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NKT Cells Provide Help for Dendritic Cell-Dependent Priming of MHC Class I-Restricted CD8\(^+\) T Cells In Vivo\(^1\)

Detlef Stober, Ieva Jomantaite, Reinhold Schirmbeck, and Jörg Reimann\(^2\)

Dendritic cells (DC)\(^3\) are potent APCs for naive T cells. DC prime in vivo MHC class I-restricted CD8\(^+\) CTL responses (1–4). Different human and murine DC subsets can efficiently prime in vitro and in vivo class I-restricted CTL responses with a wide range of specificities. Both CD8\(\alpha\)\(^+\) and CD8\(\alpha\)\(^-\) DC subsets prime CD8\(^+\) T cells in vitro and induce protective antiviral CTL responses in vivo (4–7). In many (but not all) studies on DC-mediated CTL priming in mouse and human, the establishment of a stable CTL immunity is facilitated by CD4\(^+\) T cell “help” (8–12). Highly immunogenic, minor histoincompatible DC fail to prime CTL in MHC class II-deficient mice indicating that even strong alloantigens cannot stimulate CD8\(^+\) T cell responses without help (10). CD4\(^+\) T cell help can 1) activate DC; 2) regulate the longevity of Ag presentation and of the activation status of the APC (on which the establishment of CTL immunity by DC critically depends) (13); 3) facilitate CTL infiltration into target tissues (14); and 4) facilitate in situ delivery of CTL effector functions (15). Help is not essential for establishing or maintaining CTL memory (16–18). Help operates at least partially at the level of the DC. The DC may either form a temporal bridge between CD4\(^+\) helper and CD8\(^+\) T cells thereby facilitating direct T-T interactions (19), or help may condition DC for more effective class I-restricted epitope presentation. Many studies point to CD40/CD40 ligand-dependent signals driving DC maturation (20–22). In addition to CD40-dependent CD4\(^+\) T cell help, CD40-independent DC sensitization events and direct cytokine-dependent CD4\(^+\)/CD8\(^+\) T cell interaction may provide help (23–25). An activation signal for DC is provided by CpG-containing, immune-stimulating oligodeoxynucleotides (ODN) (11, 26, 27).

NKT cells are \(\alpha\beta\) T cells with an invariant TCR and intermediate level NK1 surface expression (in appropriate mouse strains) involved in anti-microbial immunity (28). The CD1d-binding glycolipid \(\alpha\)-galactosyl-ceramide (\(\alpha\)GalCer) stimulates most NKT cells, a specific recognition highly conserved through mammalian evolution (29). A key feature of NKT cells is their prompt cytokine secretion (30). \(\alpha\)GalCer has been used as an adjuvant to facilitate priming of T cell immunity (31), an idea supported by the extensive bystander activation of T cells and NK cells after injection of this glycolipid (32, 33). Murine NKT cells activated in vivo by injecting \(\alpha\)GalCer facilitate priming of either Th2- (34, 35) or Th1-biased immunity (36–41). The interaction of NKT cells with \(\alpha\)GalCer-pulsed DC enhances the capacity of the latter to prime NK cells and CTL (42–45). We tested whether \(\alpha\)GalCer-specific NKT cells can facilitate in vivo CD4\(^+\) T cell-independent priming of CTL responses by DC that present an epitope of the hepatitis B surface Ag (HBsAg) in the context of the class I molecule K\(^\beta\).

Materials and Methods

Mice

C57BL/6J-Bom (B6) mice (H-2\(^b\)), B6 CD1d\(^{-/-}\) (46), B6 A\(\alpha\)-NK1.1\(^{-/-}\) (47), and B6 A\(\alpha\)-NK1.1\(^{-/-}\) (48, 49) mice were bred and maintained under standard pathogen-free conditions in the animal colony of Ulm University (Ulm, Germany). Male and female mice were used at 12–16 wk of age.

Peptides

The 8-mer K\(^\beta\)-binding S\(^{238-245}\) peptide ILSPFLPL of HBsAg (50), the 8-mer K\(^\beta\)-binding OVA\(^{263-271}\) peptide SIINFEKL, and the A\(^\beta\)-binding OVA\(^{233-249}\) peptide ISQAVHAAHAEINAEGR (51) of OVA were synthesized and purified by RP-HPLC by Jerini Biotools (Berlin, Germany). The peptides were stored at a concentration of 10 mg/ml and diluted before use with cell culture medium. OVA was purchased from Sigma-Aldrich (Munich, Germany; catalog no. A5503).

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\(^3\) Abbreviations used in this paper: DC, dendritic cell; BMC, bone marrow cells; HBsAg, hepatitis B surface Ag; \(\alpha\)GalCer, \(\alpha\)-galactosyl-ceramide; \(\beta_2\) m, \(\beta_2\) micro-globulin; NKT, \(\alpha\beta\) T cells expressing NK1.1; FC, flow cytometry.
The in vitro generation of DC from murine bone marrow cells (BMC) has been described (52). Briefly, BMC were depleted of CD44+, CD8−, and B220− lymphocytes and MHC class II+ cells (Ab-conjugated microbeads catalog nos. 130-049-201, 130-049-401, 130-052-401 from Miltenyi Biotec, Bergisch Gladbach, Germany) by MACS following the manufacturer’s instructions. BMC depleted of T cells, B cells, and maturing myeloid cells were cultured at 0.75 × 10^6 cells/ml in 6 ml/well in UltraCulture medium (catalog no. 12-725F; BioWhittaker, Verviers, Belgium), supplemented with 10 ng/ml GM-CSF (catalog no. 315-03; PeproTech, Offenbach, Germany), 2 mM glutamine, and (if not indicated otherwise) 5% v/v FCS (catalog no. A15-649; PAA Laboratories, Linz, Austria). Cultures were incubated at 37°C in humidified air with 5% CO₂. On day 3 and day 5, 50% of the cell culture medium was replaced by fresh, cytokine-supplemented medium. In some groups, DC were preincubated for 16 h with 2 μg/ml ODN (MWG Biotech, Ebersberg, Germany). The surface phenotype and the response to maturation-inducing stimuli of the DC used in this study have been previously described in detail (53, 54).

The in vitro generation of DC from murine bone marrow cells (BMC) has been previously described (53, 54). DC pulsed with antigenic peptide for 2–37 h with antigenic peptide were found in preliminary experiments to optimally prime CTL responses. DC (5 × 10^6 cells/ml) suspended in GM-CSF-containing medium were pulsed for 2 h at 37°C with 40 μg/ml antigenic peptide. DC harvested from serum-free cultures were preincubated with 1 μg/ml human recombinant β₂-microglobulin (β₂-m, m) for 2 h before the peptide pulse, as this enhances class I-restricted presentation of pulsed DC (54). Thereafter, cells were washed before adoptive transfer. In some groups, DC were copulsed with 2 μg/ml αGalCer (kindly provided by Dr. Y. Koezuka; Kirin Brewery, Pharmaceutical Research Laboratory, Gunma, Japan). DC pulsed with 20–40 μg/ml peptide were found in preliminary experiments to optimally prime CTL responses.

CTL lines

CTL lines were generated by repeated in vitro stimulation of splenocytes from HBsAg-immune B6 mice with peptide-pulsed, irradiated RBL5 cells in the presence of 30 U/ml recombinant human IL-2 (54). Ag-pulsed or nonpulsed DC (5 × 10^6/well) from B6 or Aa−/− mice were cocultured with CTL (5 × 10^5/well) for 24 h in 96-well U-bottom plates. Culture supernatants were harvested and analyzed for IFN-γ by ELISA.

Isolation of NKT cells

CD4+ NKT cells were isolated from spleens of Aβ−/− mice by MACS using anti-mouse CD4-microbeads (catalog no. 130-049-01; Miltenyi Biotec) following the manufacturer’s instructions. The purity of the obtained NKT cell population assessed by flow cytometry (FCM) was >95%.

FCM analyses

Cells were suspended in PBS supplemented with 0.3% w/v BSA and 0.1% w/v sodium azide. Nonspecific binding of Abs to FcR was blocked by preincubating cells with 1 μg/10^6 cells of the anti-CD16/CD32 mAb 2.4G2 (BD Biosciences; catalog no. 01240D). Cells were incubated with 0.5 μg mAb per 10^6 cells for 30 min at 4°C and were washed twice. Cells stained with biotinylated Abs were incubated for 10 min at 4°C with a second-step reagent, washed twice, and analyzed using a FACScan (BD Biosciences, Heidelberg, Germany). Dead cells were excluded by propidium iodide staining. The following anti-murine mAbs (BD Biosciences) were used: FITC-conjugated anti-CD3 (catalog no. 1084D), PE-conjugated anti-CD11c (catalog no. 09705A), anti-K(B) (catalog no. 06105A), anti-A(B) (catalog no. 06045A), anti-CD1d (catalog no. 09905A), anti-CD80 (catalog no. 09605B), biotinylated anti-CD40 (catalog no. 09622D), and streptavidin-Red670 (catalog no. 19543-024; Life Technologies, Eggenstein, Germany).

Adoptive DC transfer

DC (4 × 10^5/mouse in 100 μl) were s.c. injected into the base of the tail. In some experiments, 30 μg ODN were coinjected.

In vivo suppression of CD4+ T cells

CD4+ T cells were suppressed in mice by repeated injections of anti-CD4 mAb (clone, YTS 191.1) as described (11). One day before, at the time of, and 2, 5, and 8 days after the DC transfer, mice were i.p. injected with 200

**FIGURE 1.** DC generated from normal or MHC class II-deficient (Aa−/−) C57BL/6 (B6) mice. A. Surface marker profile by DC. CD11c+ DC from normal (B6) or Aa−/− mice were purified from day 7 cultures and stained with Abs to MHC class II (Aa), CD80, MHC class I (K(B)), CD1d, or CD40. B. Cytokine release by DC from normal or Aa−/− B6 mice. Purified CD11c+ DC from normal or Aa−/− B6 mice were stimulated for 24 h with 4 μg/ml ODN, or CD40L-expressing J558/CD40L transfectants. TNF-α- and IL-12 p40-release into the supernatant was determined by ELISA. C. Stimulation IFN-γ-release of K(B)/SPFLP (ILSPFLPL)-specific CTL by pulsed DC from normal or Aa−/− B6 mice. Purified CD11c+ DC from normal or Aa−/− B6 mice were pulsed with antigenic peptide for 2 h at 37°C, washed and cocultured with CTL for 24 h. IFN-γ in the supernatants was determined by ELISA. Mean values ± SD of triplicates are shown.
μl PBS containing 100 μg Ab. FCM analyses of PBMC and splenic mononuclear cell populations demonstrated that >99% of the CD4⁺ T cells were depleted 4 days after the last injection, and only 5–6% of the CD4⁺ T cell population reappeared within 2 wk after the last injection.

In vivo suppression of NK cells

NK cells were eliminated during CTL priming by injecting 30 μl α-asialoGM1 antiserum (catalog no. 986-10001; Wako Chemicals, Neuss, Germany) i.p. 3 days before and at the day of adoptive DC transfer. FCM analyses of spleen cell populations demonstrated that >92% of the DX5⁺ NK cells were depleted 48 h after the last injection, and >80% of the NK cells were depleted 4 days after the last injection, which confirmed our previously published data (45).

Cytokine detection by ELISA

Cytokines were detected in supernatants by double-sandwich ELISA. Cytokine detection by ELISA

FIGURE 2. Priming CTL responses in vivo by adoptive transfer of pulsed DC. A. Purified CD11c⁺ DC from normal or MHC class II-deficient (Aec⁻⁻) B6 mice were pulsed for 2 h at 37°C with the K⁺-binding S₂₃₀₋₂₁₅ peptide ILSPFLPL of HBsAg and washed. Nonpulsed or pulsed DC were injected s.c. (4 × 10⁵ cells per mouse) into naive, normal B6 mice. Groups marked with * differ, p < 0.01 (unpaired t test). B. Kinetics of appearance of specific CTL in the spleen after s.c. injection of peptide-pulsed DC. DC prepared as described in A were injected s.c., and the appearance of specific CTL in the spleen was determined at day 7, 9, 12, and 15 posttransfer. DC were pulsed either with peptide only or with peptide plus αGalCer (as described for Fig. 6). The data points represent two mice per time point (± SD).

FIGURE 3. Priming CTL by adoptive DC transfer is help-dependent. A. DC were generated in 7-day cultures of BMC precursors in medium either supplemented with 5% v/v FCS, or serum-free. Cells were harvested, pulsed, and transferred. DC generated under serum-free conditions were incubated with 1 μg/ml human recombinant β₂₉ m, washed, and subsequently pulsed with the antigenic peptide for 2 h to facilitate class I-restricted presentation. The preincubation with exogenous β₂₉ m, B. Specific CD4⁺ T cell help in the priming of CTL responses by adoptively transferred DC. Purified DC were pulsed with the K⁺-binding antigenic peptide either alone, or together with either the A₂⁻⁻ binding OVA₁₂₃₋₁₃₉ peptide, ISQAVHAAHAEINEAGR, or purified OVA. Cells were washed and transferred. On day 14 posttransfer, spleen cells were obtained and stimulated (in the presence of brefeldin A) for 6 h with the antigenic K⁺-binding peptide. Specifically inducible IFN-γ⁺ CD₈⁺ T cells were detected by FCM. Mean numbers of splenic IFN-γ⁺ CD₈⁺ T cells per 10⁵ CD₈⁺ T cells ± SD of three mice per group are given. Groups marked with * differ, p < 0.01 (unpaired t test).
Cytotoxic assay
In vitro expansion of CTL and detection of their specific cytotoxicity was performed as described (55). Briefly, 3 × 10^5 responder spleen cells and 1 × 10^6 peptide-pulsed, irradiated stimulator RBL5 cells were cocultured in 10 ml for 5 days at 37°C in humidified air with 5% CO2. Specific cytotoxicity was tested in a 3Cr-release assay. CTL were harvested and washed, and serial dilutions of effector cells were cocultured with 2 × 10^5 3Cr-labeled, peptide-pulsed targets in 200 μl round bottom wells. After a 4-h incubation at 37°C, 50 μl supernatant were collected for gamma-radiation counting. The percentage of specific lysis was calculated as [(experimental release – spontaneous release)/(total counts – spontaneous release)] × 100. Total counts were measured by resuspending target cells. Spontaneous release was always <15% of the total counts. Data shown are the mean of triplicate values.

Results
MHC class II-deficient and MHC class II-competent, bone marrow-derived DC
An established protocol was used to generate DC in serum-free or serum-supplemented cultures with GM-CSF from immature BMC precursors from either normal B6 mice or MHC class II-deficient Aβ^-/- (or Aα^-/-) B6 mice (52, 53). CD11c^+ DC generated from either MHC class II-deficient or MHC class II-competent precursors expressed a similar surface marker profile. These DC expressed MHC class I (K^b) molecules, CD80, CD40, and CD1d molecules on the surface (Fig. 1A). As expected, DC from normal, but not MHC class II (A^b)-deficient mice expressed MHC class II molecules on the surface (Fig. 1A). We further tested “spontaneous” and inducible release of cytokines by DC generated from normal or MHC class II-deficient mice. Both DC populations released IL-12 p40 and TNF-α after CD40 ligation or stimulation with immune-stimulating ODN, but not spontaneously (Fig. 1B). DC from MHC class II-deficient and normal B6 mice presented the K^b/S208-215 peptide ILSPFLPL to specific CTL lines in vitro (Fig. 1C). Presentation of the antigenic peptide by DC generated in serum-free cultures was enhanced by preincubating the DC for 2 h before the peptide pulse with β2 m (data not shown). DC from MHC class II-expressing (normal B6) or MHC class II-deficient (Aα^-/- or Aβ^-/- B6) mice thus have a similar surface phenotype, a similar inducible TNF-α or IL-12 response, and a similar capacity to present antigenic epitopes to CTL.

CD4^+ T cells facilitate CTL priming by adoptively transferred, immunogenic DC
CD11c^+ DC generated from bone marrow progenitors of MHC class II-competent (B6) or MHC class II-deficient (Aα^-/- or Aβ^-/- B6) mice were pulsed with the K^b-binding S208-215 peptide and were washed. Pulsed or nonpulsed DC were injected s.c. into naive, syngeneic B6 hosts (4 × 10^5/mouse). Fourteen days after the single injection of DC, the number of specific, splenic CTL was determined directly ex vivo. Transfer of peptide-pulsed DC from normal B6 donor mice into naive, syngeneic B6 hosts primed a HBsAg-specific CTL response, whereas CTL priming by adoptive transfer of Ag-pulsed, MHC class II-deficient DC was severely deficient (Fig. 2A). Although the number of specific CTL found after adoptive transfer of Ag-pulsed, MHC class II-deficient DC was low, it was always above background (Fig. 2A). Transfer of nonpulsed or β2 m-pulsed control DC did not induce CTL (Fig. 2A). High numbers of specific CTL were detected at day 12 to day 15 posttransfer (Fig. 2B). CTL priming by DC generated in vitro in

FIGURE 4. Priming CTL responses by adoptive DC transfer depends on CD4^+ T cells. Purified DC from normal B6 mice were either pulsed with the antigenic, K^b-binding peptide or nonpulsed. DC were transferred into naive B6 mice (4 × 10^6 cells per mouse) that were either untreated or depleted of CD4^+ T cells by in vivo Ab treatment. On day 14 posttransfer, spleen cells were obtained and stimulated (in the presence of brefeldin A) for 6 h with the antigenic K^b-binding peptide. Specifically inducible IFN-γ^+ CD8^+ T cells were detected by FCM. Mean numbers of splenic IFN-γ^+ CD8^+ T cells per 10^6 CD8^+ T cells ± SD of three mice per group are given. Groups marked with * differ, p < 0.01 (unpaired t test).

FIGURE 5. Pretreatment of DC by immune-stimulating ODN enhances their capacity to prime CTL responses in vivo. A, Pretreatment of DC with ODN in vitro makes them more potent APC for CTL precursors in vivo. DC from normal or MHC class II-deficient (Aα^-/-) B6 mice were pretreated with 2 μg/ml ODN for 16 h before the 2-h pulse at 37°C with antigenic peptide. Cells were washed and injected s.c. (4 × 10^6 DC per mouse). B, Pretreatment of DC with ODN facilitates CD4^+ T cell help-independent priming of CTL. DC from Aα^-/- B6 mice were pretreated for 16 h with 2 μg/ml ODN, washed, pulsed for 2 h at 37°C with the K^b/S208-215-specific peptide ILSPFLPL, and washed. Nonpulsed or pulsed, pretreated DC were injected s.c. (4 × 10^6 DC per mouse) into CD4^-/- T cell-competent or -deficient B6 hosts. On day 14 posttransfer, spleen cells were obtained and stimulated (in the presence of brefeldin A) for 6 h with the antigenic K^b-binding peptide. Specifically inducible IFN-γ^+ CD8^+ T cells were detected by FCM. Mean numbers of splenic IFN-γ^+ CD8^+ T cells per 10^6 CD8^+ T cells ± SD of three mice per group are given. Groups marked with * differ, p < 0.01 (unpaired t test).
serum-supplemented cultures was efficient while CTL priming by DC generated under serum-free conditions was deficient (Fig. 3A). Hence, peptides derived from heterologous serum supplements in the medium may provide help in this system. To provide specific, CD4⁺ T cell-dependent help, DC were pulsed with the K⁺-binding, antigenic S₂₀₈₋₂₁₅ peptide ILSPFLPL of HBsAg, and with either the A⁺-binding, antigenic OVA₁₂₂₋₁₃₉ peptide ISQAVHAA-HAEINEAGR or purified OVA (Fig. 3B). DC that presented additional helper determinants of OVA primed OVA-specific CD4⁺ T cells (data not shown) and facilitated CTL priming to HBsAg (Fig. 3B).

Depletion studies confirmed that specific priming of CD8⁺ CTL responses by adoptive transfer of immunogenic DC depends on CD4⁺ T cells. When peptide-pulsed DC were transferred into hosts depleted of CD4⁺ T cells (by repeated injections of anti-CD4 Ab), they did not prime a CTL response (Fig. 4). The specific activation of CD8⁺ CTL precursors by Ag-pulsed DC in vivo thus depends on help from CD4⁺ T cells supporting the interpretation that MHC class II-dependent help facilitates CTL priming by DC.

**Activating DC before transfer renders CTL priming partially help-independent**

Activation of DC with immune-stimulating ODN before adoptive transfer facilitates CTL priming and renders the induction of CTL responses helper-independent. Purified DC were treated in vitro with 2 μg/ml ODN for 16 h. This treatment stimulated release of IL-12 p40/p70 and TNF-α, and up-regulated surface expression of MHC class II and costimulator molecules (Fig. 1, data not shown) (56–60). Adoptive transfer of ODN-pretreated, Ag-pulsed DC from normal (MHC class II-competent) B6 mice primed CTL responses to the HBsAg-restricted epitope more efficiently than non-pretreated, Ag-pulsed DC (Fig. 5A). When ODN-pretreated, pulsed DC from MHC class II-deficient mice were transferred into normal B6 mice, they were ~10-fold more efficient in priming CTL than non-pretreated, pulsed DC (Fig. 5A). Thus, ODN-activated, pulsed DC efficiently primed a CTL response in the absence of CD4⁺ T cells. Pulsed, MHC class II-deficient DC were transferred into B6 host mice with either an intact CD4 T cell compartment, or a severely depleted CD4⁺ T cell compartment. CTL priming facilitated by pretreatment of DC with ODN was also detected in the absence of an intact CD4 T cell compartment (Fig. 5B). Pretreatment of DC with ODN thus delivers a helper signal that facilitates CD4⁺ T cell-independent priming of a CTL response.

**NKT cells provide help for DC-mediated CTL priming in vivo**

A T cell subset that activates DC are NKT cells (44). NKT cells rapidly deliver effector functions after recognizing the glycolipid αGalCer bound to CD1d. This MHC class I-like molecule is expressed by bone marrow-derived DC (Fig. 1A). The presentation of an antigenic peptide (by K⁺) together with αGalCer (by CD1d) by DC enhanced their potency to prime CTL responses in vivo. The peptide- and αGalCer-pulsed DC showed more efficient, specific CTL priming in host B6 mice than did only peptide- but not αGalCer-pulsed DC (Fig. 6A) with a similar kinetics (Fig. 2B). B6 mice injected with peptide- and αGalCer-pulsed DC from MHC class II-deficient (Aα⁻⁻ or Aβ⁻⁻) mice showed as efficient CTL priming as normal mice (Fig. 6B).

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**FIGURE 6.** NKT cell recruitment by peptide-pulsed DC facilitates CTL priming in vivo. Purified DC from normal (A) or MHC class II-deficient Aα⁻⁻ (B and D) B6 mice were pulsed with the antigenic K⁺/S₂₀₈₋₂₁₅ peptide plus (in some groups) 2 μg/ml of the CD1d-binding glycolipid αGalCer. DC were washed and transferred into naive, normal B6 mice. C. Purified DC from normal or Aα⁻⁻ B6 mice were pulsed with the antigenic K⁺/OVA₁₂₂₋₁₃₉ peptide and (in some groups) αGalCer. DC were washed and transferred into naive, normal B6 mice (4 × 10⁵ DC per mouse). On day 14 posttransfer, spleen cells were obtained and stimulated (in the presence of brefeldin A) for 6 h with either the antigenic K⁺/S₂₀₈₋₂₁₅ peptide ILSPFLPL (A and B), or the K⁺/OVA₁₂₂₋₁₃₉ peptide SIINFEKL (C). Specifically inducible IFN-γ⁺ CD8⁺ T cells were detected by FCM. Mean numbers of splenic IFN-γ⁺ CD8⁺ T cells per 10⁵ CD8⁺ T cells ± SD of three mice per group are given. D. Spleen cells obtained from the same mice as in B were restimulated in vitro for 5 days with irradiated, peptide-pulsed RBL5 cells. Specific lysis at an E/T of 20:1 read out in a 4-h ⁵¹Cr-release assay was determined. Mean values ± SD of triplicates are shown. Groups marked with * differ, p < 0.01 (unpaired t test).
priming to the HBsAg epitope as B6 mice injected with peptide- and αGalCer-pulsed DC from MHC class II-competent (normal) B6 mice (Fig. 6B). CTL specifically induced by adoptive transfer of peptide- and αGalCer-pulsed DC in vivo produced IFN-γ (Fig. 6, A and B), and specifically lysed peptide-pulsed targets (Fig. 6D). These data were confirmed in another Ag system, i.e., OVA. DC from MHC class II-deficient (Aα−/− or Aβ−/−) B6 mice were pulsed with the Kβ-binding peptide SIINFEKL from OVA. In some groups, DC were also pulsed with αGalCer. Following adoptive transfer into normal, naive B6 hosts, peptide-presenting DC also pulsed with αGalCer primed CTL responses more efficiently than peptide-pulsed DC not pulsed with αGalCer (Fig. 6C). NKT cells can thus provide potent help for specific CTL priming by DC in vivo, even when the conventional MHC class II-dependent CD4+ T cell help system is excluded.

We tested whether αGalCer has to be presented by the DC that also presents the antigenic peptide to facilitate CTL priming. DC were either pulsed with the antigenic peptide ILSPFLPL and αGalCer, or one DC population was pulsed with the antigenic peptide ILSPFLPL and a second DC population was pulsed with αGalCer. The cells were washed, and the different populations were mixed at a 1:1 ratio (Fig. 7A). These DC populations were transferred into naive B6 hosts. Specific CTL priming was only facilitated by αGalCer when the same DC presented the antigenic peptide and αGalCer (Fig. 7A). Furthermore, neither the injection of a mixture of 5 μg peptide and 5 μg αGalCer nor an injection of peptide-pulsed DC together with 5 μg αGalCer into the same site facilitated CTL priming (data not shown). Hence, only presentation of αGalCer and antigenic peptide by the same DC facilitates CTL priming in vivo, while just mixing the Ag and αGalCer before injection is inefficient. Help mediated by ODN and by αGalCer had additive but no synergistic effects (Fig. 7B). Treating DC with ODN and pulsing them with αGalCer and antigenic peptide enhanced CTL priming after adoptive transfer into normal B6 hosts, when compared with only αGalCer-pulsed, or only ODN-pretreated pulsed DC. This suggests that ODN-mediated and NKT cell-mediated signals that activate DC are different.

**CD1d expression in the host is required to facilitate CTL priming by αGalCer-pulsed DC**

Development and activation of the αGalCer-reactive NKT cell population depends on CD1d molecules. This NKT cell subset...
(expressing CD4 and intermediate levels of CD3ε and NK1.1 on the surface) is severely depleted in CD1d−/− knockout mice (Fig. 8A). Peptide-pulsed DC from Aβ−/− B6 mice transferred into naive, normal or CD1d−/− B6 hosts inefficiently primed CTL (Fig. 8B). DC from Aβ−/− B6 mice pretreated with ODN and pulsed with the antigenic peptide efficiently primed CTL in naive, normal, or CD1d−/− B6 hosts (Fig. 8B), indicating that facilitated, specific CTL priming in vivo by ODN-stimulated, epitope-presenting DC is NKT cell-independent. In contrast, peptide- and αGalCer-pulsed DC from Aβ−/− B6 mice transferred into naive, normal, or CD1d−/− B6 hosts efficiently primed CTL in normal but not CD1d−/− B6 hosts (Fig. 8B). Hence, NKT cell help is CD1d-dependent. These data furthermore support the notion that ODN-mediated and NKT cell-mediated signals facilitating DC-dependent CTL priming operate independently of each other.

The role of NK cells vs NKT cells in DC-dependent CTL priming

NKT cell-activated DC recruit NK cells and stimulate their IFN-γ release (44, 45, 61). We tested whether NK cells are required to prime CTL by adoptive transfer of DC presenting an antigenic, Kβ-binding peptide (Fig. 9). CTL priming was deficient, when pulsed DC were transferred into naive B6 mice depleted of NK cells before DC transfer (by repeated injections of α-asialoGM1 antiserum) (Fig. 9). Priming T cell help-dependent CTL responses by adoptively transferred, Ag-presenting DC thus requires NK cells.

MHC class II-expressing or -deficient DC pulsed with antigenic peptide together with αGalCer were injected into normal or NK cell-depleted B6 hosts. In both systems, CTL priming by DC was less efficient in the absence of NK cells (Fig. 9). NK cells may help specific activation of CTL precursors either directly (through release of cytokines such as IL-15, type I IFNs, TNF, or IL-18) or indirectly (by enhancing the immunostimulatory phenotype of DC). Although recruitment of NKT cells help makes CTL priming by DC more efficient, it does not make it independent of NK cell help. Hence, NKT cell help can only partially replace NK cell help in specific CTL priming by adoptively transferred DC.

Discussion

DC used in this study were generated from BMC precursors in 7 day GM-CSF-supplemented cultures. Similar to freshly isolated splenic or hepatic DC (44), these DC express high levels of CD1d, Kβ, and Dβ but low/intermediate levels of the class II molecule Aβ and the CD40, CD80, and CD86 costimulator molecules on the surface. No difference (except MHC class II expression) was found in the surface phenotype of bone marrow-derived DC from normal and MHC class II-deficient (Aα−/− or Aβ−/−) B6 mice. This phenotype, together with the lack of spontaneous release of IL-12 or TNF, characterizes these DC as immature. DC activated in vitro by either CpG-containing ODN or CD40 ligation up-regulated surface expression of MHC and costimulator molecules, and released IL-12 and TNF (Fig. 1). When pulsed with the Kβ-binding peptide S208-215 of HBsAg, these DC specifically stimulated IFN-γ release by cocultured CTL. When these DC were pulsed with αGalCer, they triggered IFN-γ, IL-4, and IL-13 release by cocultured NKT cells (data not shown) (44). Hence, we have a source of large numbers of a fairly uniform, mostly immature DC available for in vivo studies that are inducible to maturation and specifically present ligands to class I-restricted CTL and CD1d-restricted NKT cells.

The adoptive transfer of DC pulsed with the Kβ-binding antigenic HBsAg peptide into a syngeneic, naive recipient specifically primed a CD8+ CTL response (Fig. 2) confirming previous reports (2–4, 62). Transfer of pulsed DC into CD4− T cell-depleted hosts primed CTL responses inefficiently (Fig. 4). This result indicates that a CD4− T cell subset facilitates CTL priming by DC in this system, which could be either a conventional, Aβ-restricted CD4+ T cells or an αGalCer-reactive NKT cell. In these experiments, DC were pulsed only with the Kβ-binding, immunogenic peptide. Transfer of peptide-pulsed, MHC class II-deficient DC into normal, naive B6 hosts was inefficient in priming CTL, whereas transfer of similarly pulsed MHC class II-expressing, congenic DC into the same hosts efficiently primed CTL (Fig. 2A). This suggests that antigenic peptides from the serum supplementing the culture medium are captured by Aβ molecules on the surface of DC and mediate help in this system. This result was supported by the observation that pulsed, MHC class II-expressing DC generated in serum-free medium were inefficient in priming CTL responses in vivo (Fig. 3A). The data stress the point that helper-independence of CTL priming can be reliably assessed only with genetically engineered DC as culture or separation of DC cannot rule out noncontrolled, class II-restricted help. We confirmed the Aβ-dependent, specific CD4+ T cell help by pulsing DC from MHC class II-expressing, normal B6 mice with the Kβ-binding peptide and an Aβ-binding helper peptide. The data in Fig. 3B indicate that

![FIGURE 9.](http://www.jimmunol.org/) NK cell help and NKT cell help in DC-mediated CTL priming. Purified DC from normal or MHC class II-deficient Aα−/− B6 mice were pulsed with the antigenic Kβ/S208-215 peptide, plus (in some groups) the CD1d-binding glycolipid αGalCer. DC were washed and transferred (4 × 10⁶ DC per mouse) into normal or NK cell-depleted, naive B6 mice. B6 mice were depleted of NK cells by repeated injections of the α-asialoGM1 Ab (as described in Materials and Methods). On day 14 posttransfer, splenic cells were obtained and stimulated (in the presence of brefeldin A) for 6 h with the antigenic Kβ-binding peptide. Specifically inducible IFN-γ+ CD8+ T cells were detected by FCM. Mean numbers of splenic IFN-γ+ CD8+ T cells per 10⁶ CD8+ T cells ± SD of three mice per group are given.
specific CD4+ T cell help can facilitate CTL priming in vivo by adaptively transferred DC, as previously described (10, 11, 14). As specific help is in many vaccination situations either not well defined or specifically anergized or deleted, we asked: 1) can the requirement for class II-restricted CD4+ T cell help be bypassed by conditioning DC before transfer and 2) can the class II-restricted CD4+ T cell help be replaced or enhanced by an alternative helper cell.

Mature DC can prime CTL responses, whereas immature DC cannot (63). Immature DC acquire CTL priming capacity upon activation by Th-independent or -dependent stimuli (24). The maturation-inducing effect of CD4+ T cells operates to a large extent through CD40/CD40 ligand interactions (20, 64). As MHC class II-deficient DC do not prime CTL in vivo (22) (and shown in this study), the class II-restricted CD4+ T cell subset but not other CD40-expressing (e.g., NK, NKT, B) cells seem to be the main source of help under physiological conditions although CD8+ CTL themselves may also be helpers that induce DC maturation (65). Immune-stimulating DNA-based vaccines, CpG-containing oligonucleotides, or poly(I:C) induce DC maturation and CTL responses by a Th cell-independent mechanism (11, 23, 26, 27, 57, 58, 66–68). We confirm these data. CD4+ T cell-independent priming of CTL by DC in vivo is the target of the ODN adjuvant effect on CTL priming as 1) pulsed Aαβ−/− or Aββ−/− DC treated with ODN in vitro primed CTL as effectively as T help-supported DC in vivo, and 2) in vitro treatment of pulsed DC with ODN (followed by extensive washes), and the coinjection of pulsed DC with ODN into the mouse were equally effective in triggering the ODN adjuvant effect in vivo (suggesting that DC but not other cells recruited in vivo to the site of priming are the main target of the ODN adjuvant effect in this system).

The glycolipid αGalCer has been shown to promote adaptive immunity (reviewed in 31). NKT cells produce IFN-γ (promoting Th1 immunity) and IL-4/IL-13 (promoting Th2 immunity), and facilitate in vivo priming of specific Th1 immunity (36, 69, 70) or Th2 immunity (34, 35). NKT cells have been shown to act as class II-independent helper cells in the generation of CD8+ effector function against intracellular infection (41, 71), and NKT cells can activate splenic and hepatic DC (31, 43, 44) and are expanded by αGalCer-pulsed DC (72). The question is are DC the target for the help of NKT cells? Our data indicate that αGalCer-pulsed, MHC class II-deficient DC presenting a class I-binding peptide efficiently prime CD8+ CTL responses in vivo. αGalCer-pulsed DC induce prolonged IFN-γ-producing NKT responses (73), which may explain the exceptional adjuvant effect of this glycolipid. Under natural conditions, CD1d-binding ligands from either an exogenous source (e.g., pathogens) or an endogenous source (e.g., activated or damaged cells) may be available to facilitate CTL priming. Endogenous ligands may be either produced by DC themselves, or may be picked up by DC from the immediate vicinity. This adjuvant effect is certainly of interest for priming CTL responses in situations in which the CD4+ T cell system is either depleted (e.g., as in HIV infection), or specifically anergized (as in many chronic infections). The practical value of the approach in these clinical situations remains to be shown.

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References


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