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The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation

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The Linkage of Innate to Adaptive Immunity via Maturing Dendritic Cells In Vivo Requires CD40 Ligation in Addition to Antigen Presentation and CD80/86 Costimulation

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Abstract

Dendritic cell (DC) maturation is an innate response that leads to adaptive immunity to coadministered proteins. To begin to identify underlying mechanisms in intact lymphoid tissues, we studied α-galactosylceramide. This glycolipid activates innate $V\alpha14^+$ natural killer T cell (NKT) lymphocytes, which drive DC maturation and T cell responses to ovalbumin antigen. Hours after giving glycolipid i.v., tumor necrosis factor (TNF)–α and interferon (IFN)-γ were released primarily by DCs. These cytokines induced rapid surface remodeling of DCs, including increased CD80/86 costimulatory molecules. Surprisingly, DCs from $CD40^{-/-}$ and $CD40L^{-/-}$ mice did not elicit $CD4^+$ and $CD8^+$ T cell immunity, even though the DCs exhibited presented ovalbumin on major histocompatibility complex class I and II products and expressed high levels of CD80/86. Likewise, an injection of TNF-α up-regulated CD80/86 on DCs, but CD40 was required for immunity. CD40 was needed for DC interleukin (IL)-12 production, but IL-$12p40^{-/-}$ mice generated normal ovalbumin-specific responses. Therefore, the link between innate and adaptive immunity via splenic DCs and innate NKT cells has several components under distinct controls: antigen presentation in the steady state, increases in costimulatory molecules dependent on inflammatory cytokines, and a distinct CD40/CD40L signal that functions together with antigen presentation ("signal one") and costimulation ("signal two") to generate functioning $CD4^+$ T helper cell 1 and $CD8^+$ cytolytic T lymphocytes.

Key words: α-galactosylceramide • maturation • CD40 • NKT • TNF-α

Introduction

DCs in lymphoid tissues can initiate antigen-specific adaptive responses: both peripheral tolerance and immunity (for reviews see references 1–3). The administration of a stimulus for maturation switches DC function from tolerance to immunity, including the development of $CD4^+$ T cells of the Th1 type, and active cytolytic $CD8^+$ T cells. Although much of the early work on DC maturation was performed in culture, the process has begun to be studied in vivo, particularly in lymphoid organs (4, 5), which are the tissues involved in the generation of tolerance and immunity. In the steady state, in the absence of overt inflammatory and infectious stimuli, many DCs in lymphoid organs are defined as immature, able to endocytose (6–8), and process antigens to form peptide complexes with MHC class I and II products (8–11), but unable to initiate the differentiation of effector T cells and memory. Instead, DCs in the steady state, or subsets of DCs, elicit different forms of peripheral tolerance, such as deletion (9, 10), anergy (12), and the expansion of regulatory T cells (13). If the antigen-capturing DCs are exposed to a stimulus that leads to their maturation in vivo (5, 9, 10, 14–17), strong T cell immunity develops, especially $CD4^+$ and $CD8^+$ effector T cells capable of IFN-γ production and cytolysis. Thus, the avoidance of tolerance and the initiation of immunity are major correlates of DC maturation; it is important to identify underlying mechanisms particularly in the intact animal.

To begin to identify in vivo mechanisms that drive DC maturation and link innate with adaptive immunity, we

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Abbreviations used in this paper: α-GalCer, α-galactosylceramide; CFSE, carboxyfluorescein succinimidyl ester; MLR, mixed leukocyte reaction.

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have dissected the DC response in lymphoid tissues to the synthetic glycolipid, α-galactosylceramide (α-GalCer). This glycolipid is presented by CD1d molecules to the conserved Vα14 T cell receptor on innate NKT cells (18). α-GalCer, in an NKT cell–dependent fashion, acts as an adjuvant for T cell immunity (19) and as an inducer of maturation in splenic DCs (15, 16). The α-GalCer/NKT system is attractive to study the role of different components of DC maturation in vivo. Several cardinal features of maturation are engaged within 4–8 h of a single i.v. dose of α-GalCer. There is a marked up-regulation of CD40, 80, and 86 costimulatory and MHC class II antigen-presenting molecules, identical to that observed with toll-like receptor stimuli in vivo, such as lipopolysaccharide (4) and CpG oligonucleotides (5). The DCs also start to produce large amounts of cytokines such as IFN-γ and IL-12 (15). Importantly, if a small amount of cell-associated antigen is administered together with α-GalCer, the DCs initiate combined $CD4^+$ and $CD8^+$ T cell immunity to that protein. Specifically, a single dose of <1 μg of OVA within dying cells leads to strong immunity, including protection of OVA-transduced tumors (15); this protection persists for at least 2 mo (unpublished data). To prove that maturing DCs are directly responsible for the induction of immunity, DCs can be removed from mice 4–8 h after giving α-GalCer and antigen. When transferred to naive animals, these DCs initiate immunity without further antigen, α-GalCer, or NKT cells (15). An analogous conclusion on the importance of maturing DCs was reached by Shah et al., who adoptively transferred immunity with DCs responding to CpG oligonucleotides (20).

Therefore, the α-GalCer system provides an opportunity to identify mechanisms whereby maturing DCs convert a single low dose of antigen into strong and prolonged $CD4^+$ and $CD8^+$ T cell immunity. Interestingly, NKT lymphocytes rather than microbial stimuli forge the link between innate and adaptive immunity. We will show that different elements in the immunization process are under distinct controls, and that a particularly critical one entails CD40 ligation. It is well known that CD40 acts as a DC maturation stimulus in culture, increasing the expression of CD80 and CD86 (21, 22), enhancing antigen presentation (23, 24), and inducing IL-12 production (25, 26). Also, CD40 is required for DC function in vivo, particularly for $CD8^+$ T cell responses (27–29). Mechanistically, it has been assumed that CD40 ligation triggers DCs to express the two presumed requirements for immunity, antigen presentation or "signal one" and up-regulation of membrane and cytokine costimulators or "signal two." Here, we find that cytokines, independently of CD40 ligation, are responsible for the maturation of DCs when this is assessed by increased expression of CD80/86 in vivo. Nonetheless, CD40 and CD40L are required by DCs to induce both $CD4^+$ and $CD8^+$ T cell immunity even when the DCs are expressing high levels of MHC peptide and costimulatory molecules. At least in the α-GalCer/NKT system, it appears that more is required for DCs to initiate immunity than the combination of signals one and two.

Materials and Methods

Mice. Pathogen-free C57BL/6 (B6), IFN-$\gamma^{-/-}$, IFN-$\gamma R^{-/-}$, TNF-$\alpha^{-/-}$, and $TAP^{-/-}$ female mice at 6–7 wk were purchased from The Jackson Laboratory. Mice deleted of Jα281 (Jα18) genes were provided by M. Taniguchi (Institute of Physical and Chemical Research, Yokohama, Japan). Mice were maintained under specific pathogen-free conditions and studied in compliance with institutional guidelines.

Reagents. α-GalCer (2S,3S,4R-1-*O*(α-galactopyranosyl)-2(*N*-hexacosanoylamino)-1,3,4-octadecanetriol and vehicle were provided by the Pharmaceutical Research Laboratory and diluted in PBS. LPS-free OVA was obtained from the Seikagaku Corp. The following mAbs were obtained from BD Biosciences: FITC-conjugated α-CD4, α-CD8α or PE-conjugated α-CD8α, allophycocyanin-conjugated α-CD11c, biotinylated isotype control, α-CD40, CD80, and CD86. Biotinylated mAbs were detected with streptavidin-allophycocyanin. IFN-γ mAb (R4-6A2) was purified from hybridoma (American Type Culture Collection) supernatants.

Flow Cytometry for DC Surface Markers. Cells were preincubated with 2.4G2 culture medium to block FcγR, washed, incubated with mAb conjugates for 30 min, washed, and analyzed on a FACSCalibur™ flow cytometer (Becton Dickinson).

DC Preparation from Spleen. Using methods described previously (30), splenocytes were released by teasing and treatment with collagenase D (Roche Diagnostics Corp.). For flow cytometry, DC-enriched cells were obtained as a low-density fraction on BSA columns (30). In functional assays, we separated $CD11c^+$ fractions with α-CD11c–coated magnetic beads immediately after collagenase treatment, followed by labeling with α-CD11b–PE and PE-magnetic beads (MACS).

Cytokine Production by DCs. $CD11c^+$ DC enriched, $CD11c^-$ $CD11b^+$ monocyte-enriched, and $CD11c^-CD11b^-$ lymphocytes, isolated as aforementioned with magnetic beads 2 h after mice were given i.v. α-GalCer or vehicle, were cultured at 2×10^5 cells/well for 24 h in 96-well plates. The supernatants were assayed with the following ELISA kits for cytokines: IFN-γ and IL-12p40 (Opti EIA; BD Biosciences); TNF-α and IL-12p70 (Quantikine; R&D Systems); and IFN-α (PBL Biomedical Labs).

Antigen Uptake and Presentation In Vivo. To measure splenic DC presentation of cell-associated antigens to T cells, we used assays described previously (7, 8) in which mice were injected with 2×10^7 OVA-pulsed and osmotically shocked, syngeneic $TAP^{-/-}$ splenocytes (7). Osmotic shock causes cells to die, and these cells are taken up by $CD8^+$ splenic DCs. To monitor OVA presentation, mice were adoptively transferred with carboxyfluorescein succinimidyl ester (CFSE)–labeled, $CD8^+$ (OT-I) and $CD4^+$ (OT-II) OVA-specific TCR transgenic T cells. 3 d later, single cell suspensions of spleen and lymph nodes were taken to monitor T cell proliferation by dilution of CFSE, and T cell expansion was expressed as the number of transgenic T cells in the spleen. OT-I T cells were marked with CD45.1 and OT-II T cells were marked by expression of Vβ5.1/5.2 and Vα2.

Initiation (Priming) of T Cell Immunity. 7 d after cell-associated OVA injection as aforementioned, recipient spleen cells were tested for $CD4^+$ and $CD8^+$ T cell priming. 5×10^6 cells were cultured 6 h in 24-well plates ±1 μM $OVA_{257\text{-}264}$ peptide (for $CD8^+$ T cells) or 2 μM $OVA_{323\text{-}339}$ peptide (for $CD4^+$ T cells) with brefeldin A (BD Biosciences) to accumulate IFN-γ intracellularly. Cells were incubated for 15 min at 4°C with 2.4G2 α-FcγR mAb to block nonspecific staining and with FITC α-CD4 or CD8 for 20 min at room temperature. After Cytofix/Cytoperm Plus™ permeabilization (BD Biosciences), we stained

cells with PE-conjugated α–IFN-γ (XMG1.2) mAb for 15 min at room temperature and analyzed them with a FACSCalibur™ and CELLQuest™ (BD Biosciences) or FlowJo (Tree Star) software. To monitor proliferation by immunized T cells, splenocytes were labeled with 1 μM CFSE for 10 min at 37°C on day 7 and challenged for 3 d in vitro with 500 μg/ml OVA protein to assess successive halving of CFSE/cell. Cytolytic activity of $CD8^+$ T cells in vivo was tested with a 1:1 mix of spleen cells, labeled $CSFE^{high}$, and pulsed with $OVA_{257-264}$; unpulsed cells were labeled $CFSE^{low}$. After washing, the cells were coinjected i.v. to immunized B6 mice. 16 h later, we assessed a selective loss of peptide-positive $CFSE^{high}$ cells in spleen by FACS®.

DC Stimulation of the Mixed Leukocyte Reaction (MLR). Spleen $CD11c^+$ DCs were isolated 8 h after administration of α-GalCer or vehicle. Graded numbers of C57BL/6 DCs were irradiated, added to 2×10^5 allogeneic BALB/c or syngeneic (C57BL/6) T cells, and isolated using T cell enrichment columns (R&D Systems) in 96-well flat-bottom plates for 88 h. During the final 16 h, [^{3}H]thymidine (1 μCi/well) was added. In some experiments, DCs were fixed with 0.75% paraformaldehyde (Electron Microscopy Science) for 30 min on ice before coculture with T cells.

Online Supplemental Material. Six figures comprise this paper's online supplemental material. In Fig. S1, cytokine production by splenic DCs after i.v. α-GalCer is shown. Fig. S2 depicts the weak contribution of individual inflammatory cytokines in the α-GalCer–induced remodeling of the DC surface. Fig. S3 shows the role of CD40L in the response to α-GalCer. In Fig. S4, the critical role of a bone marrow–derived cell in presentation of OVA associated with injected $TAP^{-/-}$ splenocytes is indicated. Fig. S5 shows that IL-12p40 is not required to induce OVA-specific immunity by DCs maturing to α-GalCer. Fig. S6 contains a summary diagram of the observed responses to a single i.v. dose of α-GalCer. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20040317/DC1.

Results

Cytokine Production by DCs after i.v. Injection of α-GalCer. To account for the rapid maturation of most DCs in the spleens of mice given a single dose of α-GalCer, we assessed the production of inflammatory cytokines because these

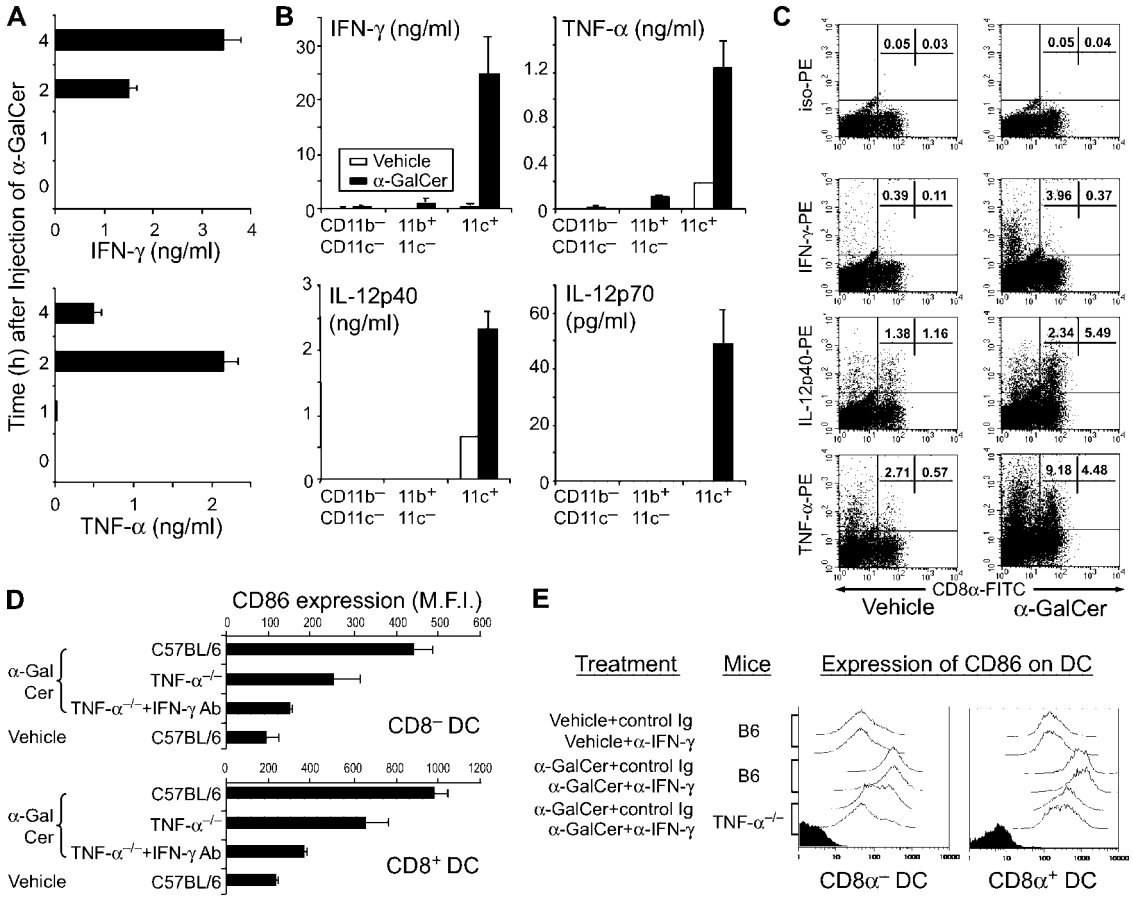

Figure 1. Cytokine production by splenic DCs in response to i.v. α-GalCer (A) Serum IFN-γ and TNF-α levels 1, 2, and 4 h after mice received 2 μg α-GalCer i.v.; no cytokines were detected in mice given vehicle only. (B) Production of cytokines by the indicated subsets (x axis) of splenocytes isolated 2 h after vehicle or α-GalCer i.v., which is before the time that NK cells are activated. (C) FACS® for intracellular cytokines produced by splenic $CD11c^+$ DCs taken from mice 2 h after injection of vehicle or α-GalCer and cultured for 4 h in brefeldin A; numbers are the percentage of $CD8^+$ and $CD8^-$ $CD11c^+$ cells producing cytokines. (D and E) Maturation of splenic DCs, as assessed by CD86 expression, after blockade of both TNF-α and IFN-γ. Vehicle or α-GalCer was given to wild-type or $TNF\text{-}\alpha^{-/-}$ mice treated with α-IFN-γ antibody or control Ig (300 μg/mouse) 3 h before injection of α-GalCer. Mean values for CD86 expression are shown for three experiments in four groups of mice. All results represent the mean of three or more independent experiments (A, B, and D) or are representative (C and E).

drive DC differentiation (31–33). Both TNF-α and IFN-γ were detected in the serum 2–4 h after α-GalCer, but not in mice given vehicle control (Fig. 1 A). To look for the cellular sources of cytokines, we prepared different cell populations 2, 8, and 24 h after i.v. α-GalCer and cultured the cells without known stimuli for 24 h (in an earlier analysis, we cultured the DCs with anti-CD40 for 72 h; reference 15). IFN-γ, TNF-α, IL-12p40, and IL-12p70 were each released in substantial amounts (ELISA assay), but only by enriched $CD11c^+$ DCs and not by enriched $CD11b^+$ $CD11c^-$ macrophages and $CD11b^-$ $CD11c^-$ lymphocytes (Fig. 1 B). We did not see a contribution of NKT cells in our assays because IL-4 was not detected in four out of four experiments. Also, it is known that IFN-γ–producing NKT cells are evident at 2 h, but not 5 h, after α-GalCer (34). DC cytokine production was also evident by intracellular cytokine staining, permitting analyses of the contribution of $CD3^-$ $CD11c^+$ DC subsets (Fig. 1 C). Interestingly, $CD8\alpha^-$ DCs were the principal source of IFN-γ and $CD8\alpha^+$ DCs for IL-12p40 as in other studies (35), whereas both subsets made TNF-α (Fig. 1 C). Cytokine production by FACS® was increased if the DCs were assayed after longer culture periods (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20040317/DC1). IFN-α, which can mature DCs, was not detectably made by splenic $CD8\alpha^+$ or $CD8^-$ $CD11c^+$ DCs (unpublished data). Also, when plasmacytoid DCs were sorted at 2, 4, and 6 h after α-GalCer administration, we detected no IFN-α and lower IL-12p70 and p40 (<100 pg/ml) in the supernatants. Thus, nonplasmacytoid DC subsets act in concert to produce inflammatory cytokines and seem to be their major source early after i.v. α-GalCer.

Cytokines Drive Increased Costimulatory Molecules on DCs Maturing in Response to α-GalCer. To determine the consequences of TNF-α and IFN-γ on DC maturation, we analyzed the DC surface from $TNF\text{-}\alpha^{-/-}$, $IFN\text{-}\gamma^{-/-}$, and $IFN\text{-}\gamma\ R^{-/-}$ mice 4–8 h after α-GalCer. CD40, CD80, and CD86 increased in the individual knockout mice much like wild type, although there was slightly less up-regulation of CD86 in $TNF\text{-}\alpha^{-/-}$ knockouts (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20040317/DC1). In contrast, when we blocked both cytokines by injecting a neutralizing α-IFN-γ antibody (300 μg/mouse) into $TNF\text{-}\alpha^{-/-}$ mice, DC surface maturation was almost completely abrogated (Fig. 1, D and E). Because B cells up-regulate their surface markers in α-GalCer–treated mice (15, 36), we tested if B cells contributed to DC maturation. However, DCs from B cell–deficient $J_H^{-/-}$ mice responded comparably to wild-type α-GalCer (unpublished data). Thus, TNF-α and IFN-γ are largely responsible for the changes in the DC surface induced by α-GalCer.

The Response of Splenic DCs to Cytokine Administration In Vivo. To further assess the role of cytokines, we gave mice TNF-α and IFN-γ i.v. and examined the DCs 4–8 h later. At sufficiently high doses, we observed an up-regulation of costimulatory molecules, but the increases were smaller than induced by α-GalCer, especially on the $CD8^+$ DC subset (Fig. 2 A, left). TNF-α was primarily active because IFN-γ only had a modest effect and only on $CD8^+$ DCs (Fig. 2 A, right). To test if the cytokine-stimulated DCs were immunogenic, we administered OVA as a protein antigen. Instead of free OVA, where i.v. milligram doses are required for capture by splenic DCs, we used OVA associated with osmotically shocked splenocytes because submicrogram amounts of cell-associated OVA are presented by splenic DCs (7, 8). The injected splenocytes were always from $TAP^{-/-}$ mice, so that the recipient DCs, which capture the injected cells within 30–60 min, were responsible for OVA presentation on MHC class I (7, 8). 7 d later, spleen cells were cultured for 6 h without or with MHC class I and II binding peptides, $OVA_{257\text{-}264}$, or $OVA_{323\text{-}339}$ in the presence of brefeldin A to

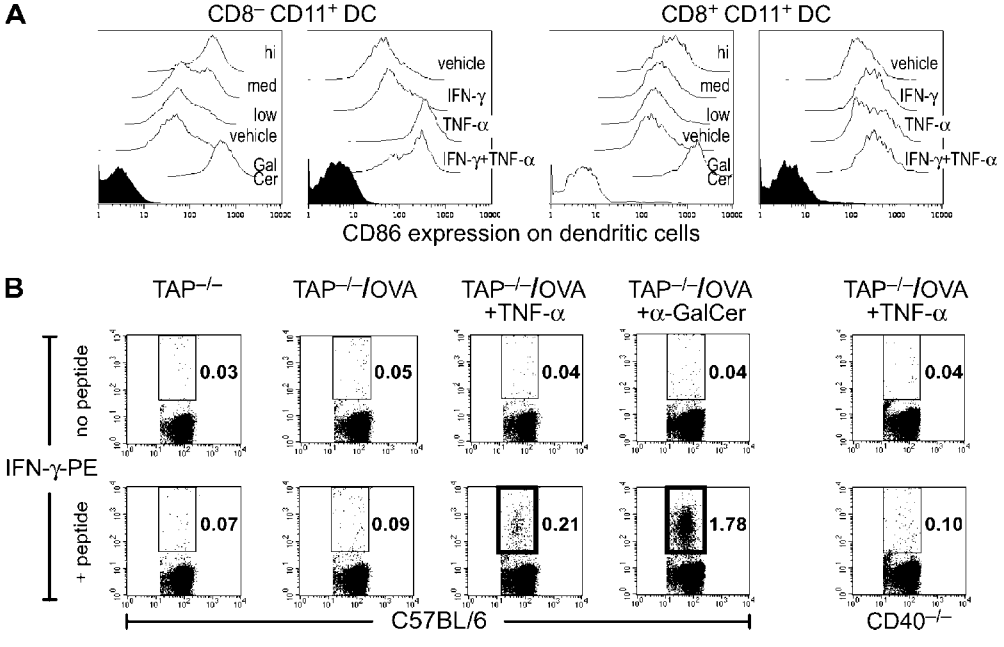

Figure 2. Function of DCs in response to IFN-γ and TNF-α. (A) Up-regulation of CD86 expression on splenic DC subsets 4 h after administration of a single low (20 ng IFN-γ and 2 ng TNF-α/mouse), medium (200 ng IFN-γ and 20 ng TNF-α), or high (2 μg IFN-γ and 200 ng TNF-α) dose of TNF-α (left) or a combination of high dose TNF-α and IFN-γ (right). (B) Induction of some $CD8^+$ T cell immunity when OVA-loaded $TAP^{-/-}$ splenocytes were given to mice followed 2 h later by TNF-α (inset). Immunity was monitored at 7 d by formation of IFN-γ–secreting, OVA-specific $CD8^+$ T cells.

quantify newly formed, IFN-γ–producing $CD8^+$ and $CD4^+$ "effector" T cells by intracellular cytokine staining.

We found a lack of OVA-specific immunity in mice given TNF-α, IFN-γ, or both cytokines together with OVA-loaded splenocytes (not depicted), in spite of the DC up-regulation of CD80/86 (Fig. 2 A). We were concerned that the TNF-α stimulus may have diminished DC uptake and/or processing of OVA, so we did the experiment another way, giving the OVA 2 h before the TNF-α. At that point, some T cell immunity developed. At 1 wk, ~0.2% of the $CD8^+$ T cells could secrete IFN-γ (Fig. 2 B, inset). However, if we gave OVA-loaded splenocytes followed by TNF-α to $CD40^{-/-}$ mice, a T cell response did not develop (Fig. 2 B), even though CD80/86 costimulatory molecules were elevated comparably to wild-type mice. These data indicated that CD80/86 up-regulation by cytokines did not lead to immunity per se; a CD40-dependent step was needed.

CD40 on Maturing DCs, Activated by CD40L, Is Critical for Immunogenicity Initiated by α-GalCer and NKT Cells. We returned to the basic α-GalCer model and assessed the effects of CD40 ablation on immunogenicity. It is known that activation of NKT cells is intact in $CD40^{-/-}$ mice given α-GalCer, using CD1d/α-GalCer tetramers to identify NKT cells producing IFN-γ and IL-4 (37). However, we noted a major requirement of CD40 for $CD8^+$ and $CD4^+$ T cell immunity because $CD40^{-/-}$ mice failed to generate responses to the coadministered OVA (Fig. 3 A). Mean values of three such tests are shown in Fig. 3 B. To establish that CD40 was operating at the level of the DCs, we used CD11c selection to isolate these cells from immunized wild-type and $CD40^{-/-}$ mice and transferred the DCs into naive mice that were not given additional OVA or α-GalCer. $CD11c^+$ DCs from α-GalCer–treated mice could initiate immunity when transferred to naive mice as reported previously (15), but DCs from $CD40^{-/-}$ mice were inactive (Fig. 3 C). This implied that CD40L was essential for full DC maturation. In fact, we found a 90% drop in the response to OVA plus α-GalCer in $CD40L^{-/-}$ mice (Fig. S3 A, available at http://www.jem.org/cgi/content/full/jem.20040317/DC1).

We confirmed the data (38) that 2 h after administration of α-GalCer, CD40L increases on some NKT cells (gated as $CD3^+$ $NKT1.1^+$ lymphocytes), and that this increased CD40L occurred in mice lacking CD40 or subject to cytokine blockade (Fig. S3 B). To test if CD40L on OVA-activated $CD4^+$ T cells might play a role in $CD8^+$ T cell priming, we used $CD4^{-/-}$ mice. When wild-type mice were given cell-associated OVA and α-GalCer, and 4 h later, DCs from these mice were obtained and transferred to naive $CD4^{-/-}$ mice (thus lacking a $CD4^+$ T cell source of CD40L), we observed OVA-specific $CD8^+$ T cell priming 1 wk later. These results suggest that an early CD40L–CD40 interaction, presumably from NKT cells interacting with DCs presenting glycolipids, critically allows immunity to be induced by maturing DCs.

We repeated the immunogenicity experiments using more criteria for T cell immunity, and we compared cytokine-

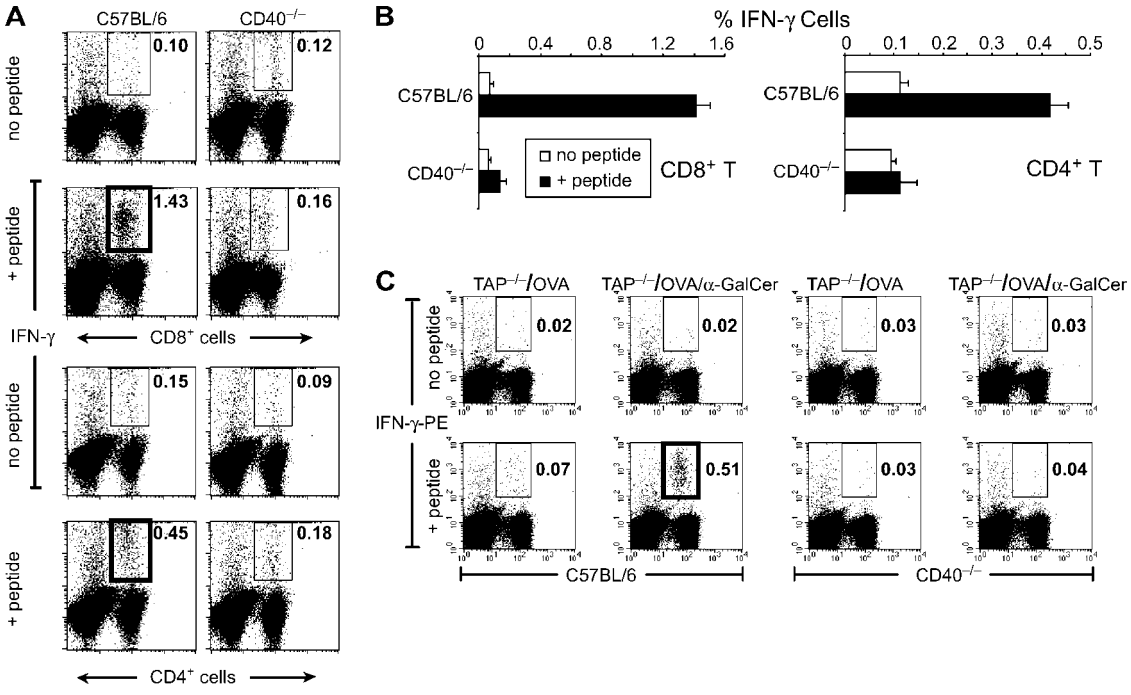

Figure 3. CD40 is required for the immunogenicity of DCs maturing in response to α-GalCer. (A) $CD40^{-/-}$ or wild-type B6 mice were primed with 2×10^7 OVA-loaded $TAP^{-/-}$ spleen cells plus i.v. α-GalCer 7 d later. Spleen cells were evaluated for the production of IFN-γ in response to restimulation by MHC class I ($CD8^+$ T)– or MHC class II ($CD4^+$ T)–binding OVA peptides. Insets indicate newly formed $CD8^+$ and $CD4^+$ T cells that produced IFN-γ upon OVA peptide reexposure. (B) As in A, except that the mean values for three experiments are shown. (C) Immunization of naive mice with 10^6 $CD11c^+$ DCs taken from wild-type or $CD40^{-/-}$ mice 4 h after administration of α-GalCer together with $TAP^{-/-}$ OVA-loaded splenocytes. Immunity was monitored 7 d later by enumerating OVA-responsive $CD4^+$ and $CD8^+$ T cells as in A.

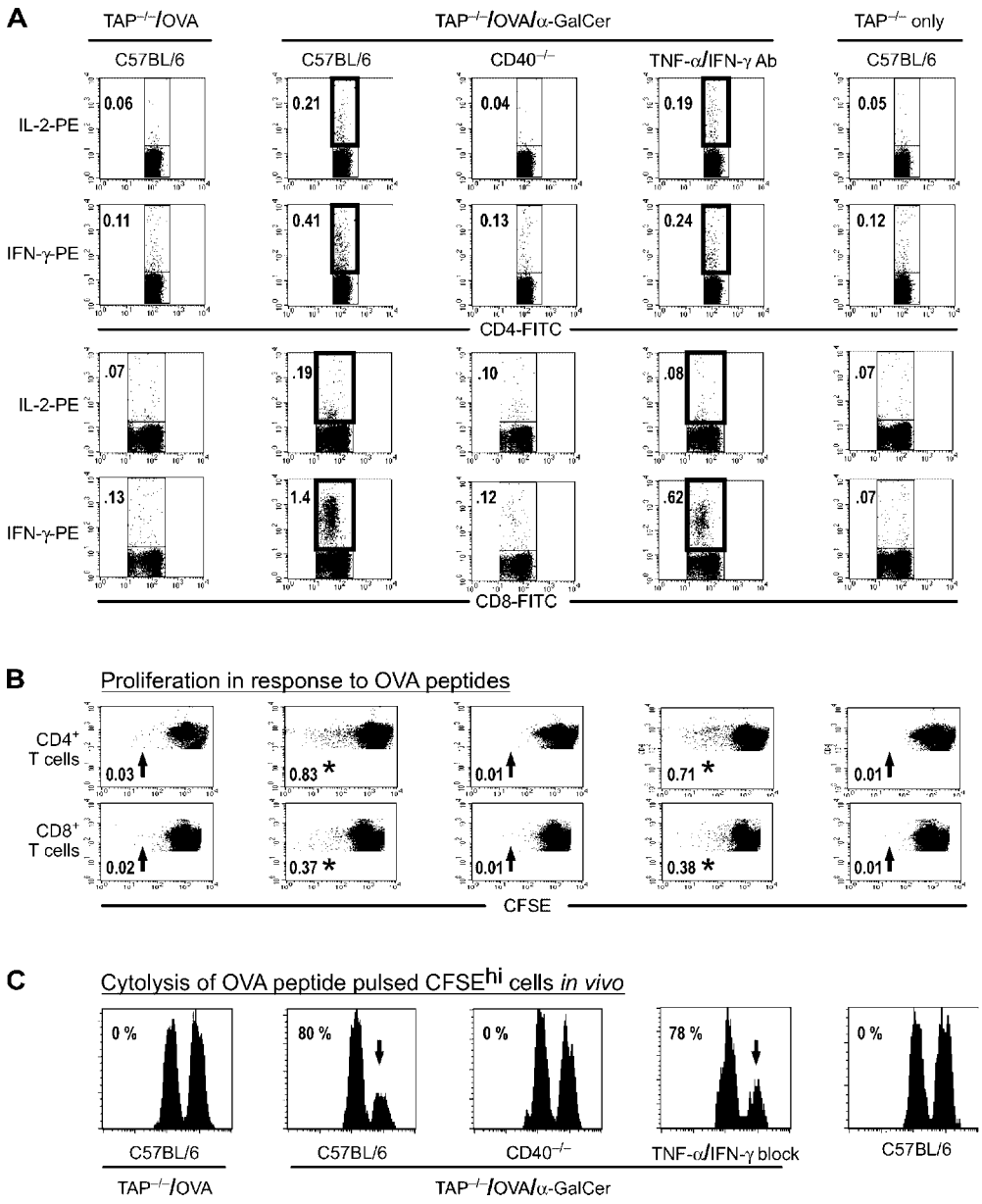

Figure 4. Several forms of $CD4^+$ and $CD8^+$ T cell priming are blocked in mice lacking CD40, but occur substantially during blockade of TNF-α and IFN-γ (mouse groups defined in the top row of labels). (A) As in Fig. 3 A, mice were immunized with 2×10^7 OVA-loaded $TAP^{-/-}$ spleen cells and i.v. α-GalCer, and 7 d later, antigen (OVA peptide)-responsive, cytokine (IL-2 and IFN-γ)-producing T cells were monitored in spleen and expressed as a percentage of $CD4^+$ (top) or $CD8^+$ (bottom) T cells (insets). (B) As in A, but recall proliferative responses (*) were used to monitor T cell priming. CFSE-labeled spleen cells from the different groups of mice were challenged during 3 d of culture with 500 μg/ml OVA protein. (C) As in A, but the development of cytolytic T cells was monitored 7 d after immunization. The groups of mice were given a mixture of CFSE spleen cells, 10^7 each loaded with $OVA_{257-264}$ peptide (high CFSE) or not (low CFSE). 12 h later, killing of antigen-loaded spleen cells was detected (arrow) in wild-type mice and in $TNF^{-/-}$ mice treated with anti–IFN-γ antibody, but not in $CD40^{-/-}$ mice. Data are representative of three individual mice.

blocked and $CD40^{-/-}$ mice in parallel (Fig. 4). First, the $CD40^{-/-}$ mice failed to prime for IL-2 and IFN-γ–producing $CD4^+$ and $CD8^+$ T cells (Fig. 4 A, middle), whereas cytokine blockade only had a partial inhibitory effect (Fig. 4 A, insets). Second, the $CD40^{-/-}$ mice failed to prime for T cell proliferative responses in response to OVA restimulation, whereas cytokine blockade had no inhibitory effect (Fig. 4 B; compare arrows with asterisks). We used CFSE dilution to monitor T cell proliferation because this is a more sensitive method than [^{3}H]thymidine uptake and is associated with little or no background (dilution of CFSE in the absence of antigen). Third, $CD40^{-/-}$ mice failed to generate $CD8^+$ cytolytic effector cells in vivo, whereas cytokine blockade did not detectably alter killer activity relative to control mice (Fig. 4 C, arrows). These results indicate that CD40 ligation is vital for initiating several manifestations of T cell–mediated immunity.

CD40 Is Not Required for the Development of Signal One and Signal Two on DCs Responding to α-GalCer In Vivo. Because CD40 ligation increases MHC class I–peptide complex formation in bone marrow derived DCs (23, 24), we assessed if DCs in $CD40^{-/-}$ mice were able to present OVA. To do this, CFSE-labeled, OVA-specific $CD8^+$ and $CD4^+$ TCR transgenic T cells were injected separately. 18 h later, OVA-loaded dying splenocytes were injected i.v. without or with α-GalCer. First, we verified the need for bone marrow–derived cells in the presentation of cell-associated protein because it has been reported that nonhematopoietic, liver sinusoidal endothelial cells were also able to present OVA on MHC class I by a TAP-dependent pathway (39). However, using bone marrow chimeras, we confirmed analogous experiments (40), finding that hematopoietic cells were essential for presentation of $TAP^{-/-}$

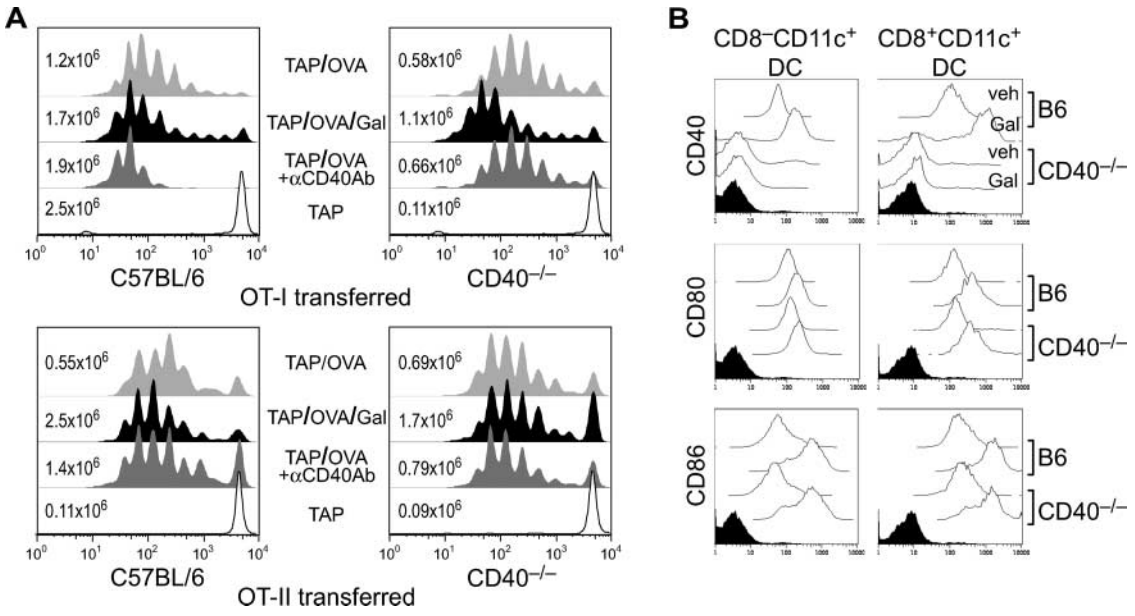

Figure 5. CD40 ablation does not interfere with efficient DC expression of signal one (antigen presentation) and signal two (CD80/86 costimulation). (A) Presentation of cell-associated OVA to OT-I, $CD8^+$, and OT-II, $CD4^+$ TCR transgenic T cells in wild-type C57BL/6 or $CD40^{-/-}$ mice in the absence or presence of α-GalCer. Total numbers of transgenic T cells (the mean of two experiments) are shown in each panel. (B) As in Fig. 1 E, 8 h after i.v. α-GalCer to C57BL/6 or $CD40^{-/-}$ mice, the maturation of spleen $CD11c^+$ DC subsets was assessed at the level of three surface markers.

OVA splenocytes (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20040317/DC1), presumably DCs as described previously (8). When we compared presentation of cell-associated OVA to TCR transgenic T cells transferred to wild-type and $CD40^{-/-}$ mice, in the absence or presence of α-GalCer, the proliferation of $CD8^+$ and $CD4^+$ T cells was identical, as indicated by CFSE dilution and the increase in total T cell numbers in the spleen (Fig. 5 A). Likewise, we found that $CD40^{-/-}$ (Fig. 5 B) and $CD40L^{-/-}$ (Fig. S3 C) mice still up-regulated CD80 and CD86 comparably to wild type upon challenge with α-GalCer. In addition, $CD40^{-/-}$ mice had similar numbers of splenic DCs relative to wild type, with or without stimulation by α-GalCer. These results indicate that CD40 and CD40L are not required for splenic DCs to present antigen and express CD80 and 86 costimulators;

Table I. *Cytokine Production Requirements for Dendritic Cells from Mice Given α-GalCer*

	Cytokine release from $CD11c^+$ splenic DCs			
	IFN-γ	TNF-α	IL-12p40	IL-12p70
	ng/ml	*pg/ml*	*pg/ml*	*pg/ml*
C57BL/6, vehicle	0.7 ± 0.4	119 ± 9.6	693 ± 71	0
α-GalCer treated				
C57BL/6	23 ± 0.8[a]	1,057 ± 77[a]	2,338 ± 124[a]	52 ± 4.1[a]
TNF-α/IFN-γ block	4.0 ± 1.0[b]	0	966 ± 50[b]	0
$CD40^{-/-}$	13 ± 1.4[a]	306 ± 15[b]	1,074 ± 174[b]	0
$CD80/CD86^{-/-}$	17 ± 3.5[a]	679 ± 1[b]	1,147 ± 172[b]	27 ± 12[b]
$J\alpha18^{-/-}$	0.4 ± 0.2	86 ± 9[c]	772 ± 103[c]	0[c]

2 h after giving mice vehicle or α-GalCer, $CD11c^+$ DCs were selected from the spleens and cultured for 24 h. Supernatants were analyzed by ELISA for secretion of the indicated cytokines. Data are representative of four independent experiments. The p-value of the groups relative to the control value was determined by a Student's *t* test.

[a]$P < 0.005$.

[b]$P < 0.05$.

[c]No significant difference from C57BL/6 vehicle control ($P > 0.05$).

i.e., signals one and two in the steady state and during activation with α-GalCer and NKT lymphocytes.

Inflammatory Cytokines and CD40 Ligation in Concert Lead to IL-12 p70 Release. We considered the possibility that the need for CD40 in adaptive immunity reflected its role in the production of IL-12 (41). In fact, IL-12p40 and p70 release from $CD11c^+$ splenic DCs was greatly reduced when either inflammatory cytokines (TNF-α, IFN-γ) or CD40 were nullified. In wild-type mice, α-GalCer increased IL-12p40 threefold and induced the active IL-12p70 heterodimer de novo, whereas the cytokine-blocked and $CD40^{-/-}$ mice only increased IL-12p40 by 50% and did not produce any IL-12p70 (Table I, compare second with third and fourth rows). In contrast, substantial production of cytokines including IL-12p70 took place in $CD80/86^{-/-}$ mice. We also verified that NKT cells were needed for α-GalCer to induce cytokine production by DCs (Table I, compare second and bottom rows). We tested if IL-12p40 contributed to immunity in response to α-GalCer plus OVA. However, $IL\text{-}12p40^{-/-}$ mice were fully competent to initiate immunity, both cytokine-producing $CD8^+$ and $CD4^+$ effectors and cytolytic T cells (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20040317/DC1). Together, these data and the results in Fig. 2 indicate that some features of DC maturation in response to α-GalCer are dependent on the production of TNF-α and IFN-γ (i.e., the up-regulation of costimulatory molecules [Fig. 1] and production of IL-12p70 [Table I]), but neither leads directly to T cell–mediated immunity.

The Need for CD80 and CD86 Costimulatory Molecules. Although our data showed that the marked CD80/86 up-regulation during DC maturation did not initiate immunity independently, we wanted to know if these costimulators were required, perhaps at the lower but significant levels observed on DCs in lymphoid tissues. Therefore, we extended our studies to $CD80/86^{-/-}$ mice. Interestingly, DCs from these mice, such as $CD40^{-/-}$ mice, could remodel their surface in response to α-GalCer, as shown by the up-regulation of the endocytic receptor DEC-205 (CD205) and down-regulation of the IFN-γ receptor (CD119; Fig. 6 A, gray). Nevertheless, $CD80/86^{-/-}$ mice were unable to initiate an immune response to α-GalCer and OVA (Fig. 6 B). Therefore, the immunogenic properties of DCs maturing to α-GalCer requires at least basal levels of CD80 and CD86.

Stimulation of the MLR by Maturing DCs Also Requires Both Cytokines and CD40. Another assay for the capacity of maturing DCs to activate resting T cells is MLR stimulation. Splenic DCs from α-GalCer–treated mice have greatly increased MLR stimulatory activity (15). $CD11c^+$ DCs from $IFN\text{-}\gamma^{-/-}$ mice were comparable MLR stimulators to those from α-GalCer treated wild-type mice, whereas the DCs from $TNF\text{-}\alpha^{-/-}$ mice were slightly less active (Fig. 7 A, left). Because DCs could undergo additional maturation during the MLR assay itself, we tested cells that were inhibited by

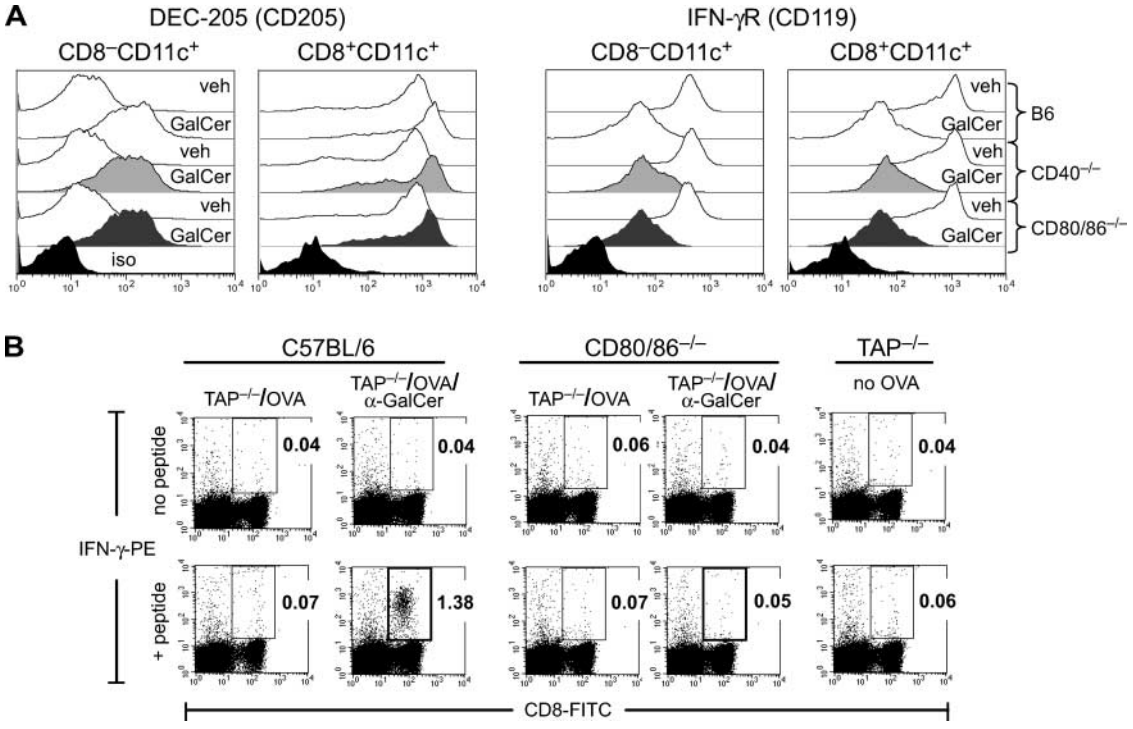

Figure 6. Requirement for CD80/86 for the adjuvant action of α-GalCer. (A) As in Fig. 1 E, 8 h after i.v. administration of α-GalCer to C57BL/6, $CD40^{-/-}$, or $CD80/86^{-/-}$ mice, the maturation of spleen $CD11c^+$ DC subsets was assessed at the level of surface markers using α-CD205 and α-CD119 antibodies. (B) As in Fig. 3, $CD80/86^{-/-}$ or wild-type B6 mice were primed with a combination of 2×10^7 OVA-loaded $TAP^{-/-}$ spleen cells and i.v. α-GalCer. 7 d later, spleen cells were evaluated for the production of IFN-γ in response to restimulation by MHC class I ($CD8^+$ T cells)– or MHC class II ($CD4^+$ T cells)–binding OVA peptides. The results (insets) expressed as the percentage of $CD4^+$ or $CD8^+$ T cells producing IFN-γ, are representative of four individual mice.

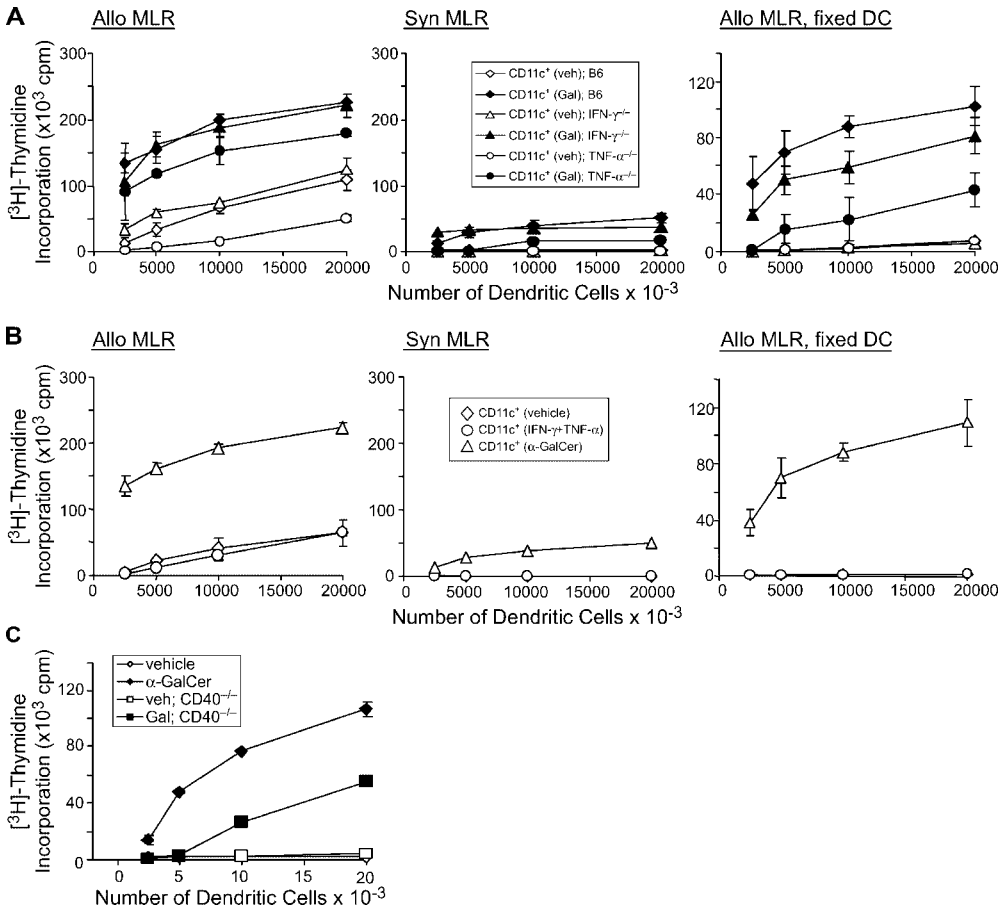

Figure 7. Cytokine and CD40 requirements for increased MLR stimulatory activity. (A) 8 h after i.v. administration of α-GalCer, $CD11c^+$ DCs were used to stimulate T cell proliferation (y axis) in the MLR. Graded numbers of spleen DCs from wild-type B6, TNF-$\alpha^{-/-}$, or IFN-$\gamma^{-/-}$ mice were irradiated (30 Gy) and added for 3 d in flat bottomed 96-well plates to 2×10^5 allogeneic BALB/c T cells (Allo MLR, left) and C57BL/6 T cells (Syn MLR, middle). To block DC maturation in the T cell coculture, the DCs were fixed with paraformaldehyde for 30 min and irradiated followed by the addition to allogeneic T cells (right). T cell proliferation was measured by [^{3}H]thymidine incorporation; representative results of three independent experiments are shown. (B) As in A, DCs from mice given TNF-α and IFN-γ i.v. were fixed with paraformaldehyde for 30 min and irradiated followed by the addition of allogeneic T cells, and [^{3}H]thymidine uptake was measured at 88–96 h. (C) As in A, DCs from α-GalCer–treated wild-type and $CD40^{-/-}$ mice were fixed, irradiated, and used to stimulate the MLR.

paraformaldehyde fixation immediately upon isolation. The splenic $CD11c^+$ DCs from TNF-$\alpha^{-/-}$ mice now showed less MLR stimulatory activity ($P < 0.05$; Fig. 7 A, right). When DCs were examined from TNF-$\alpha^{-/-}$ mice that also received α-IFN-γ antibody, there was no further reduction in MLR stimulation in response to α-GalCer (unpublished data). In contrast, when we tested DCs from mice injected with TNF-α and/or IFN-γ, no MLR stimulation was observed, in contrast with the strong MLR induced by DCs from α-GalCer–treated mice (Fig. 7 B). This implied that other factors, in addition to cytokines and CD80/86 up-regulation, were required for increased MLR stimulation by DCs. Again, CD40 was playing a role because MLR stimulation was reduced in its absence (Fig. 7 C). Therefore, the induction of immunity by DCs, as assessed by stimulation of the MLR, also requires cytokines and CD40 in concert.

Discussion

The α-GalCer Model for DC Maturation In Vivo. α-GalCer was discovered to be an adjuvant for protective T cell immunity with an important antigen, irradiated malaria sporozoites (19). The antigenic system we chose to study involved a single intravenous injection of OVA-loaded dying splenocytes (7, 8). These were rapidly captured (uptake was evident within 30 min) and presented by a subset of $CD8\alpha^+$ splenic DCs in vivo, the consequence of which is tolerance in the steady state (8). We used OVA-loaded dying cells to model the handling by DCs of dying cells from self tissues, tumors, and infections. When given together with α-GalCer, uptake of a single submicrogram dose of cell-associated OVA elicits combined $CD4^+$ and $CD8^+$ T cell immunity (15, 16), which is long lived and imparts resistance to OVA-transduced tumors for at least 2 mo (unpublished data). To do this, α-GalCer activates innate NKT lymphocytes that are responsible for most of the major features of DC maturation, such as remodeling of the DC surface to express high levels of CD80 and CD86 costimulators and the production of numerous cytokines (Table I and references 15, 16). These consequences of innate NKT lymphocyte responses to α-GalCer parallel what takes place with several microbial ligands for toll-like receptors in vivo (4, 5, 42, 43).

Previous studies with ex vivo–derived and antigen-loaded DCs showed that mature DCs were more immunogenic (32, 44). Likewise, immunity is induced in vivo by administering antigen together with maturation stimuli such as microbial products (5), infection (14), and agonistic α-CD40 antibodies (9, 10, 17). Here, we have used the α-GalCer/NKT model to begin to dissect the components of maturation responsible for the induction of effective T cell–mediated immunity. In contrast, much of the literature on DC maturation from its very beginnings (45–48) has involved surrogate criteria, especially a marked up-regulation of costimulatory molecules and production of cytokines.

Distinct Controls for the Components of DC Function during Immunogenicity In Vivo. There are several components to the function of maturing DCs in initiating immunity, and these proved to be under distinct controls in lymphoid organs (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20040317/DC1). Antigen presentation, or signal one, occurred in the steady state, in the ostensible absence of a maturation stimulus (Fig. 5). Interestingly, bone marrow–derived DCs in culture showed greatly enhanced presentation on MHC class I as a result of CD40 ligation (23, 24). Such up-regulation also might take place in vivo, but our data indicate that DCs within lymphoid organs efficiently formed MHC class I and II–peptide complexes in the steady state (e.g., Fig. 5 A). We found previously that in the steady state, the processing of dying cells resulted in tolerance in vivo. Other studies in vitro concluded that DC maturation (maturation being defined as cell surface remodeling with increased expression of CD86) was required for deletional tolerance (49), although in our studies of cell-associated OVA in vivo, such tolerance occurs in the steady state (8).

The production of many cytokines by splenic DCs did require the α-GalCer/NKT maturation stimulus. There was strong and rapid production of IFN-γ, TNF-α, and IL-12p70 beginning within 2 h of glycolipid injection. We found that these cytokines led to the major surrogate marker for maturation, expression of high levels of CD80 and 86 costimulatory molecules (signal two) on most DCs. CD80 and 86 were clearly required for immunity to a coadministered antigen in the α-GalCer system (Fig. 6). In contrast, NKT cell–dependent cytokine production by DCs occurred to a substantial extent in CD80/86-deficient mice (Table I). However, the greatly increased expression of costimulatory molecules on maturing DCs did not independently link the innate response between DCs and NKT cells to the adaptive response, between DCs and αβT cells; CD40 also was essential. Likewise, when TNF-α was given to mice, or when MLR stimulation was used to assess immune responses to DCs (Figs. 2 and 7), we again observed that inflammation and increased expression of CD80 and 86 did not lead independently to immunity; CD40 ligation played a major role.

CD40L likely was provided by NKT cells early in the DC–NKT interaction initiated by α-GalCer, but CD40L might additionally contribute to the DC–T cell interaction role when expressed by antigen-activated T cells. Interestingly CD40L-bearing $CD4^+$ T cells were not essential for the initiation of OVA-specific $CD8^+$ T cell immunity in our model. Other potential sources of a CD40L signal for DCs would include activated platelets, mast cells, and possibly, heat shock proteins (50, 51). However, at this time, we have not identified a means to selectively remove CD40L from different types of cells, especially NKT cells and TCRαβ T cells, to directly assess the relative importance of each source of CD40L during the different stages of immunogenicity.

A valuable feature of the α-GalCer system was to be able to dissect the consequences of different components of DC maturation within a single system, and in vivo, rather than by comparing different complex stimuli in vitro, as previously was the case. The different stimuli for DC maturation (inflammatory cytokines, CD40 ligands, and microbial stimuli) each induce myriad distinct transcriptional changes, so that it is hard to draw conclusions concerning any one component by comparing cells stimulated in different ways. By dissecting the controls of DC maturation within a single system, we could observe that efficient expression of MHC–peptide complexes and CD86 costimulators did not lead to immunity in the absence of a CD40–CD40L interaction.

Roles for CD40–CD40L in DC Maturation. The effects of CD40–CD40L in the α-GalCer system extend earlier work on this TNF receptor family member. Previous reports had concluded that agonistic α-CD40 antibodies acted through DCs to bypass the need for $CD4^+$ helper cells in $CD8^+$ T cell responses (27–29). The implication of these and other studies (9, 10) was that CD40 was increasing immunity by increasing formation of MHC–peptide complexes and/or increasing expression of costimulatory molecules, either membrane bound like CD80 and CD86, or soluble cytokines including IL-12. The formation of these T cell costimulatory molecules is a well known feature of DCs maturing in response to CD40 ligation (21, 22, 25, 26). CD40 also influences DC survival (52), migration (53), and avoidance of suppression (54, 55). The surprise in the current work was the major dependence of both $CD4^+$ and $CD8^+$ T cell responses on CD40 expression by DCs, under circumstances when active CD40-independent antigen presentation and costimulation were already in place. Identification of the CD40-based changes in DCs should contribute to further understanding of DC function and immunogenicity mechanisms.

A distinctive role for CD40 was envisaged by other studies in which CD40-ligated, bone marrow–derived DCs proved to be better inducers of T cell responses than DCs matured by other stimuli in culture (56, 57). Our results show that CD40 ablation in vivo does not retard antigen presentation and the expression of high levels of costimulators by DCs in lymphoid tissues. Yet CD40 still has a major role in linking innate to adaptive immunity and for both $CD4^+$ and $CD8^+$ T cell responses. These observations suggest changes in the standard signal one–signal two theory as a sufficient mechanism used by DCs to link innate with adaptive immunity.

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