fig. 4B and C). In addition, treatment with anti-TNF-α reduced the α-GalCer-induced increase in CD86 expression by CD8$^+$ and CD8$^-$ DCs (Fig. 4D). A similar decreased DC activation has been reported by others using TNF-α-deficient hosts [32]. These results suggest that TNF-α plays a critical role in the activation and loss of CD8$^+$ DCs after α-GalCer treatment, although other factors may also be involved.

## Treatment with immunostimulatory CpG ODN induces a phenotypic loss of CD8$^+$ DCs

We examined whether other stimuli that induce DC activation in vivo might also result in the loss of CD8$^+$ DCs. C57BL/6 mice were treated with 20 nmol CpG ODN 1668 via the i.p. route, and their spleens were harvested 18 h later. **Fig. 5A** shows that treatment with CpG ODN induced a significant decrease in the percentage of splenic CD8$^+$ DCs, and CD8$^-$ DCs were not affected. However, unlike the case with α-GalCer, the decrease in CD8$^+$ DCs was not a result of a true cell loss but of a decreased surface expression of CD11c and CD8. The observed decrease in the number of CD11c$^+$CD8$^+$ cells was accounted for by an increase in a CD11c$^{lo}$CD8$^{lo}$ population in CpG ODN-treated mice (Fig. 5A, and data not shown). Down-regulation of CD8 and CD11c after CpG ODN treatment did not require the presence of iNKT cells, as it was observed in Jα18$^{-/-}$ or CD1d$^{-/-}$ mice that lack iNKT cells (Fig. 5B). In addition, treatment of TNF-α$^{-/-}$ mice with CpG ODN resulted in down-regulation of CD11c and CD8 expression (Fig. 5B), and DC up-regulation of CD80 and CD86 was not affected (not shown). Therefore, although treatments with α-GalCer and CpG ODN induce a decrease in the number of CD8$^+$ DCs, these decreases are phenotypically distinct events and occur via different mechanisms.

## CD8$^+$Langerin$^+$ DCs are not required for the initial activation of iNKT cells after α-GalCer treatment

To determine the role of CD8$^+$ DCs in the response of iNKT cells to α-GalCer, we examined the effect of CD8$^+$ DC depletion on iNKT cell activation. We used Langerin-DTReGFP and (Langerin-DTReGFP×Langerin-eGFP)F1 mice, which express the DTR under the control of the Langerin promoter. In these mice, DT administration selectively eliminates all Langerin-expressing cells (**Fig. 6A**), including a population of CD8$^+$Langerin$^+$ DCs that is found in the marginal zone of the spleen [33]. iNKT cell activation after α-GalCer administration was evaluated by measuring serum IL-4 and TNF-α, as iNKT cells are known to secrete copious amounts of these cytokines early after α-GalCer administration [22]. Serum IFN-γ was not examined, as this cytokine is also released by NK cells responding to IL-12 produced by Langerin$^+$ DCs [34, 35]. At 3 h after α-GalCer administration, IL-4 and TNF-α could be detected in the serum of all mice, and their levels in Langerin-DTReGFP mice were not decreased by DT treatment (Fig. 6B). The modest increase in TNF-α and IL-4 in DT-treated Langerin-DTReGFP mice was not statistically significant and was less pronounced in repeat experiments. Thus, early cytokine production after iNKT cell activation does not require the presence of CD8$^+$Langerin$^+$ DCs.

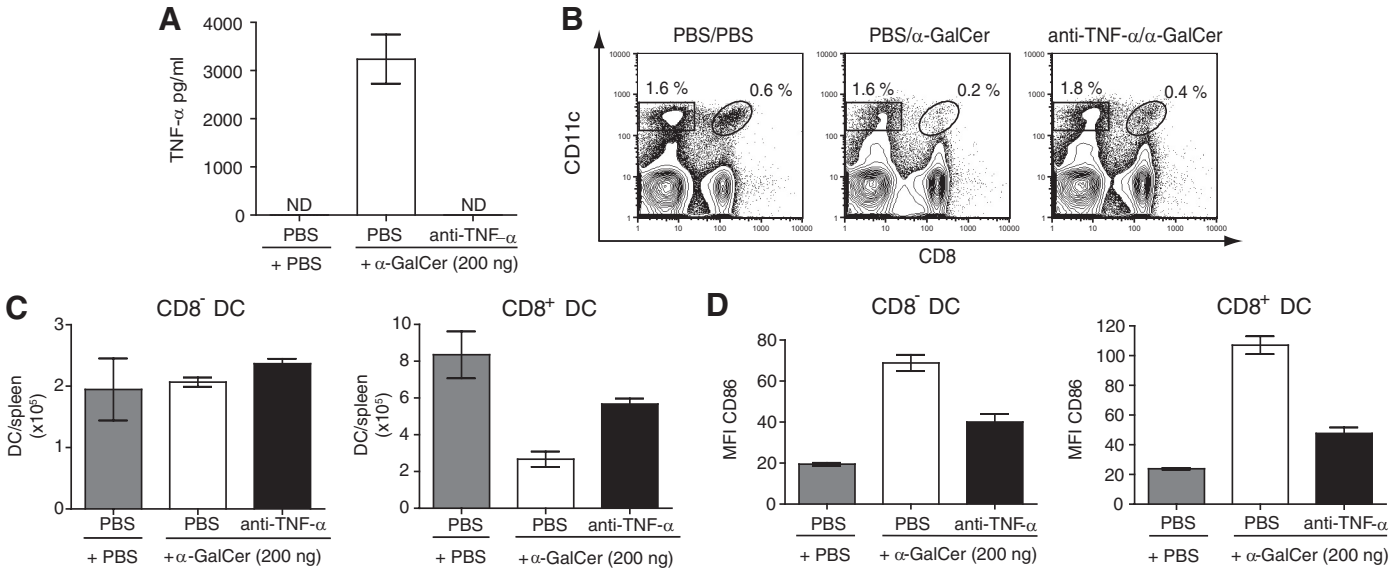


**Figure 4. The loss of CD8$^+$ DCs following α-GalCer treatment requires TNF-α.** C57BL/6 mice were injected i.p. with PBS or 10 mg/kg anti-TNF-α, treated with α-GalCer 20 h later, and analyzed as described in Fig. 1. (A) Levels of serum TNF-α 3 h after α-GalCer treatment were determined using a bead multiplex immunoassay. ND, Not detectable. (B) Mice were treated as indicated and examined 48 h after α-GalCer treatment. Representative dot plots of PI$^-$B220$^-$ events are shown. (C) The numbers of CD11c$^+$CD8$^-$ (left) and CD11c$^+$CD8$^+$ (right) spleen cells in the indicated treatment groups were determined 48 h after α-GalCer injection. (D) The expression of CD86 on CD11c$^+$CD8$^-$ (left) and CD11c$^+$CD8$^+$ (right) spleen cells in the indicated treatment groups was determined 24 h after α-GalCer injection. Bars show mean and range for two mice/group. Data are representative of two independent experiments.

## Treatment with α-GalCer induces the loss of cross-presenting splenic DCs

CD8$^+$ DCs perform a specialized function in vivo by cross-presenting blood-derived antigens to CD8$^+$ T cells [34, 36]. To determine whether the loss of CD8$^+$ DCs after α-GalCer treatment also affects antigen cross-presentation, we compared the ability of DCs exposed to OVA and α-GalCer in vivo to stimulate the proliferation of specific CD8$^+$ T cells in vitro. As shown in **Fig. 7**, mice were injected i.v. with OVA only or with OVA and α-GalCer at different time-points and were killed 42 h after OVA injection. DCs from mice injected with OVA only were unable to induce CD8$^+$ T cell proliferation in vitro. Treatment with α-GalCer 2 h before sacrifice substantially increased the ability of DCs to induce OVA-specific CD8$^+$ T cell proliferation in vitro, possibly by increasing the expression of costimulatory molecules that support CD8$^+$ T cell proliferation in vitro [7, 12, 37] or the ability of DCs to cross-present. In contrast, DCs from mice treated with OVA and α-GalCer 40 h before sacrifice induced minimal CD8$^+$ T cell proliferation. In these mice, α-GalCer treatment had resulted in the

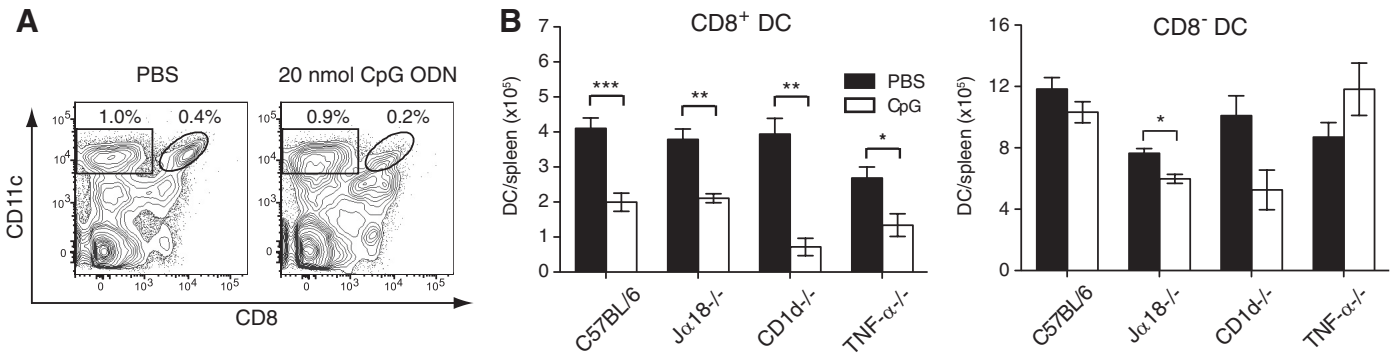


**Figure 5. Treatment with CpG ODN induces phenotypic loss of splenic CD8$^+$ DCs by an iNKT and TNF-α-independent mechanism.** Mice were treated i.p. with PBS or 20 nmol CpG ODN 1668 and killed 18 h later. (A) Representative dot plots of CD8 and CD11c expression in spleens of mock-treated mice and mice treated with CpG ODN 18 h earlier. Only PI$^-$CD3$^-$CD19$^-$NK1.1$^-$ events are shown. (B) Numbers of CD11c$^+$CD8$^+$ and CD11c$^+$CD8$^-$ cells/spleen are shown. Bars show mean ± SEM for groups of three to 12 mice. *P* values are for comparisons with the respective PBS control group and were calculated using a Mann-Whitney test. ***$P < 0.001$; **$0.001 < P < 0.01$; *$0.01 < P < 0.05$.

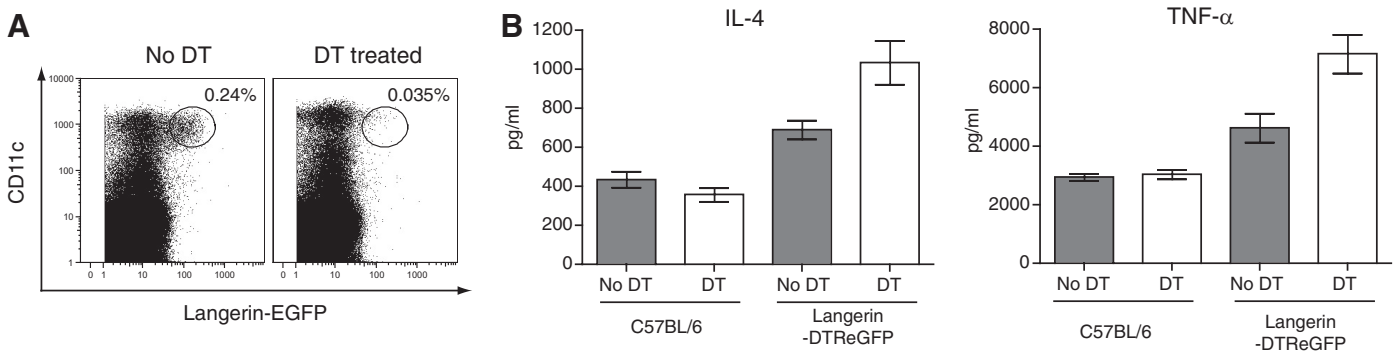


**Figure 6. The depletion of CD8$^+$Langerin$^+$ DCs in vivo does not prevent iNKT cell activation.** (A) (Langerin-DTReGFP×Langerin-eGFP)F1 mice were treated with DT or PBS, and spleen cell suspensions were examined 24 h later by flow cytometry. Panels show a representative sample from each group, and only PI$^-$ events are included. (B) C57BL/6 and Langerin-DTReGFP mice were pretreated with DT and injected i.v. with 200 ng α-GalCer. Mice were bled 3 h after α-GalCer treatment, and serum TNF-α and IL-4 were measured using a bead multiplex immunoassay. Bars represent mean ± SEM from three mice/group. Data are representative of two independent experiments.

loss of the CD8$^+$ DC subpopulation. Thus, loss of CD8$^+$ DCs after α-GalCer treatment correlates with a decreased ability of spleen cells to cross-present soluble antigens.

## DISCUSSION

In this paper, we report that iNKT cell activation induced by treatment with α-GalCer results in a transient but dramatic reduction in the numbers of CD8$^+$Langerin$^+$ DCs and in the cross-presentation of blood-borne antigen in the spleen. The reduction in CD8$^+$ DC numbers did not appear to be related to a preferential interaction between iNKT cells and CD8$^+$ DCs during the immune response to α-GalCer but was mediated at least in part by TNF-α, which is secreted rapidly and abundantly by iNKT cells after α-GalCer recognition [22].

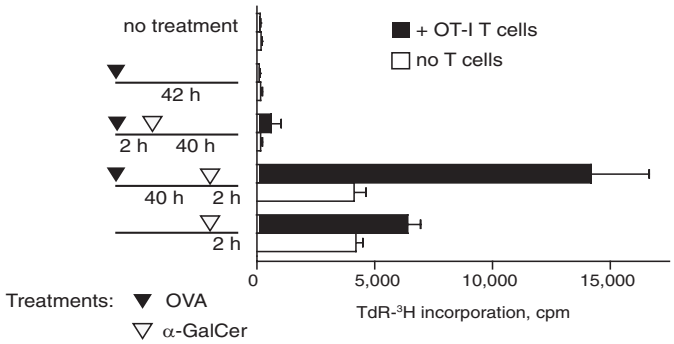


**Figure 7. Treatment with α-GalCer leads to the loss of cross-presenting DCs from the spleen.** C57BL/6 mice were left untreated or injected i.v. with 2 mg OVA and/or 200 ng α-GalCer, according to the schedule shown on the left. Spleen cells from control and treated mice were enriched for CD11c$^+$ DCs by magnetic selection, and 30,000 CD11c-enriched cells were incubated with OT-I T cells or no T cells for 3 days. T cell proliferation was assessed by thymidine (TdR) incorporation. Bars show mean thymidine incorporation + SEM for groups of two to four mice.

Several lines of evidence suggest that the reduction in CD8$^+$ DC numbers observed after α-GalCer treatment was a result of cell loss and not of decreased expression of the CD8 marker. First, the number of CD8$^-$ DCs was not increased in α-GalCer-treated mice, as would be expected if the DCs that are normally CD8$^+$ become CD8$^-$. Second, loss of CD8$^+$ DCs was accompanied by a decrease in the numbers of cells that are Langerin-GFP$^+$ and CD24$^+$. Langerin and CD24 are expressed by CD8$^+$ DCs, and their simultaneous decrease is highly suggestive of cell loss. Interestingly, the depleted population was best identified by its expression of Langerin-GFP$^+$, as essentially no Langerin-GFP$^+$ cells were detected in α-GalCer-treated mice, and some CD8$^+$ and CD24$^+$ cells remained, albeit in significantly reduced numbers compared with untreated mice. Lastly, the decline in CD8$^+$ DCs was accompanied by a decreased capacity of spleen cells to cross-present soluble antigens injected i.v., a function that depends on the presence of CD8$^+$ DCs [38]. Together, these results suggest that α-GalCer treatment induces the loss of CD8$^+$ DCs, rather than down-regulation of CD8 expression.

It is tempting to assume that the selective loss of CD8$^+$Langerin$^+$ DCs might be a result of these cells being uniquely involved in presenting α-GalCer to iNKT cells. CD8$^+$ DCs express approximately threefold higher levels of CD1d compared with the CD8$^-$ DC subpopulation (ref. [39], and data not shown) and might preferentially interact with iNKT cells during presentation of α-GalCer. However, experiments using DT to deplete CD8$^+$Langerin$^+$ DCs selectively before α-GalCer injection showed that CD8$^+$Langerin$^+$ DCs were dispensable for the early response to α-GalCer. Serum levels of TNF-α and IL-4 were not decreased in DT-treated mice, and the frequencies of iNKT cells expressing intracellular IL-4 or IFN-γ were also not affected [34]. Therefore, CD8$^+$Langerin$^+$ DCs do not appear to have a specialized function in presenting α-GalCer to iNKT cells in vivo, although they remain important contributors to other responses induced by α-GalCer, such as IL-12 production [34].

Depletion of CD8$^+$ DCs after α-GalCer treatment required iNKT cells but did not appear to involve selective killing of CD8$^+$

DCs in vivo, as it was not reversed in PKO or Fas-deficient mice, which are reportedly defective in iNKT-mediated cytotoxicity [20]. In contrast, neutralization of TNF-α reversed most of the DC loss and, as reported by other authors using TNF-$\alpha^{-/-}$- or TNF-$\alpha R^{-/-}$-deficient mice [32], also prevented the α-GalCer-induced activation of $CD8^+$ and $CD8^-$ DCs in vivo. Additional cytokines/factors were probably involved, as treatment with anti-TNF-α antibodies did not prevent $CD8^+$ DC loss completely. We examined the role of IFN-γ, but the $CD8^+$ DC population was similarly reduced in α-GalCer-treated control and IFN-$\gamma^{-/-}$ mice and in mice treated with the CD1d ligand OCH, which is a weak inducer of IFN-γ secretion [9]. Together, these data suggest that IFN-γ was not a major contributor to the loss of $CD8^+$ DCs induced by α-GalCer treatment.

The loss of $CD8^+$ DCs could not be explained by preferential expression of TNF-αRs on this DC subpopulation, in the steady state or after α-GalCer treatment (data not shown). Loss of $CD8^+$ DCs has been observed in a model of polymicrobial sepsis [40], but this was in the absence of detectable up-regulation of activation markers on all DC subpopulations, was accompanied by a loss of T cells, and thus was likely to involve different mechanisms. Loss of $CD8^+$ DCs and decreased IL-12 production have been observed after in vivo treatment with cholera toxin [41]; however, DC activation was not characterized in that model. Lastly, loss of $Langerin^+CD8^+$ DCs was also reported after treatment with TLR ligands, such as polyinosinic:polycytidylic acid and low-dose LPS [33, 42]. Receptors for the TLR ligand LPS are expressed on all mouse DC subpopulations, but as in our experiments with α-GalCer, only the $CD8^+$ DC subpopulation was affected [33]. Taken together, these data may suggest that compared with other DC subpopulations the $CD8^+$ DCs are especially prone to undergoing apoptosis or activation-induced cell death.

Interestingly, data in this paper also suggest that DC activation is not necessarily followed by cell death. Similar to α-GalCer, treatment with CpG ODN induced DC activation and a decrease in the number of $CD8^+$ DCs, but this decrease was a result of down-regulation of the CD8 and CD11c markers and not of a true cell loss. Down-regulation of CD11c and CD8 on DCs after CpG ODN administration did not require iNKT cells or TNF-α, confirming that it occurred by a mechanism different from the mechanism leading to $CD8^+$ DC loss after α-GalCer treatment.

It is possible that the differential response of $CD8^+$ and $CD8^-$ DCs to α-GalCer treatment was a result of DCs receiving multiple signals, some of which lead to activation, while others lead to survival or death. We attempted to separate the activation and death signals by injecting decreasing doses of α-GalCer and by using the α-GalCer analog OCH, which has a lower binding affinity for mouse iNKT cells [30]. We were unable to identify conditions where DC activation and loss could be separated, and whenever DC activation was observed, it was followed by the selective loss of the $CD8^+$ DC subpopulation. This loss appears compatible with activation-induced cell death, which has been reported by other authors in DCs responding to LPS [33, 43]. However, such a mechanism of death would be surprising in light of the known role of CD40-CD40L-mediated signals in the response to α-GalCer. In vitro and in vivo evidence suggests that CD40-CD40L-mediated signals support the survival of DCs [44–48] and may reverse activation-induced cell death following LPS treatment [44]. CD40-mediated signaling is clearly involved in the response to α-GalCer: $CD40^{-/-}$ mice treated with α-GalCer fail to produce high levels of IL-12p70, and fail to generate robust $CD8^+$ immune responses to coadministered OVA [7, 32], despite up-regulating expression of costimulatory molecules. These observations suggest that iNKT cells form cognate interactions with at least some $CD8^+$ DCs during the response to α-GalCer, a possibility that is supported by the observation that iNKT can promote $CD8^+$ T cell responses when antigen and CD40 are expressed on the same APC [37]. These interactions may involve only rare, surviving $CD8^+$ DCs, which are nonetheless sufficient to induce productive $CD8^+$ T cell immune responses.

The use of α-GalCer as an adjuvant has been proposed for multiple applications, as coadministration of α-GalCer and protein antigen can support the activation of T and B cell responses [6, 7, 49]. Our results are relevant to these proposed applications in two main respects. First, our data suggest that treatment with α-GalCer is followed by a degree of immune impairment as a result of the transient loss of the $CD8^+$ DC population. In support of this possibility, we show that cross-presentation of i.v.-injected OVA is decreased, and recent work by Qiu et al. [33] suggests that tolerance induction might also be affected. These consequences need to be taken into account when designing protocols using α-GalCer. Second, the loss of a substantial proportion of $CD8^+$ DCs, which have a specialized role in antigen cross-presentation [36], might impair the full expansion and/or effector differentiation of $CD8^+$ T cells specific for coadministered protein antigens. As this issue is especially relevant to the proposed use of α-GalCer in tumor immunotherapy, we sought to establish whether preventing the loss of $CD8^+$ DCs by treatment with a TNF-α-neutralizing antibody might result in increased $CD8^+$ T cell responses. Our results indicated that the frequency of antigen-specific $CD8^+$ T cells was only transiently increased in anti-TNF-α-treated mice compared with untreated controls (data not shown). Thus, we conclude that preventing the loss of $CD8^+$ DCs during treatment with α-GalCer may not lead to significant improvements of the resulting $CD8^+$ T cell response. These results are consistent with studies showing that a brief period of antigen presentation is sufficient for the induction of strong $CD8^+$ T cell responses [50].

Taken together, our data offer interesting insights into the physiology of DCs during the immune response to α-GalCer. They also suggest that although the precise consequences of the loss of $CD8^+Langerin^+$ DCs require further characterization, they should not prevent the use of this ligand as a powerful adjuvant for T cell-based immunotherapies.

## AUTHORSHIP

H.M.A.S., E.H., K.J.F., and M.L.O. designed and carried out experiments; I.F.H., M.A.D-E., and F.R. designed experiments; and H.M.A.S. and F.R. wrote the manuscript.

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