

# Mechanisms of Mouse Spleen Dendritic Cell Function in the Generation of Influenza-specific, Cytolytic T Lymphocytes

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## Summary

We have evaluated the capacity of dendritic cells to function as antigen-presenting cells (APCs) for influenza and have examined their mechanism of action. Virus-pulsed dendritic cells were 100 times more efficient than bulk spleen cells in stimulating cytotoxic T lymphocyte (CTL) formation. The induction of CTLs required neither exogenous lymphokines nor APCs in the responding T cell population. Infectious virus entered dendritic cells through intracellular acidic vacuoles and directed the synthesis of several viral proteins. If ultraviolet (UV)-inactivated or bromelain-treated viruses were used, viral protein synthesis could not be detected, and there was poor induction of CTLs. This indicated that dendritic cells were not capable of processing noninfectious virus onto major histocompatibility complex (MHC) class I molecules. However, UV-inactivated and bromelain-treated viruses were presented efficiently to class II-restricted CD4<sup>+</sup> T cells. The CD4<sup>+</sup> T cells crossreacted with different strains of influenza and markedly amplified CTL formation. Cell lines that lacked MHC class II, and consequently the capacity to stimulate CD4<sup>+</sup> T cells, failed to induce CTLs unless helper lymphokines were added. Similarly, dendritic cells pulsed with the MHC class I-restricted nucleoprotein 147-155 peptide were poor stimulators in the absence of exogenous helper factors. We conclude that the function of dendritic cells as APCs for the generation of virus-specific CTLs *in vitro* depends measurably upon: (a) charging class I molecules with peptides derived from endogenously synthesized viral antigens, and (b) stimulating a strong CD4<sup>+</sup> helper T cell response.

As part of the host defense against influenza, MHC class I-restricted CD8<sup>+</sup> CTLs are generated that kill virus-infected targets. Whereas neutralizing antibodies recognize the highly variable envelope glycoproteins of the virus, CTLs are directed primarily against highly conserved internal proteins like the nucleoprotein (NP).<sup>1</sup> As a result, most influenza-specific CTLs are broadly crossreactive and kill target cells infected with viruses belonging to different subtypes (1-4). By eliminating infected cells before infectious viral progeny can be released, these killer cells appear to be important in recovery from infection. In mice, adoptively transferred CTLs can promote viral clearance and can protect against lethal infection (5-7). In infected humans, McMichael et al. (8) noted a negative correlation between CTL activity and lung virus titers.

The study of virus-specific CTLs has yielded several key concepts in cell-mediated immunity. Using these CTLs, Zinkernagel and Doherty (9, 10) first demonstrated the presentation of antigens in association with products of genes within the MHC. Subsequently, Townsend et al. (11) demonstrated the recognition of processed influenza proteins or peptides by class I-restricted T cells, providing a basis for the corecognition of antigen and MHC. Virus-specific CTL systems helped two different pathways for antigen processing, one exogenous and the other endogenous (12). Internalized exogenous antigens undergo acid-dependent degradation in endocytic vacuoles, and peptides generated in this manner are presented in association with MHC class II molecules to CD4<sup>+</sup> CTLs. In contrast, viral antigens synthesized within cells are most likely processed within the cytoplasm, and the immunogenetic peptides are presented with newly synthesized class I MHC molecules to CD8<sup>+</sup> CTLs.

These studies on the recognition of viral antigens by CTLs have relied upon activated or chronically stimulated T cells. Less is known about the generation of functional class I-re-

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<sup>1</sup> Abbreviations used in this paper: HA, hemagglutinin; NP, nucleoprotein; PD, phosphate saline.

stricted CTLs from quiescent precursors. Dendritic cells are potent stimulators of class II-restricted T cell responses to a variety of antigens *in vitro* and *in vivo*, and can stimulate vigorous CTL responses to influenza (13) and other viruses (14–16). To approach the mechanism of dendritic cell function, we have studied the development of influenza-specific CTLs. Two major issues have interested us. The first is whether splenic dendritic cells must support endogenous viral protein synthesis to stimulate CD8<sup>+</sup> CTLs, or can these APCs present viral antigens using the exogenous processing pathway? Recent reports suggest that APCs exist within the spleen with the capacity to present native OVA to class I-restricted CTLs (17, 18), and other studies have documented the presentation of class I-restricted minor antigens carried on donor cells by host APCs (19, 20). A second area of interest is the role of helper T cells. Dendritic cells can support the generation of allospecific CD8<sup>+</sup> CTL in the absence of CD4<sup>+</sup> helper T cells (21–23). We show here that viral protein synthesis and helper T cells are both significant quantitative elements in the dendritic cell-mediated induction of influenza-specific CTLs *in vitro*.

## Materials and Methods

**Mice.** Male or female (BALB/c × DBA/2)<sub>F</sub><sub>1</sub> (C × D2; H-2<sup>d</sup>), (C3H × DBA/2)<sub>F</sub><sub>1</sub> (C3D2; H-2<sup>k</sup> × H-2<sup>d</sup>), C3H (H-2<sup>k</sup>), and DBA/2 (H-2<sup>d</sup>) mice, 6–8 wk of age, were purchased from the Trudeau Institute (Saranac Lake, NY). To prime with influenza virus, the animals were anesthetized with metofane, and 5 hemagglutination units (HAU) of NT60 virus in a volume of 50 μl were applied to the nose pad. The mice were kept in a specific pathogen-free facility and used 3–6 wk later.

**Virus.** The PR8 (A/Puerto Rico/8/1934; H1N1 subtype) and NT60 (A/Northern Territory/60/1968; H3N2 subtype) strains were kindly provided by Dr. P. Palese (Mount Sinai School of Medicine, New York) and were grown in the allantoic cavity of 10-d embryonated chicken eggs (SPAFAS, Norwich, CT). The allantoic fluid was harvested after 2 d and stored at –70°C. To purify the virus, the allantoic fluid was centrifuged at 100,000 g for 2 h at 4°C. The virus pellet was resuspended in SM buffer (0.1 M NaCl, 0.05 M Tris, 10 mM MgSO<sub>4</sub>, pH 7.4), layered on a 10/35/45% cesium chloride step gradient (Bethesda Research Laboratories, Gaithersburg, MD) in SM buffer, and centrifuged in a rotor (SW41; Beckman Instruments, Inc., Fullerton, CA) at 22,000 rpm for 2 h at 4°C. After centrifugation the virus band at the 10 and 35% interface was collected, diluted in phosphate saline (PD), and pelleted. The virus pellet was then resuspended in PD and stored in aliquots at –70°C.

The enzyme bromelain (Sigma Chemical Co., St. Louis, MO) was used to remove hemagglutinin (HA) and neuraminidase glycoproteins from the virus particle (24). Pelleted virus, prepared as above, was resuspended in bromelain buffer (0.1 M Tris, pH 7.2, with 0.05 M β-mercaptoethanol and 0.001 M EDTA) at a viral protein concentration of 0.2 mg/ml, and bromelain at 2 mg/ml. After an 18–20-h incubation at 37°C, the virus was diluted in PD and pelleted. The pelleted virus was resuspended in a small volume of PD and applied to a CsCl step gradient as above. Virus cores were collected from the interface between the 10 and 35% layers, pelleted, resuspended in PD, and stored in aliquots at –70°C. Removal of the HA was confirmed using a hemagglutination assay.

Virus was inactivated by diluting the allantoic fluid 1:10 in HBSS with Ca<sup>2+</sup> and Mg<sup>2+</sup>, and exposing it to shortwave UV radiation from a Mineralight UV lamp (UVGL58; Ultraviolet Products, San Gabriel, CA) for 10 min at a distance of 10 cm with constant stirring. Virus inactivation was confirmed with a modified plaque assay.

**Culture Medium.** The culture medium for cells was RPMI 1640 (Gibco Laboratories, Grand Island, NY) containing 5% heat-inactivated FCS (JRH-Biosciences, Lenexa, KS), 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin, and 10 μM β-mercaptoethanol.

**Responder T Cells.** Spleen cell suspensions were prepared and passed over nylon wool columns (Polysciences, Inc., Warrington, PA). T cells were further purified by labeling the nylon wool-nonadherent cells with rat mAbs from the American Type Culture Collection (ATCC; Rockville, MD) (B21-2, anti-Ia; M1/70, anti-Mac 1; RA3-6B2.1, anti-B220) and two rounds of panning. Bacteriological plates (Falcon Labware, Oxnard, CA) were coated with goat anti-rat IgG (H + L) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) at 10 μg/ml for 1 h at room temperature and washed. The cells were added and the pans centrifuged in a centrifuge (RC-3B; Sorvall) at 500 rpm for 5 min. After this spin, the plates were swirled, rotated 180°, and centrifuged again. The cells were harvested by gently washing the plates with cold RPMI. T cells prepared in this manner were 85–90% Thy-1<sup>+</sup>. Depletion of APCs was evident by the fact that the T cells did not respond to stimulation with anti-CD3 mAb or with Con A.

To prepare CD4<sup>+</sup> or CD8<sup>+</sup> T cell subsets, nylon wool-nonadherent cells were treated with complement (Pel-Freeze Biologicals, Rogers, AR) plus anti-Ia<sup>b,d</sup> (B21-2) and either anti-CD8 mAbs (TIB 150 and TIB 211; ATCC) or anti-CD4 mAb (GK1.5). CD8<sup>+</sup> T cells were further purified by labeling the complement-treated cells with a FITC rat anti-mouse CD8 (Becton Dickinson & Co., Mountain View, CA) at 0.2 μg/ml and then sorting positively stained cells in a FACStar Plus<sup>®</sup> instrument (Becton Dickinson & Co.). Cells prepared with this protocol were >98% CD8<sup>+</sup>.

**APCs.** Spleens were injected with 100 U/ml collagenase D (Boehringer Mannheim Biochemicals, Indianapolis, IN) and teased apart with forceps (25). The released cells were transferred to a tube and the remaining fragments incubated in 400 U/ml collagenase for 45–60 min at 37°C. After this incubation, the spleen fragments were passed through a steel mesh, and the cells were pooled with the cells collected earlier and centrifuged. The resulting pellet was resuspended in dense BSA (ρ = 1.080) and centrifuged in a rotor (HS-4; Sorvall) at 7,000 rpm (9,500 g) for 15 min. The dendritic cell-enriched, low-density cells at the interface were collected, washed, and plated on 60-mm tissue culture dishes (Falcon Labware) allowing two to three spleen equivalents per plate. After 90 min at 37°C, the nonadherent cells, mainly lymphocyte, were removed by pipetting over the plates with warm RPMI. The adherent cells were cultured overnight in medium at 37°C. Dendritic cells, which become nonadherent upon culture, were collected and contaminating macrophages removed by readhering the cells to tissue culture plates for 1 h at 37°C. Dendritic cells purified in this manner were 90–95% pure and contaminated primarily with a few B cells (25).

**Cell Lines.** The P815 mastocytoma (H-2<sup>d</sup>) and the L929 fibroblast lines (H-2<sup>k</sup>) were grown in DME (Gibco Laboratories) supplemented with 5% FCS, penicillin, streptomycin, and gentamicin. To use the L929 cells as targets in <sup>51</sup>Cr release assays, they were detached by treating with 0.025% trypsin and cultured overnight in medium in bacteriological dishes to which they do not adhere. An L929 cell line transduced with the influenza NP gene

via a retroviral vector was kindly provided by Dr. E. Gilboa and colleagues (Sloan-Kettering Institute, New York).

**Infection of Cells with Influenza *In Vitro*.** APCs and targets were resuspended in serum-free RPMI at  $10^7$  cell/ml, containing 1,000 HAU of virus/ml, for 90 min at  $37^\circ\text{C}$ . In the case of freshly isolated dendritic cells, the low-density adherent spleen cells were infected by adding serum-free RPMI plus virus directly to the plate. To test if influenza infection required an acidic compartment, the cells were incubated in ammonium chloride for 30 min before adding virus and throughout the subsequent infection (26). 30 and 10 mM  $\text{NH}_4\text{Cl}$  blocked infection in P815 and dendritic cells, respectively. The block was reversible upon removing the  $\text{NH}_4\text{Cl}$ , as assessed by staining the cells for newly synthesized viral protein.

**Generation of CTLs.** Purified T cells from virus-primed animals ( $1-3 \times 10^5$ /well) were cultured with graded doses of APCs in round-bottomed 96-well tissue culture plates (Corning Glass Works, Corning, NY) in a total volume of 200  $\mu\text{l}$  medium. The APCs were irradiated with a  $^{137}\text{Cs}$  source (1,000 rad for primary APCs and 10,000 rad for cell lines). After 5–6 d of culture, CTL activity was assayed by  $^{51}\text{Cr}$ -release from P815 (H-2<sup>d</sup>) and L929 (H-2<sup>k</sup>) targets. When viable cell counts were obtained, specific cytotoxicity of 80–100% was obtained at E/T ratios of 30:1.

**Cytotoxicity Assay.**  $2 \times 10^6$  targets at a concentration of  $10^7$  cells/ml in serum-free RPMI were labeled with 100  $\mu\text{Ci}$   $\text{Na}^{51}\text{CrO}_4$  (New England Nuclear, Boston, MA) and simultaneously infected with 200 HAU PR8 virus for 2 h at  $37^\circ\text{C}$ . The targets were washed four times and resuspended in medium at  $4 \times 10^5$  cells/ml. 50  $\mu\text{l}$  of targets ( $2 \times 10^4$  cells) was added to each microwell in triplicate. Spontaneous and total release samples were prepared by adding the targets to wells containing only RPMI or 0.2% SDS, respectively. The plates were centrifuged at 100 *g* for 5 min and incubated at  $37^\circ\text{C}$  for 5 h. 25  $\mu\text{l}$  of supernatant was collected, deposited onto a glass fiber filter mat, and counted in a Betaplate Liquid Scintillation Counter (LKB Wallac, Wallac Oy, Finland). Percent specific  $^{51}\text{Cr}$  release was calculated as follows:  $100 \times (\text{release by CTL} - \text{spontaneous release}) / (\text{total release} - \text{spontaneous release})$ . Spontaneous release was generally 10–15% of the total release.

**T Cell Proliferation Assay.** T cells ( $3 \times 10^5$ /well) were cultured in flat-bottomed microwells with graded doses of APCs. After 72–80 h, proliferation was assessed by pulsing the cultures with 1  $\mu\text{Ci}$ /well [ $^3\text{H}$ ]thymidine (ICN Radiochemicals, Irvine, CA) for 8–16 h. The plates were harvested using an Automatic Harvester (Skatron Inc., Sterling, VA), and the radioactivity was bound to filters counted in a Betaplate counter.

**Preparation of Lymphokine-rich Supernatant.**  $3 \times 10^6$   $\text{CD4}^+$  T cells were combined with  $10^5$  allogeneic dendritic cells in 16-mm macrowells. Supernatants were collected after 60–72 h and contained 50–100 U IL-2/ml.

**Nucleoprotein Peptides.** Peptides corresponding to residues 147–155 (TYQRTRALV) and 147–161 (TYQRTRALVRTGMDP) of the 1968 influenza NP (27) were synthesized with the solid-phase procedure (28) using the tertbutoxycarbonyl chemistry on 0.5 g of Boc-aminoacyl-OCH<sub>2</sub>-Pam resin (Applied Biosystems, Inc., Foster City, CA). The peptide was cleaved off from the resin support by the high hydrofluoric acid (HF) method and purified using reverse-phase C<sub>18</sub> on a preparative column eluted with a linear gradient from 5–60% of acetonitrile at a flow rate of 15 ml/min. The major peak was pooled. The acetonitrile was removed and lyophilized to give a white powder. Amino acid analyses of both peptides after 6 N HCl hydrolysis were in good agreement with the theoretical values of the peptide sequences.

**Immunohistochemistry.** Suspensions of  $3-10 \times 10^5$  cells/ml were

cytospun in a Cytospin 2 cytocentrifuge (Shandon, Sewickley, PA) and stored with dessiccant at  $-20^\circ\text{C}$ . The slides were brought to room temperature, fixed in acetone for 10 min, incubated with hybridoma supernatant for 45–60 min, washed, and then incubated with a 1:500 dilution of affinity-purified peroxidase-conjugated rabbit anti-mouse IgG (H + L) (Zymed Laboratories, San Francisco, CA) for 45–60 min. The cytopspins were washed in PD/1% BSA and incubated with substrate, 3,3'-diaminobenzidine tetrahydrochloride (Polysciences, Inc., Warrington, PA). After 7–10 min the slides were washed and mounted in PD-glycerol. The mAbs to influenza proteins were kindly provided by Dr. J. Yewdell (National Institutes of Health) and included anti-NP (H16-L10-4R5; ATCC HB65), anti-HA (H28E23), and anti-NS1 (29).

**IL-2 and IL-6 Assays.** Serial dilutions of conditioned medium were prepared and added to microwells containing  $5 \times 10^3$  CTL2 cells (IL-2) or 7TD1 cells (IL-6). After 24 h, the cultures were pulsed with 1  $\mu\text{Ci}$ /well of [ $^3\text{H}$ ]thymidine for 8–12 h and units calculated relative to standard recombinant lymphokines.

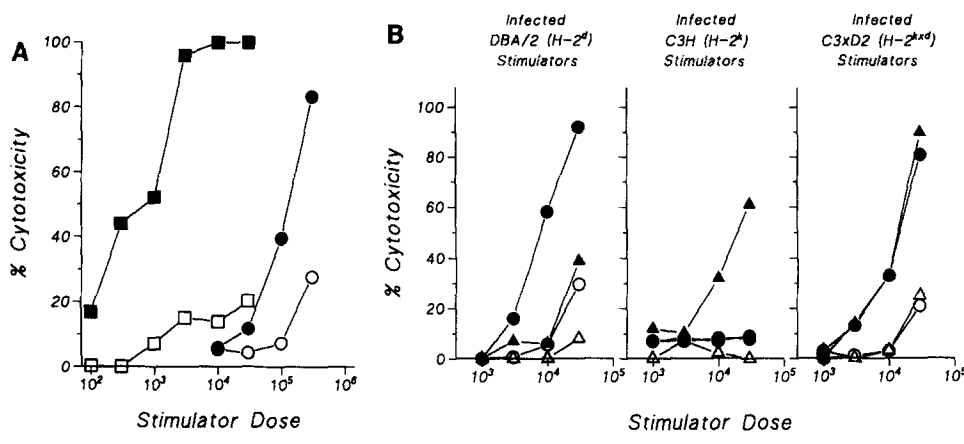
## Results

**The Capacity of Dendritic Cells to Induce Influenza-specific CTL.** Recent studies have demonstrated that mouse spleen dendritic cells are potent stimulators of the CTL response to influenza (13). We made similar observations using responding T cells that had been extensively depleted of APCs (see Materials and Methods). When compared with a standard APC population of bulk spleen cells, virus-pulsed dendritic cells were  $\sim 100$  times more efficient as stimulators (Fig. 1 A). A significant response was generated even when the dendritic cell/T cell ratio was as low as 1:1,000. No CTLs were induced with virus-pulsed splenic B cells, T cells, or peritoneal macrophages (data not shown).

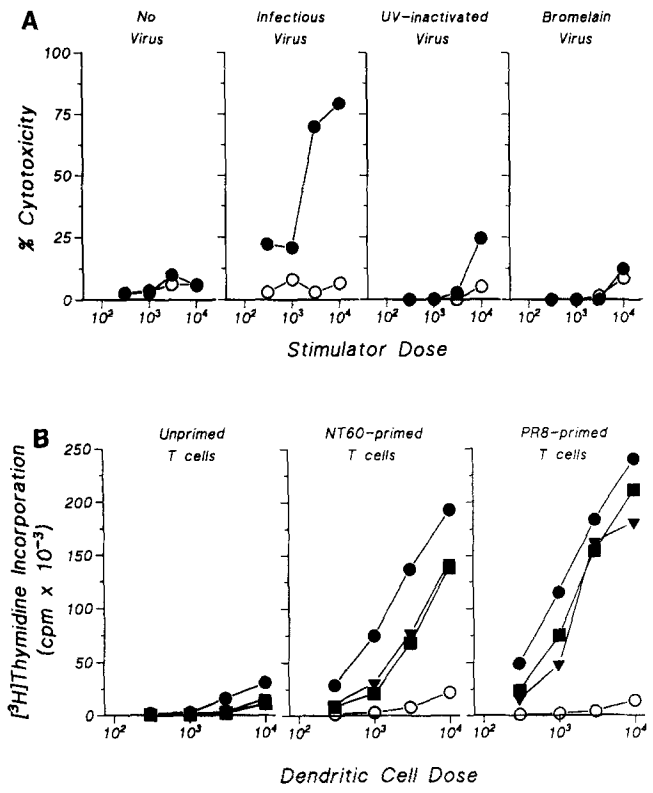
Contaminants within the enriched dendritic cell preparation could conceivably be responsible for the stimulation of CTLs. However, removal of the trace B cell contaminants with mAb RA3-6B2.1 plus complement did not diminish the stimulatory capacity, while removal of dendritic cells with 33D1 plus complement dramatically reduced APC function (data not shown) as in other T-dependent responses (30, 31).

In additional experiments, which are not shown, we verified that the killer cells induced by dendritic cells in culture were typical of CTLs generated in more heterogeneous systems. Specifically, the killer cells were sensitive to lysis with anti-Thy-1 or anti-CD8 mAbs and complement, but not anti-CD4 mAbs and complement. The CTL response was directed in large part to epitopes in the viral NP, since targets coated with the dominant NP147-155 peptide were recognized as efficiently as virus-infected targets.

It could be argued that viral antigens released by the infected dendritic cells were presented by contaminating APCs within the responder T cell population. To test this possibility, T cells from primed (C3H  $\times$  DBA/2) $F_1$  mice (H-2<sup>k</sup>  $\times$  H-2<sup>d</sup>) were restimulated with virus-pulsed dendritic cells prepared from either parental or  $F_1$  strains. We then measured lysis on both H-2<sup>k</sup> (L929) and H-2<sup>d</sup> (P815) targets. When  $F_1$  responders were stimulated with  $F_1$  dendritic cells, we detected the lysis of both L929 and P815 targets (Fig.



**Figure 1.** Dendritic cells are potent stimulators of influenza-specific CTLs. (A)  $3 \times 10^5$  purified T cells from virus-primed C  $\times$  D2 mice were cultured with graded doses of infected spleen cells (O and ●) or dendritic cells (□ and ■). After 5 d, CTL activity was assayed with uninfected (O and □) and infected (● and ■) P815 targets. (B) T cells were prepared from the spleens of virus-primed (C3H  $\times$  DBA/2) $F_1$  (C3  $\times$  D2) animals.  $3 \times 10^5$  T cells were cultured with graded doses of infected DBA/2 (H-2<sup>d</sup>; left), C3H (H-2<sup>k</sup>; middle), or C3  $\times$  D2 (right) dendritic cells. Cytotoxicity was assayed on uninfected (open symbols) and infected (filled symbols) L929 (H-2<sup>k</sup>;  $\Delta$  and  $\blacktriangle$ ) and P815 (H-2<sup>d</sup>; O and ●) targets.



**Figure 2.** Dendritic cells pulsed with bromelain-treated or UV-inactivated virus do not stimulate class I-restricted CTL but do induce proliferation of purified CD4<sup>+</sup> T cells. Dendritic cells were incubated overnight with no addition, 1,000 HAU/ml purified virus, bromelain-treated virus, or UV-inactivated virus. The number of the different virus particles added to the APCs was equivalent. (A)  $10^5$  virus-primed T cells were cultured with graded doses of APCs, and cytotoxicity was assayed on uninfected (O) and infected (●) P815 targets. (B)  $3 \times 10^5$  T cells from unprimed (left), NT60-primed (middle), and PR8-primed (right) mice were stimulated with dendritic cells pulsed with the following: no virus (O), infectious virus (●), bromelain-treated virus (▼), and UV-inactivated virus (■). The cultures were pulsed with [<sup>3</sup>H]thymidine from 80 to 92 h.

1 B). In contrast, when the F<sub>1</sub> T cells were stimulated with C3H (H-2<sup>k</sup>) dendritic cells, only H-2<sup>k</sup>-restricted CTLs recognizing infected L929 cells were induced. Restimulation with DBA/2 (H-2<sup>d</sup>) dendritic cells generated CTLs that were largely restricted to infected P815 (H-2<sup>d</sup>) targets. Therefore, dendritic cells efficiently present viral antigens directly to developing CTL.

*The Need for Viral Infection of Dendritic Cells for CTL Induction.* The viral antigens presented by dendritic cells could theoretically be derived either from the input virus or from proteins newly synthesized within the APCs. To explore these alternatives, we compared presentation of wild-type influenza with two types of noninfectious virus. UV irradiation blocks viral RNA replication, while bromelain proteolytically removes the external glycoproteins, including the HA that is essential for fusion with intracellular membranes and virus entry. These forms of noninfectious influenza did not yield viral progeny, as determined using a modified plaque assay with MDCK cells, and did not direct viral protein synthesis in P815 cells as detected by staining with antiviral mAbs (see below).

Dendritic cells pulsed with UV-inactivated virus did not stimulate a strong CTL response (Fig. 2 A). At the highest dose of APCs, there was a low level of specific lysis on infected target cells ranging from 5% to 25%, but in all experiments, dendritic cells pulsed with infectious virus were much more potent than APCs pulsed with UV-inactivated virus. Dendritic cells pulsed with bromelain-treated virus also failed to stimulate influenza-specific CTLs (Fig. 2 A). The UV- and bromelain-treated virus did not contain “preprocessed” peptides, since neither could sensitize targets for lysis by CTL (data not shown).

The inability of dendritic cells to present noninfectious virus to class I-restricted T cells could reflect poor internalization of viral antigens. However, dendritic cells readily presented either UV-inactivated or bromelain-treated virus to class II-re-

stricted T cells, as evidenced by strong proliferative responses of purified CD4<sup>+</sup> T cells (Fig. 2 B). In fact, dendritic cells pulsed with noninfectious virus stimulated the proliferation of CD4<sup>+</sup> T cells almost as effectively as dendritic cells exposed to infectious virus (Fig. 2 B). The response was blocked by >80–90% with anti-class II or anti-CD4 mAbs (data not shown).

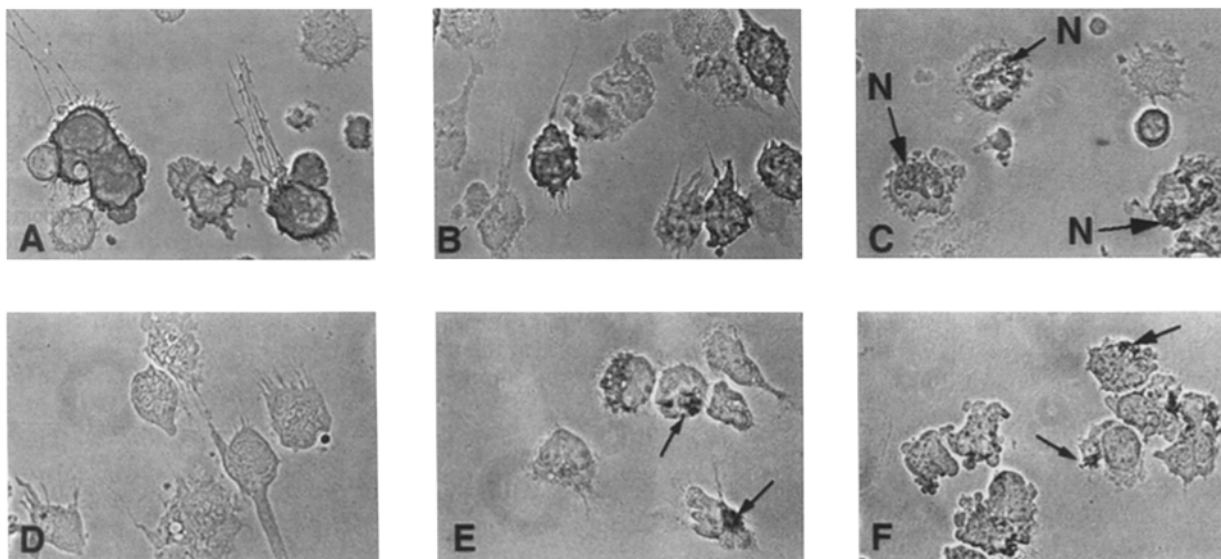
Unprimed CD4<sup>+</sup> T cells did not proliferate in response to infected dendritic cells (Figs. 2 B, left). Superantigens can stimulate unprimed T cells, so our data suggests that influenza virus does not carry significant superantigens for BALB/c × DBA/2 T cells. The helper T cell response we observed was for the most part crossreactive (Fig. 2 B). T cells prepared from mice primed with the NT60 strain (H3N2 subtype) or the PR8 strain (H1N1 subtype) responded similarly to antigens in PR8 virus, including bromelain-treated virus. We conclude that internal virion components in noninfectious influenza are presented in a potent fashion to CD4<sup>+</sup> but not CD8<sup>+</sup> T cells.

**Visualization of Viral Uptake and Infection in Dendritic Cells.** We used immunocytochemical techniques to determine directly if dendritic cells were capable of supporting the synthesis of influenza proteins. Standard direct metabolic labeling methods using [<sup>35</sup>S]methionine did demonstrate the synthesis of NP in infected dendritic cells (data not shown), but the approach was not pursued because very large numbers of dendritic cells ( $5 \times 10^6$ ) were needed for each experiment. Instead, we found that dendritic cells exposed to infectious virus could be stained positively with mAbs to the influenza NP or HA. Both mAbs stained the dendritic cells diffusely, consistent with endogenous viral protein synthesis

(Fig. 3, A and B). The number of positively stained cells varied, ranging from 20 to 50%. We also stained the cells with a mAb to the nonstructural protein, NS1. NS1 is not part of incoming virions but is a virally encoded protein synthesized within infected cells. The NS1 staining consisted of granular deposits in the nucleus (Fig. 3 C). Control studies indicated that infected dendritic cells did not stain with an isotype-matched antibody against human CD8 (Fig. 3 D). Uninfected dendritic cells did not stain with any of the mAbs tested (data not shown).

We were not able to detect the uptake of influenza virus particles by light microscopy. However, when infection was blocked with ammonium chloride (32), which neutralizes acidic vesicles needed for viral envelope fusion and delivery of RNA to the cytoplasm, the dendritic cells accumulated dense granules that stained with anti-NP mAb (Fig. 3 E) We presume the granules represent endocytic vesicles that were neutralized with ammonium chloride. Similarly, when we exposed dendritic cells to bromelain-treated virus overnight, the cytoplasm again contained granules of anti-NP antibody staining, and the number of these granules increased if ammonium chloride was added (Fig. 3 F). If dendritic cells were pulsed with UV-inactivated virus, there was no diffuse staining with mAbs to NP and HA (data not shown). Therefore, influenza seems to gain access to dendritic cells via an acid-dependent endocytic route, although for unknown reasons, only a fraction of the APCs show detectable levels of viral protein synthesis.

**The Role of CD4<sup>+</sup> Helper Cells and Lymphokine in the Induction of CTLs.** Primed CD4<sup>+</sup> T cells proliferate vigorously to both infectious and noninfectious influenza (Fig.



**Figure 3.** Detection of influenza NP, HA, and NS1 proteins in infected dendritic cells. Dendritic cells were incubated with virus for 90 min and then cultured for 16 h. Cytospins were prepared and stained with the following mAbs: anti-HA (A), anti-NP (B), anti-NS1 (C), and anti-human CD8 (D). In C, the NS1 staining is confined to the nucleus and consists of granular deposits (arrows). In E, dendritic cells were infected in the presence of 10 mM NH<sub>4</sub>Cl for 16 h and stained with anti-NP mAb. In F, cells were pulsed with bromelain-treated virus for 16 h in the presence of 10 mM of NH<sub>4</sub>Cl, and were stained with anti-NP mAb. In both E and F, granular presumptive endocytic vesicles are seen (arrows) (× 500).

2 B). The role of CD4<sup>+</sup> helper cells in influenza-specific antibody response is established, but we wished to evaluate the importance of helper T cells and lymphokines in the CTL response. In prior studies of alloreactive CTLs, CD4<sup>+</sup> helper cells were not required when dendritic cells were the APCs (21–23).

When purified CD8<sup>+</sup> T cells (see Materials and Methods) were stimulated with infected dendritic cells, little (10–20% killing) or no induction of virus-specific cytotoxicity was observed (Fig. 4 A). The response could be restored by adding a lymphokine-rich supernatant to the culture. The required soluble factors were not antigen specific since they could be generated in dendritic cell–CD4<sup>+</sup> T cell, allogeneic MLRs (33).

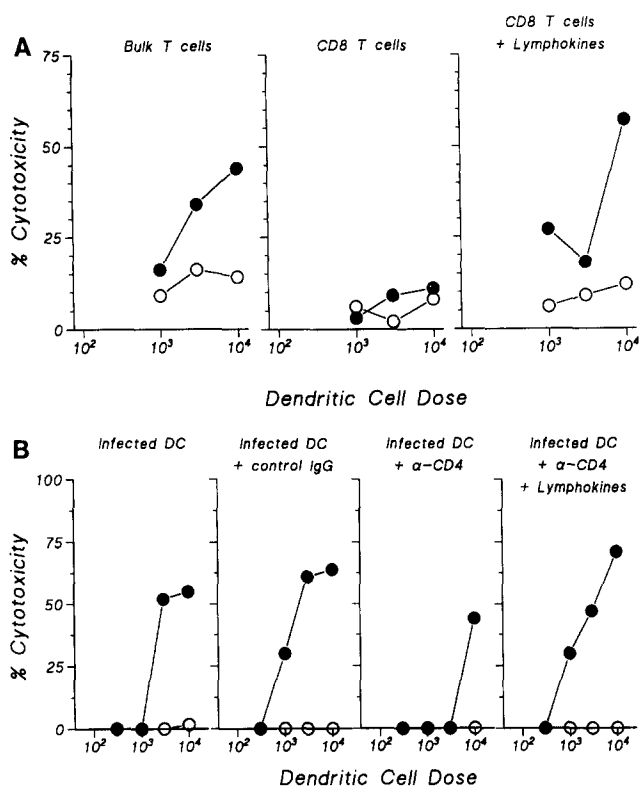
The importance of CD4<sup>+</sup> T cells for the generation of virus-specific CTLs could be demonstrated using blocking mAbs (Fig. 4 B). Anti-CD4 mAb GK1.5 significantly reduced the response, and the block was reversed by adding exogenous lymphokines. At the highest stimulator dose, GK1.5 did not totally block the CTL response. This is con-

sistent with the fact that GK1.5 did not completely block CD4 proliferation to infected APCs (data not shown).

**Capacity of Class II-Negative Cells to Stimulate CTL.** The above data suggested that one reason for the intense stimulatory capacity of dendritic cells is their capacity to stimulate class II-restricted helper T cells and the production of requisite lymphokines. We therefore tested the APC function of cells that lack class II MHC molecules, choosing the P815 cell line, since this line acts as a target for class I-restricted CTLs. In contrast to dendritic cells, infected P815 cells did not stimulate CTL development from inactive precursors (Fig. 5 A). P815 cells functioned as excellent stimulators when supplemented with a lymphokine-rich supernatant.

We observed similar results when we used the H-2<sup>k</sup> L929 cell line as the stimulator. Here, instead of using infected cells, we used a variant that had been transduced with the influenza NP gene carried by a retroviral vector (34). By using this L929-NP cell line, we could rule out any inhibitory effects of influenza virus, a cytotoxic virus, on APC function. L929-NP cells were stained positively with anti-NP mAb (data not shown) and were efficiently lysed by H-2<sup>k</sup>-restricted CTLs; however, NP-expressing L cells were poor stimulators of a CTL response unless supplemented with exogenous lymphokines (Fig. 5 B).

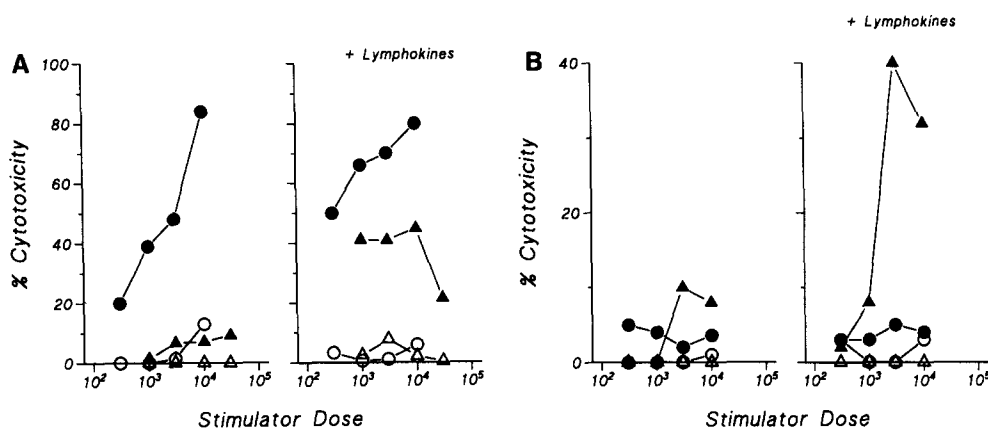
**Stimulation of CTLs with a Synthetic NP Peptide.** Given the need for CD4<sup>+</sup> helper cells, it seemed unlikely that dendritic cells pulsed with a class I-restricted NP peptide could direct the formation of CTLs. This proved to be the case with either NP147-161 or NP147-155 peptides. CTLs could only be induced by peptide-pulsed APCs if the cultures were supplemented with lymphokines (Fig. 6 A). These findings suggested that dendritic cells pulsed with peptide were poor stimulators of helper lymphokine production. To assess this directly, we examined the production of IL-2 and IL-6, which have been shown to enhance CTL development by dendritic cells (35). Virus-infected dendritic cells stimulated high levels of IL-2 and IL-6 secretion from bulk T cells, but peptide-pulsed dendritic cells were inactive (Fig. 6 B). It is to be pointed out that at higher APC doses, both infected and uninfected dendritic cells induce a significant syngeneic MLR from primed or unprimed CD4<sup>+</sup> T cells, resulting in the production of helper lymphokines (36).



**Figure 4.** Induction of influenza-specific CTLs by dendritic cells requires functional CD4<sup>+</sup> helper cells or their lymphokine products. (A) 10<sup>5</sup> bulk T or CD8<sup>+</sup> T cells were cultured with graded doses of infected dendritic cells. (B) 3 × 10<sup>5</sup> purified T cells were cultured with graded doses of dendritic cells in flat-bottomed wells. The control mAb to Thy-1, B5-5, and the anti-CD4 mAb GK1.5 were both used as ascites, at a final dilution of 1:300. Cultures were supplemented with 25% allogeneic MLR supernatant as a source of lymphokines. After 5 d, cytotoxic activity was assayed on uninfected (○) and infected (●) P815 targets.

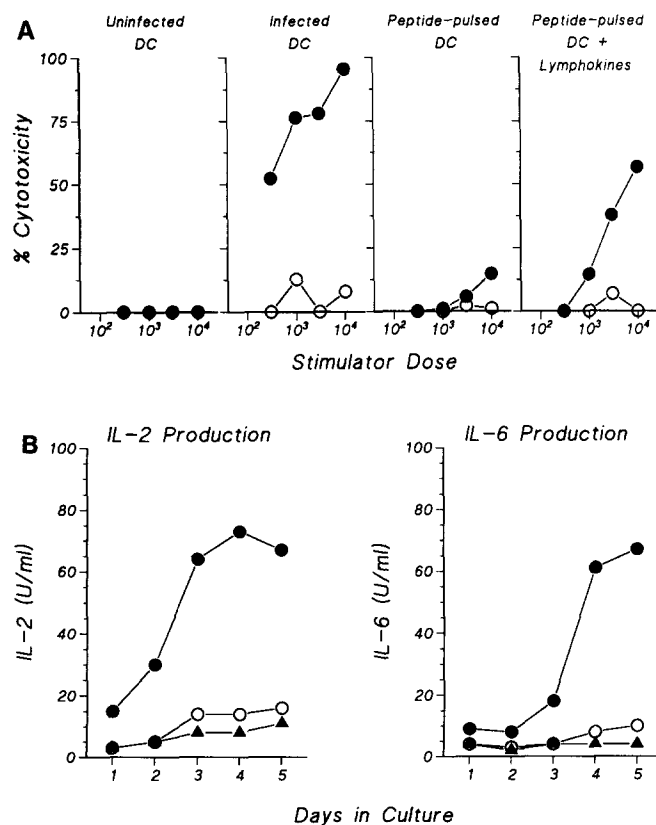
## Discussion

The recognition of viral antigens by CTLs has been studied intensively, so that presentation of viral peptides on MHC class I products is now understood in some detail (11). In such studies, the CTLs have already been activated, typically as long-term cell lines or in the case of primary cultures, by stimulating heterogeneous cell populations with virus and exogenous lymphokines. In this report, we have confirmed prior findings that dendritic cells are specialized APCs for generating virus-specific CTLs from inactive precursors (13–16). Given the apparently critical role of dendritic cells in inducing CTLs (Fig. 1), we have focused on two aspects of mechanism: the need for viral protein synthesis and the contribution of helper T cells.



**Figure 5.** Cell lines lacking class II MHC molecules are poor stimulators of CTL formation. (A)  $10^5$  purified C  $\times$  D2 T cells were cultured with graded doses of infected P815 (triangles) or dendritic cells (circles). (Right) Cultures were supplemented with 25% allogeneic MLR supernatant. After 5 d, cytotoxic activity was measured in a  $^{51}\text{Cr}$  release assay with uninfected (open symbols) and infected (filled symbols) P815 targets. At the highest dose of P815 stimulators used, the cultures became very acidic, which could explain the decrease in proliferation and cytotoxicity. (B) T cells from primed (C3H  $\times$  DBA/2)F<sub>1</sub>

mice were cultured with graded doses of L929-NP stimulators. (Right) Cultures were supplemented with 25% allogeneic MLR supernatant. After 5 d cytotoxicity was assayed on uninfected (open symbols) and infected (filled symbols) L929 (H-2<sup>k</sup>; triangles) and P815 (H-2<sup>d</sup>; circles) targets.



**Figure 6.** Dendritic cells pulsed with the NP147-155 peptide do not induce CTL or lymphokine secretion. Dendritic cells were left untreated, infected, or incubated with  $10\ \mu\text{M}$  NP147-155 peptide overnight. (A)  $10^5$  purified T cells were cultured with graded doses of these dendritic cells. Cultures were supplemented with 25% allogeneic MLR supernatant as a source of lymphokines. Cytotoxic activity was measured on uninfected (O) and infected (●) P815 targets. (B)  $3 \times 10^6$  purified T cells were cultured in macrowells with  $10^5$  uninfected (O), infected (●), or peptide-pulsed (▲) dendritic cells. On the days indicated in the figure, the culture supernatants were collected and assayed for IL-2 and IL-6.

*The Need for Viral Protein Synthesis within Dendritic Cells.* MHC class I molecules acquire peptides derived from the proteolysis of antigens within the cytoplasm (reviewed in reference 37). A pathway for the presentation of exogenous proteins on class I, without the need for de novo synthesis of the antigen within the APC, has also been described (17, 18). We wondered if dendritic cells, being specialized APCs, had such a mechanism for charging class I MHC in the absence of de novo viral protein synthesis. If exogenous processing could occur, dendritic cells would not have to be infected to act as APCs for many different viruses.

However, an exogenous pathway could not be demonstrated. In agreement with the findings of Morrison et al., and Braciale and Yap (12, 38) in other cells, UV inactivation of the virus, which inhibits viral replication and protein synthesis, led to a dramatic reduction in presentation (Fig. 2). Dendritic cells also did not present bromelain-treated virus, which lacks the HA needed for the delivery of viral RNA into the cytoplasm. The UV- and bromelain-treated forms of influenza were taken up efficiently, because dendritic cells pulsed with these exogenous viral antigens stimulated vigorous proliferation of CD4<sup>+</sup> T cells (Fig. 3). Therefore, dendritic cells do not appear to possess an alternative processing pathway by which exogenous antigens can be presented on class I MHC molecules. Nair et al. (39) have recently demonstrated that exogenous antigens can be presented by dendritic cells only if delivered directly to the cytoplasm using acid-sensitive liposomes. It is not yet clear if this pathway can deliver sufficient quantities of antigen to generate CTLs as efficiently as infectious virus.

*Helper Cell Requirements and the Immunogenicity of Class I-restricted Peptides.* A second issue relates to the need for helper T cells during dendritic cell-mediated CTL development. Prior data in the allogeneic MLR showed that dendritic cells could induce CD8<sup>+</sup> CTLs in the apparent absence of helper cells (21-23). CD4<sup>+</sup> helper T cells are known to potentiate virus-specific CTL responses in vitro (40) and in

vivo (41–43), although prior studies were not carried out using dendritic cells as stimulators.

We noted a marked amplification of the CTL response to virus in vitro by CD4<sup>+</sup> helper T cells and their lymphokine products, even when dendritic cells were used as stimulators (Fig. 4). The helper factors were antigen nonspecific, since active factors were present in supernatants derived from CD4<sup>+</sup> T cells that were not primed with influenza but instead were stimulated with allogeneic APCs. There are contrasting studies in which virus-specific CTL develop in the absence of CD4<sup>+</sup> T cells (44–47). Possibly helper cells are not essential but accelerate the CTL response, allowing the host to develop resistance more quickly or in the face of larger viral inocula. Stimulated CD8<sup>+</sup> cells can secrete IL-2 and IFN- $\gamma$  (48–50), but the adequacy of lymphokine secretion may be influenced by the proportion of responding T cells. In a CD4-independent MLR, >1% of the CD8<sup>+</sup> cells respond to antigens presented by dendritic cells. These large numbers may produce enough lymphokine to provide help (22). In contrast, only 1:14,000 to 1:2,400 of influenza-primed populations are responsive to influenza antigens (51, 52).

We also examined the stimulatory capacity of cells that could not stimulate class II-restricted T cell help. Class II-negative cell lines, either infected with live virus or transfected with the gene for influenza NP, were tested as APCs. A need for helper lymphokines was observed (Fig. 5), even though these cells were excellent targets for mature CTLs induced by dendritic cells. Similarly, dendritic cells, when pulsed with peptides that could be presented on class I but not class II MHC molecules, did not induce CTLs (Fig. 6 A) or helper lymphokines (Fig. 6 B). Our studies indicate that an important mechanism of dendritic cell function is the capacity to present antigens to both CD8<sup>+</sup> cytotoxic and CD4<sup>+</sup> helper T cells.

The direct stimulation of class I-restricted CTLs with peptide appears to be difficult. The NP147-161 peptide, as well as peptides representing other CTL epitopes, fail to prime CTLs in vivo (53–55). Recently, two groups studied in vivo priming of CTLs with peptides derived from the lymphocytic choriomeningitis virus and influenza NPs (56, 57). Both concluded that responses required CD4<sup>+</sup> helper T cells. In some instances, peptides that are effective immunogens are longer than those required to sensitize targets for lysis by CTLs (54). Perhaps increasing the length has included a helper epitope.

Although the addition of lymphokines increased the potency of peptide-pulsed dendritic cells, these stimulators were never as efficient as virus-infected dendritic cells (Fig. 6 A). When we added lymphokines to T cells that had been stimulated by infected APCs in the presence of anti-CD4 blocking mAbs, we were able to restore vigorous CTL responses (Fig. 4 B). Surprisingly, our preliminary data are that the concentration of peptide required by dendritic cells for presentation to developing CTLs is >1,000 times greater than that required to sensitize target cell lines for lysis by mature CTLs. Infectious virus likely differs from peptides as a means for charging APCs with antigen. More peptide may gain access to den-

dritic cell class I molecules via the endogenous route, and/or live influenza may affect APC adhesion molecules and costimulatory functions.

Previous studies of influenza-specific helper T cells have focused on external proteins as antigens, particularly the viral HA. Both strain-specific and broadly crossreactive helper epitopes have been described (58–60). By priming mice in vivo with the NT60 strain (H3N2) and then restimulating in vitro with the PR8 strain (H1N1), we generated T cells responding to determinants shared between virus strains; therefore, help had to be derived from crossreactive helper T cells. Indeed, bromelain-treated virus that lacked envelope proteins stimulated the vigorous proliferation of crossreactive CD4<sup>+</sup> T cells (Fig. 3). The influenza-specific, crossreactive CD4<sup>+</sup> T cell response is a powerful one. It can be detected in spleen after intranasal priming, whereas most studies on murine helper T cell responses require the use of draining lymph nodes and local foot pad immunization. Also, the helper response persists for months after infection, whereas lymph node priming wanes in weeks.

*Pathways for Generating CTL In Vivo.* During the course of infection, influenza productively infects the epithelial cells lining the respiratory tract, and 3–7 d later, influenza-specific CTLs can be detected in lung tissue and in the airway (61). There are several possible mechanisms for the generation of these CTLs in vivo. First, CTLs may be induced by virus-infected, respiratory epithelial cells. However, class II-negative cell lines cannot stimulate CTLs in vitro without lymphokines derived from activated CD4<sup>+</sup> T cells (Fig. 5). Although infected epithelial cells can probably be lysed by CTLs, their lack of class II MHC molecules, and/or the inability to activate CD4<sup>+</sup> helper T cells, could obviate the induction of CTL responses.

A second possible pathway is that dendritic cells acting alone initiate influenza-specific CTLs. Dendritic cells can acquire antigens in the lung (62), where the APCs form an impressive array in the airway epithelium (63), much like the dendritic cells of the epidermis. Dendritic cells in the respiratory tract, like those in other tissues, may migrate to the draining lymph nodes (64), where they can present viral antigens simultaneously to class II-restricted helper T cells and to class I-restricted CTL precursors found in the recirculating pool of quiescent lymphocytes.

A third pathway, which pertains more to viruses that do not infect dendritic cells, has two stages in which dendritic cells take up virus and stimulate helper T cells. The latter synthesize the lymphokines needed for the development of CTL by those infected, class I-expressing cells, which are the natural targets for a given virus. Since dendritic cells are potent inducers of helper T cells that are broadly crossreactive (Fig. 2 B), this pathway might be exploited to generate more effective vaccines to influenza. Crossreactive helper T cells can secrete the antiviral agent IFN- $\gamma$ , augment the production of CTLs as described here, and amplify the formation of neutralizing antibodies through the mechanism of intermolecular help (65).



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## References

1. Kees, U., and P.H. Krammer. 1984. Most influenza A virus-specific memory cytotoxic T lymphocytes react with antigenic epitopes associated with internal virus determinants. *J. Exp. Med.* 159:365.
2. Braciale, T.J. 1977. Immunologic recognition of influenza virus-infected. 1. Generation of a virus-strain specific and a cross-reactive subpopulation of cytotoxic T cells in the response to type A influenza viruses of different subtypes. *Cell. Immunol.* 33:423.
3. Zweerink, H.J., B.A. Askonas, D. Millican, S.A. Courthedge, and J.J. Skehel. 1977. Cytotoxic T cells to type A hemagglutinin: Viral hemagglutinin induces A-strain-specificity while infected cells confer cross-reactive cytotoxicity. *Eur. J. Immunol.* 7:630.
4. McMichael, A.J., and B.A. Askonas. 1978. Influenza virus-specific cytotoxic T cells in man: induction and properties of the cytotoxic cell. *Eur. J. Immunol.* 8:705.
5. Lukacher, A.E., V.L. Braciale, and T.J. Braciale. 1984. In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. *J. Exp. Med.* 160:814.
6. Lin, Y.L., and B. Askonas. 1981. Biological properties of an influenza A virus-specific killer T cell clone: inhibition of virus replication in vivo and induction of delayed-type hypersensitivity reactions. *J. Exp. Med.* 154:225.
7. Yap, K.L., G.L. Ada, and I.F.G. McKenzie. 1978. Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. *Nature (Lond.)* 273:238.
8. McMichael, A.J., F.M. Gotch, G.R. Noble, and P.A.S. Beare. 1983. Cytotoxic T-cell immunity to influenza. *N. Engl. J. Med.* 309:13.
9. Zinkernagel, R.M., and P.C. Doherty. 1974. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature (Lond.)* 248:701.
10. Doherty, P.C., and R.M. Zinkernagel. 1975. H-2 compatibility is required for T cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus. *J. Exp. Med.* 141:502.
11. Townsend, A.R.M., F.M. Gotch, and J. Davey. 1985. Cytotoxic T cells recognize fragments of the influenza nucleoprotein. *Cell.* 42:457.
12. Morrison, L.A., A.E. Lukacher, V.L. Braciale, D.P. Fan, and T.J. Braciale. 1986. Differences in antigen presentation to MHC class I- and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. *J. Exp. Med.* 163:903.
13. Macatonia, S.E., P.M. Taylor, S.D. Knight, and B.A. Askonas. 1989. Primary stimulation by dendritic cells induces anti-viral proliferative and cytotoxic T cell responses in vitro. *J. Exp. Med.* 169:1255.
14. Hengel, H., M. Lindner, H. Wagner, and K. Heeg. 1987. Frequency of herpes simplex virus-specific murine cytotoxic T lymphocyte precursors in mitogen- and antigen-driven primary in vitro T cell responses. *J. Immunol.* 139:4196.
15. Kast, W.M., C.J.P. Wong, B.O. Roep, A.C. Voordouw, and C.J.M. Melief. 1988. Failure or success in the restoration of virus-specific cytotoxic T lymphocyte response defects by dendritic cells. *J. Immunol.* 140:3186.
16. Macatonia, S.E., S. Patterson, and S.C. Knight. 1991. Primary proliferative and cytotoxic T cell response to HIV induced in vitro by human dendritic cells. *Immunology.* 74:399.
17. Rock, K.L., S. Gamble, and L. Rothstein. 1990. Presentation of exogenous antigen with class I major histocompatibility complex molecules. *Science (Wash. DC)* 249:918.
18. Carbone, F.R., and M.J. Bevan. 1990. Class I-restricted processing and presentation of exogenous cell-associated antigen in vivo. *J. Exp. Med.* 171:377.
19. Bevan, M.J. 1976. Cross-priming for a secondary cytotoxic response to minor H. antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J. Exp. Med.* 143:1283.
20. Korngold, R., and J. Sprent. 1980. Selection of cytotoxic T-cell precursors specific for minor histocompatibility determinants. 1. Negative selection across H-2 barriers induced with disrupted cells but not with glutaraldehyde-treated cells: evidence for antigen processing. *J. Exp. Med.* 151:314.
21. Inaba, K., J.W. Young, and R.M. Steinman. 1987. Direct activation of CD8<sup>+</sup> cytotoxic T lymphocytes by dendritic cells. *J. Exp. Med.* 166:182.
22. Young, J.W., and R.M. Steinman. 1990. Dendritic cells stimulate primary human cytolytic lymphocyte responses in the absence of CD4<sup>+</sup> helper T cells. *J. Exp. Med.* 171:1315.
23. Boog, C.J.P., J. Boes, and C.J.M. Melief. 1988. Stimulation with dendritic cells decreases or obviates the CD4<sup>+</sup> helper cell requirement in cytotoxic T lymphocyte responses. *Eur. J. Immunol.* 18:219.
24. Brand, C.M., and J.J. Skehel. 1972. Crystalline antigen from the influenza virus envelope. *Nature (Lond.)* 238:145.
25. Crowley, M., K. Inaba, M. Witmer-Pack, and R.M. Steinman. 1989. The cell surface of mouse dendritic cells: FACS analyses

- of dendritic cells from different tissues including thymus. *Cell Immunol.* 118:108.
26. Wibo, M., and B. Poole. 1974. Protein degradation in cultured cells. II. The uptake of chloroquine by rat fibroblasts and the inhibition of cellular protein degradation and cathepsin B. *J. Cell Biol.* 63:430.
  27. Rotzschke, O., K. Falk, K. Deres, H. Schild, M. Norda, J. Metzger, G. Jung, and H.-G. Rammensee. 1990. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature (Lond.)* 348:252.
  28. Merrifield, B. 1986. Solid phase synthesis. *Science (Wash. DC)* 232:341.
  29. Yewdell, J.W., A. Yellen, and T. Bachi. 1988. Monoclonal antibodies localize events in the folding, assembly, and intracellular transport of the influenza virus hemagglutinin glycoprotein. *Cell.* 52:843.
  30. Steinman, R.M., B. Gutchinov, M.D. Witmer, and M.C. Nussenzweig. 1983. Dendritic cells are the principal stimulators of the primary mixed leukocyte reaction in mice. *J. Exp. Med.* 157:613.
  31. Inaba, K., R.M. Steinman, W.C. Van Voorhis, and S. Muramatsu. 1983. Dendritic cells are critical accessory cells for thymus-dependent antibody responses in mouse and man. *Proc. Natl. Acad. Sci. USA.* 80:6041.
  32. Matlin, K.S., H. Reggio, A. Helenius, and K. Simons. 1981. Infectious entry pathway of influenza virus in a canine kidney cell line. *J. Cell Biol.* 91:601.
  33. Inaba, K., and R.M. Steinman. 1984. Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen-processing cell) requirements for growth and lymphokine release. *J. Exp. Med.* 160:1717.
  34. Fetteen, J.V., N. Roy, and E. Gilboa. 1991. A frameshift mutation at the NH2 terminus of the nucleoprotein gene does not affect generation of cytotoxic T lymphocyte epitopes. *J. Immunol.* 147:2697.
  35. Ming, J.E., R.M. Steinman, and A. Granelli-Piperno. 1992. Interleukin 6 enhances the generation of CTL in the allogenic mixed leukocyte reaction. *Clin. Exp. Immunol.* In press.
  36. Inaba, K., A. Granelli-Piperno, and R.M. Steinman. 1983. Dendritic cells induce T lymphocytes to release B cell-stimulating factors by an interleukin 2-dependent mechanism. *J. Exp. Med.* 158:2040.
  37. Townsend, A., and H. Bodmer. 1989. Antigen recognition by class I-restricted T lymphocytes. *Annu. Rev. Immunol.* 7:601.
  38. Braciale, T.J., and K.L. Yap. 1978. Role of viral infectivity in the induction of influenza virus-specific cytotoxic T cells. *J. Exp. Med.* 147:1236.
  39. Nair, S., F. Zhou, R. Reddy, L. Huang, and B.T. Rouse. 1992. Soluble proteins delivered to dendritic cells via pH-sensitive liposomes induce primary cytotoxic T lymphocyte responses in vitro. *J. Exp. Med.* 175:609.
  40. Ashman, R.B., and A. Mullbacher. 1979. A T helper cell for anti-viral cytotoxic T-cell responses. *J. Exp. Med.* 150:1277.
  41. Zinkernagel, R.M., G.N. Callahan, A. Althage, S. Cooper, J.W. Streilein, and J. Klein. 1978. The lymphoreticular system in triggering virus plus self-specific cytotoxic T cells: evidence for T help. *J. Exp. Med.* 147:897.
  42. Kast, M., A.M. Bronkhorst, L.P. deWaal, and C.J.M. Melief. 1986. Cooperation between cytotoxic and helper T lymphocytes in protection against lethal sendai virus infection. *J. Exp. Med.* 164:723.
  43. Leist, I.P., M. Kohler, and R.M. Zinkernagel. 1989. Impaired generation of anti-viral cytotoxicity against lymphocytic choriomeningitis and vaccinia virus in mice treated with CD4-specific monoclonal antibody. *Scand. J. Immunol.* 30:679.
  44. Nash, A.A., A. Jayasuriya, J. Phelan, S.P. Cobbold, H. Waldmann, and T. Prospero. 1987. Different roles for L3T4<sup>+</sup> and Lyt 2<sup>+</sup> T cell subsets in the control of an acute herpes simplex virus infection of the skin and nervous system. *J. Virol.* 68:825.
  45. Ahmed, R., L.D. Butler, and L. Bhatti. 1988. T4<sup>+</sup> T helper cell function in vivo: differential requirement for induction of antiviral cytotoxic T-cell and antibody responses. *J. Virol.* 62:2102.
  46. Buller, M.L., K.L. Holmes, A. Hugin, T.N. Frederickson, and H.C. Morse III. 1987. Induction of cytotoxic T-cell response in vivo in the absence of CD4 helper cells. *Nature (Lond.)* 328:77.
  47. Rahemtulla, A., W.P. Fung-Leung, M.W. Schilham, T.M. Kundig, S.R. Sambhara, A. Narendran, A. Arabian, A. Wakeham, C.J. Paige, R.M. Zinkernagel, R.G. Miller, and T.W. Mak. 1991. Normal development and function of CD8<sup>+</sup> cells but markedly decreased helper cell activity in mice lacking CD4. *Nature (Lond.)* 353:180.
  48. Andrus, L., A. Granelli-Piperno, and E. Reich. 1984. Cytotoxic T cells both produce and respond to interleukin 2. *J. Exp. Med.* 159:647.
  49. Mizuochi, T., S. Ono, T.R. Malek, and A. Singer. 1986. Characterization of two distinct primary T cell populations that secrete interleukin 2 upon recognition of class I or class II major histocompatibility antigens. *J. Exp. Med.* 163:603.
  50. Klein, J.R., D.H. Raulet, M.S. Pasternack, and M.J. Bevan. 1982. Cytotoxic T lymphocytes produce immune interferon in response to antigen or mitogen. *J. Exp. Med.* 155:1198.
  51. Wysocka, M., and J.P. Bennis. 1988. Limiting dilution analysis of memory cytotoxic T lymphocytes specific for individual influenza virus gene products. *Cell Immunol.* 112:425.
  52. Owen, J.A., K.I. Dudzik, L. Klein, and D.R. Dorer. 1988. The kinetics of generation of influenza-specific cytotoxic T-lymphocyte precursor cells. *Cell Immunol.* 111:247.
  53. Deres, K., H. Schild, K.-H. Wiesmuller, G. Jung, and H.-G. Rammensee. 1989. In vivo priming of virus-specific cytotoxic T lymphocytes with synthetic lipopeptide vaccine. *Nature (Lond.)* 342:561.
  54. Carbone, F.R., and M.J. Bevan. 1989. Induction of ovalbumin-specific cytotoxic T cells by in vivo peptide immunization. *J. Exp. Med.* 169:603.
  55. Watari, E., B. Dietzschold, G. Szokan, and E. Heber-Katz. 1987. A synthetic peptide induces long-term protection from lethal infection with herpes simplex virus 2. *J. Exp. Med.* 165:459.
  56. Fayolle, C., E. Deriaud, and C. Leclerc. 1992. In vivo induction of cytotoxic T cell response by a free synthetic peptide requires CD4<sup>+</sup> T cell help. *J. Immunol.* 147:4069.
  57. Gao, X.-M., B. Zheng, F.Y. Liew, S. Brett, and J. Tite. 1991. Priming of influenza virus-specific cytotoxic T lymphocytes in vivo by short synthetic peptides. *J. Immunol.* 147:3268.
  58. Hurwitz, J.L., C.J. Hackett, E.C. McAndrew, and W. Gerhard. 1985. Murine TH response to influenza virus: recognition of hemagglutinin, neuraminidase, matrix, and nucleoproteins. *J. Immunol.* 134:1994.
  59. Mills, K.H.G., J.J. Skehel, and D.B. Thomas. 1986. Extensive diversity in the recognition of influenza virus hemagglutinin by murine T helper clones. *J. Exp. Med.* 163:1477.
  60. Burt, D.S., K.H.G. Mills, J.J. Skehel, and D.B. Thomas. 1989. Diversity of the class II (I-Ak/I-Ek)-restricted T cell repertoire for influenza hemagglutinin and antigenic drift. *J. Exp. Med.*

- 170:383.
61. Ennis, F.A., M.A. Wells, G.M. Butchko, and P. Albrecht. 1978. Evidence that cytotoxic T cells are part of the host response to influenza pneumonia. *J. Exp. Med.* 148:1241.
  62. Holt, P.G., M.A. Schon-Hegrad, and J. Oliver. 1987. MHC class II antigen-bearing dendritic cells in pulmonary tissues of the rat. Regulation of antigen presentation activity by endogenous macrophage populations. *J. Exp. Med.* 167:262.
  63. Schon-Hegrad, M.A., J. Oliver, P.G. McMennamin, and P.G. Holt. 1991. Studies on the density, distribution, and surface phenotype of intraepithelial class II major histocompatibility complex antigen (Ia)-bearing dendritic cells (DC) in the conducting airways. *J. Exp. Med.* 173:1345.
  64. Austyn, J.M., J.W. Kupiec-Weglinski, D.F. Hankins, and P.J. Morris. 1988. Migration patterns of dendritic cells in the mouse. Homing to T cell-dependent areas of spleen, and binding within marginal zone. *J. Exp. Med.* 167:646.
  65. Russell, S.M., and F.Y. Liew. 1979. T cells primed by influenza virion internal components can cooperate in the antibody response to haemagglutinin. *Nature (Lond.)* 280:147.