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*J Immunol* 2000; 165:1182-1190; doi: 10.4049/jimmunol.165.3.1182

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Requirement of Mature Dendritic Cells for Efficient Activation of Influenza A-Specific Memory CD8+ T Cells

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It is critical to identify the developmental stage of dendritic cells (DCs) that is most efficient at inducing CD8+ T cell responses. Immature DCs can be generated from monocytes with GM-CSF and IL-4, while maturation is accomplished by the addition of stimuli such as monocyte-conditioned medium, CD40 ligand, and LPS. We evaluated the ability of human monocytes and immature and mature DCs to induce CD8+ effector responses to influenza virus Ags from resting memory cells. We studied replicating virus, nonreplicating virus, and the HLA-A*0201-restricted influenza matrix protein peptide. Sensitive and quantitative assays were used to measure influenza A-specific immune responses, including MHC class I tetramer binding assays, enzyme-linked immunospot assays for IFN-γ production, and generation of cytokotoxic T cells. Mature DCs were demonstrated to be superior to immature DC in eliciting IFN-γ production from CD8+ effector cells. Furthermore, only mature DCs, not immature DCs, could expand and differentiate CTL precursors into cytokotoxic effector cells over 7 days. An exception to this was immature DCs infected with live influenza virus, because of the virus’s known maturation effect. Finally, mature DCs pulsed with matrix peptide induced CTLs from highly purified CD8+ T cells without requiring CD4+ T cell help. These differences between DC stages were independent of Ag concentrations or the number of immature DCs. In contrast to DCs, monocytes were markedly inferior or completely ineffective stimulators of T cell immunity. Our data with several qualitatively different assays of the memory CD8+ T cell response suggest that mature cells should be considered as immunotherapeutic adjuvants for Ag delivery.


Dendritic cells (DCs) are potent APCs for the initiation of both T cell- and B cell-dependent immunity. In the periphery DCs exist in an immature state, which is characterized by an ability to effectively capture Ags via phagocytosis, macrophagocytosis, and receptor-mediated endocytosis (1–4). Following Ag acquisition, DCs become activated, secrete macrophage inflammatory protein-3α and express its receptor CCR7, migrate to lymph nodes, and undergo further maturation (5, 6). During maturation DCs also down-regulate Ag capture capacity, up-regulate the expression of MHC class I and II and costimulatory molecules, acquire the ability to produce IL-12, and become potent initiators of both primary and secondary T cell responses (reviewed in Ref. 7).

Given the central role of DCs in the initiation of immunity, there has been considerable effort to harness their adjuvant properties to prime and boost CD8+ T cell responses against tumors and viruses responsible for chronic infections (8, 9). However, there is no consensus regarding the DC developmental stage that will be most efficient in stimulating immunity in humans. Immature DCs derived from monocytes cultured in the presence of GM-CSF and IL-4 have been explored for treatment of patients with lymphoma, melanoma, and prostate cancer (10–12). Recently, we showed that a single injection of monocyte-derived mature DCs in healthy volunteers elicited rapid and broad T cell immunity to keyhole limpet hemocyanin, tetanus toxoid, and influenza matrix protein (MP) (13). Surprisingly, immature and mature human DC have yet to be compared in depth, in vitro and in vivo, for their capacity to activate CD8+ effectors from resting cells. Therefore, it is critical to identify the most potent stage of human DCs for the design of the most efficient immunotherapy protocol. This is especially true in light of recent in vivo studies in mice indicating that activation and maturation of the DC via CD40 are required for the development of optimal antiviral and anti-tumor immunities (14–17).

In the present work we took advantage of the influenza A virus system, which has been well characterized (18), to compare the abilities of various APCs to elicit CD8+ effector responses from resting memory cells, IFN-γ production measured by ELISPOT assay, cytotoxic function assayed by chromium release, and MHC class I tetramer binding assay were used to compare the abilities of monocytes and monocyte-derived immature and mature DC to activate influenza A-specific CD8+ T cells. The APCs were exposed to different forms of influenza Ags, such as the immunodominant HLA-A*0201-restricted matrix protein peptide (19) and...
nonreplicating and replicating influenza viruses. While all APCs were capable of inducing IFN-γ production from memory CD8+ T cells in the ELISSPOT assay, mature DCs were up to 30-fold more efficient than immature DCs and monocytes. Furthermore, only the mature DCs were capable of expanding Ag-specific cytotoxic effector CD8+ T cells in a 7-day culture assay. Thus, the present and our previous in vivo data (13) support the use of mature DCs as vaccine and immunotherapy adjuvants where the goal is efficient differentiation of CD8+ T cells into cytotoxic effector cells.

Materials and Methods

Culture medium and cytokines

RPMI 1640 (Cellgro, Herdon, VA) was supplemented with 20 μg/ml gentamicin (Life Technologies, Gaithersburg, MD), 1 mM HEPES (Mediatech, Herndon, VA), and 1% human plasma, 5% heat-inactivated pooled human sera (PHS; c-Six Diagnostics, Mequon, WI), or 5% heat-inactivated single donor serum. Recombinant human GM-CSF (1 × 10^6 U/μg; Immunex, Seattle, WA) and recombinant human IL-4 (0.5 × 10^6 U/μg; Schering-Plough, Kenilworth, NJ) were used for in vitro use.

Cell isolation

Leukocyte-enriched buffy coats were obtained from the New York Blood Center. PBMCs were separated by density on Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ). Monocyte-enriched and T cell-enriched fractions were prepared by rosetting PBMCs with neuraminidase (Calbiochem-Novabiochem, La Jolla, CA)-treated SRBC (20).

T cells

Bulk CD8+ and CD4+ T cells were isolated from T cell-enriched fractions by negative selection as previously described (21, 22).

HLA *A0201 matrix peptide-specific T cell clone

A matrix peptide-specific CD8+ T cell clone was prepared according to published methods (23, 24). Mature DC and PBMC from an HLA A*0201-positive donor were pulsed with matrix peptide (10 μM/ml) and cultured for 7 days. Responding cells were cloned by limiting dilution in the presence of irradiated PBMC, EBV-transformed B lymphocytes, PHA, and IL-2 (Chiron Therapeutics, Emeryville, CA). The clone was shown to recognize MP at concentrations as low as 1 nM and by 100% binding to tetramerically complexes of MP and HLA A*0201.

Dendritic cells

DCs were generated by culturing the monocyte-enriched fraction with 1000 U/ml GM-CSF (Immunex) and 500 U/ml IL-4 (Schering-Plough) for 7 days. The cytokines were added to the cultures on days 0, 2, and 4. On day 5, nonadherent cells were collected and transferred to new plates, and the cultures were supplemented with monocyte-conditioned medium (MCM) at 50% (v/v) to induce maturation. Mature DCs were collected on day 7. Immature DCs were maintained in cultured GM-CSF and IL-4 for 4–5 days and washed extensively before use. MCM was prepared as previously described (25, 26). Briefly, 35 × 10^8 T cell-depleted cells were layered onto Ig-coated bacteriological plates for 1 h at 37°C in RPMI 1640 with 5% PHS. Nonadherent cells were removed, and the adherent cells were incubated in medium containing 1% plasma at 37°C for 24 h, after which the medium was collected for use as MCM. In some experiments 20 ng/ml LPS (Sigma, St. Louis, MO) was used as a maturation stimulus.

Influenza A virus infection

Cells were washed free of medium containing serum, resuspended to 1 × 10^7/ml in serum-free RPMI 1640, infected for 1 h at 37°C, and then washed three times with RPMI 1640 with 5% PHS. In all experiments cells were infected with 1000 hemagglutination U/ml of influenza A strain PR/8/34 (SPAFAS, Preston, CT) corresponding to a multiplicity of infection (MOI) of 2.

Monoclonal Abs

mAbs against the following 10 Ags were used: HLA DR, CD14, and CD25, (Becton Dickinson, Mountain View, CA); CD86 (IgG1, PharMingen San Diego, CA); CD83, CD40, and IgG2b (Immunotech, Coulter, Hialeah, FL); DC lysosomal-associated membrane protein (DC-LAMP) (gift from Dr. S. Lebecq, Schering-Plough, Dardilly, France); and BB7.2 (American Type Culture Collection, Manassas, VA). Secondary Ab was PE-conjugated F(ab')2 goat anti-mouse IgG (H and L chain; TAGO, Burlingame, CA). DC populations were phenotyped with the panel of mAbs listed above, and samples were analyzed on a Becton Dickinson FACScan using CellQuest software. Dead cells and contaminating lymphocytes were excluded by forward and side scatter properties.

Intracellular staining for MxA and DC-LAMP

Cells were fixed in 4% paraformaldehyde for 10 min by following wash twice in PBS with 1% FCS, 1% PHS, and 0.1% sodium azide. Saponin (1%; Calbiochem, La Jolla, CA) was added for 20 min to permeabilize the cells. Rabbit Ab to MxA and control IgG (Chemicon, Temecula, CA) were diluted with 0.1% saponin and added to the cells for 20 min. The cells were washed three times, followed 30-min incubation with 1/300 diluted goat anti-rabbit Ab (H+L chain; FITC conjugate, Jackson Immunoresearch Laboratories, West Grove, PA) at 4°C.

Assessment of cell viability

Uninfected or influenza virus-infected DCs were washed three times in PBS containing 1% PHS and 1% FCS followed by a Ca2+-enriched buffer, then incubated for 3 min with the annexin V-FITC and/or propidium iodide (Kayima Biomedical, Seattle, WA) at 4°C.

Induction of influenza virus-specific CTL

Monocytes and immature and mature DC, were washed in serum-free RPMI 1640 and resupplemented to 1 × 10^7 cells/ml. Live or heat-inactivated influenza virus (56°C, 35 min) was added at a final concentration of 1000 hemagglutination U/ml for 1 h at 37°C as previously described (21). Alternatively, APCs were pulse for 1 h with different doses of the HLA-A*0201-restricted matrix peptide (GILGFVFTL). DCs were then added in graded doses to constant numbers of purified T cells in 96- or 48-well plates (Costar, Cambridge, MA). After 7 days, the T cells were assayed for cytoxic activity on a TAP-"-aten, HLA-A*0201 MHC class II-negative T2 cell line (American Type Culture Collection) pulsed with 1 μM matrix peptide using a conventional 51Cr release assay (22). Target cells were labeled with Na2CrO4, as previously described (22). Control HLA-A*0201-restricted peptide was the HIV-1 gag epitope (SLYNTVATL). The specific lysis was determined by subtracting the percent killing of unpulsed T2 cells or gag peptide-pulsed from that of matrix peptide-pulsed T2 cells.

ELISPOT assay for IFN-γ release from single Ag-specific T cells

Ninety-six-well plates (Millititer, Millipore, Bedford, MA) were coated overnight at 4°C with 5 μg/ml of the primary anti-IFN-γ mAb (Mabtech, Stockholm, Sweden). The Ab-coated plates were washed four times with PBS and blocked with RPMI 1640 containing 5% PHS for 1 h at 37°C. Uninfected, peptide-pulsed, or influenza-infected monocytes and mature and immature DC were added to the wells together with T cells (1 × 10^5) and incubated for 6 h or overnight (14–18 h) at 37°C. Peptides were either pulsed or added at concentrations of 1–10 μM directly to wells. The peptides were titrated to give maximal responses. Wells were washed four times with PBS containing 0.05% Tween-20 (Sigma) followed by 2-h incubation with 1 μg/ml of the secondary Ab (biotin-conjugated anti-IFN-γ mAb; Mabtech, Stockholm, Sweden). Plates were washed four times in PBS with 0.1% Tween 20. Avidin-bound biotinylated HRP H (Vectastain Elite kit, Vector, Burlingame, CA) was added to the wells for 1 h at room temperature. The plates were washed four times in PBS with 0.1% Tween 20 followed by a 5-min incubation in stable diaminobenzidine (Research Genetics, Huntsville, AL) to develop the reaction. Tap water was added to stop the reaction. The spots were counted with a stereomicroscope (Stemi 2000 stereo microscope, Carl Zeiss, New York, NY) under magnifications of ×20–40. Only spots with a fuzzy border and a brown color were counted (27). Responses were considered positive if a minimum of 10 spot-forming cells/1 × 10^5 cells were counted after the control had been subtracted.

ELISPOT assay for IFN-γ measuring expansion of Ag-specific cells

Besides measuring cytotoxic effector function after 7 days, the expansion of Ag-specific T cells was also assessed in IFN-γ ELISPOT assays. The day 7 expanded T cells were harvested and added to the ELISPOT plate at 50,000 T cells/well and restimulated with DCs infected with live influenza virus, pulsed with MP or no Ag. In some assays MP was added without APCs. The assays were conducted as described above.
HLA class I tetramer complexes

Soluble peptide-MHC tetramers for HLA A*0201 were produced using the methods described by Dunbar et al. (28). Six or 7 days after coculture with Ag-pulsed monocytes or immature or mature DCs, responding T cells were incubated for 30 min at 37°C in 0.5 μl of MP HLA-A*0201 class I tetramers (PE conjugated) and washed. The cells were stained with anti-CD8 mAb conjugated with PerCP (Caltag, South San Francisco, CA) and FITC-conjugated anti-CD3 mAb (Dako, Carpinteria, CA), for 30 min at 4°C and then washed twice. Washing was followed by fixation in 1% formaldehyde with 1% FCS. Samples were analyzed on a Becton Dickinson FACS using CellQuest software.

Results

Effects of influenza A virus infection on immature and mature human DCs

The effects of influenza infection on immature DCs and mature DCs were first examined. In vitro, immature DCs can be generated from blood monocytes by culturing them in GM-CSF and IL-4. These cells are matured with the addition of 50% (v/v) MCM for 2 days and can be distinguished from immature cells by the expression of CD83 and the lysosomal marker DC-LAMP (29, 30). DC maturation also results in higher expression levels of MHC and costimulatory molecules (7). Twenty-four hours after infection with live influenza virus at a MOI of 2, >90% of both immature and mature DCs expressed viral hemagglutinin protein measured by FACS (Fig. 1A). We next evaluated the effects of influenza virus infection on the viability and phenotype of the two DC populations. Twelve hours following infection, extensive apoptosis was evident in the immature DC population (41% annexin V positive, 9% annexin V/PI double positive). In contrast, only 14% of mature DCs were annexin V positive and 9% were annexin V/PI double positive (Fig. 1B). Thus, at early times of infection, immature DCs appear to be more susceptible to the cytopathic effects of influenza infection than mature DCs. When lower MOIs were used, e.g., 0.2–0.02, the frequency of infection declined coordinately, while viability improved (data not shown).

Despite the extent of cell death observed in immature DC populations, we found that influenza infection of immature DCs induced maturation in a portion of the population as measured by the expression of the maturation-associated marker DC-LAMP (Fig. 1C). Consistent with previous observations (31), maturation was accompanied by an increase in the expression of MHC class I, MHC class II, and the costimulatory molecules CD40 and CD86 (data not shown). Infection with live virus, but not with nonreplicating heat-inactivated virus or exposure to the HLA-A*0201-restricted matrix peptide, resulted in the up-regulation of CD83 and DC-LAMP. In the DC populations matured with MCM, influenza A infection further enhanced the expression of these markers in cells that had not undergone full maturation. To assess whether influenza A virus infection directly induced DC maturation, immature DCs were infected with influenza, and cells expressing hemagglutinin were positively selected with magnetic beads, followed by analysis of surface markers. In this population up to 40%
of DCs expressed CD83 (data not shown). Similar findings of these maturation-inducing effects of live influenza virus were recently reported (31).

Finally, we examined the expression of MxA, a GTPase that inhibits cytoplasmic viral transcription and is known to mediate resistance to influenza (32, 33). Susceptibility to influenza virus infection in DCs is modulated by the expression of MxA, which is induced by LPS as well as infection with influenza virus (Fig. 1D) (31). Notably, MxA was not expressed in monocytes or in immature or MCM-matured DCs before influenza infection or LPS treatment; the latter served as a positive control for MxA up-regulation. Induction of MxA in immature DCs is thought to be due to the autocrine production of type I IFNs in response to influenza infection (31). As MCM itself failed to induce detectable levels at least by flow cytometric analysis of MxA (Fig. 1D), it is likely that the low levels of IFN-α described in these preparations (2–8 U/ml) (26) are insufficient to induce it. However, MCM-matured DCs are more resistant to the cytopathic effects of influenza, indicating that mature DCs may have additional, possibly MxA independent, antiviral mechanisms.

*Mature DCs are superior to immature DCs in inducing IFN-γ-secreting CD8+ effector cells*

We next compared the abilities of monocytes, immature DCs, and mature DCs to present different forms of influenza Ags and at varying ratios. When MP was the Ag, mature DCs consistently activated significant numbers of IFN-γ-producing T cells (Fig. 2B) even when one DC was used per 100 T cells. Influenza virus that is treated for 35 min at 56°C is rendered nonreplicating. We have previously shown that infection with heat-inactivated virus permits CD11c-positive DCs to induce virus-specific CTLs (22). When we evaluated presentation of nonreplicating heat-inactivated influenza virus, monocyte-derived mature DCs were superior to both immature DCs and monocytes by 3- to 15-fold, and significant numbers of IFN-γ-secreting effectors could be elicited with low numbers of mature DCs (Fig. 2C, representative of five experiments). The immature DCs induced responses, but only when one immature DC was used to every 10 T cells. This indicates that at very low levels of virus Ag, which are obtained when using nonreplicating virus, only the mature DCs are capable of presenting Ag efficiently.
Finally, we evaluated T cell stimulatory effects of live influenza virus-infected cells (Fig. 2D). Monocytes induced IFN-\(\gamma\)-producing effector cells when used at the relatively high monocyte:T cell ratio 1:10. Following infection with live influenza virus, the immature DCs demonstrated an enhanced capacity to activate IFN-\(\gamma\) production from T cells. We attribute this difference to the maturation impact of influenza virus. However, the cells were never as effective as live virus-infected mature DCs, presumably because many immature DCs undergo apoptosis following infection with influenza virus (Fig. 1B). Similar results were obtained when different donors were analyzed (n = 5; data not shown).

To verify that the responding T cells in the ELISPOT assay were indeed CD8\(^{+}\) T cells, we depleted bulk T cell populations of either CD8\(^{+}\) or CD4\(^{+}\) T cells. The bulk population as well as pure CD8\(^{+}\) and CD4\(^{+}\) T cell populations were cocultured with live influenza virus-infected monocytes (T:APC ratio = 1:1) or mature DC (T:APC ratio = 1:10). IFN-\(\gamma\) production was primarily elicited in bulk or CD8\(^{+}\) T cell-enriched populations (Fig. 3). More than 80% of the responses were lost with depletion of CD8\(^{+}\) T cells. These results indicate that within the 24-h span of our assay, the cells responsible for IFN-\(\gamma\) production are CD8\(^{+}\) T cells.

To further compare the abilities of monocytes, immature DCs, and mature DCs to present different forms of influenza Ags, we took advantage of an HLA A*0201 matrix peptide-specific CD8\(^{+}\) T cell clone with high avidity for MP. This clone responds to 1 nM MP on T2 cells by producing IFN-\(\gamma\) and IL-2 and exerting

**FIGURE 3.** CD8\(^{+}\) T cells are the major producers of IFN-\(\gamma\). Monocytes and mature DCs were infected with live influenza virus and cocultured with bulk T cells or negatively selected CD8\(^{+}\) or CD4\(^{+}\) T cells. The APC:T cell ratio for monocytes was 1:1, and that for mature DCs was 1:10. The controls were APCs and T cells without Ag and APCs or T cells alone. These values ranged from 0 to 20 SFC/2 \times 10^5 T cells and were subtracted from the experimental values. Data are shown as the number of SFC per 10^6 cells.

**FIGURE 4.** Mature DCs present influenza Ags to a MP-specific CD8\(^{+}\) T cell clone more efficiently than monocytes and immature DCs. Monocytes (MO), immature DCs (IDC), and mature DCs (MDC) were pulsed with 1 nM (A) or 10 \(\mu\)M (B) HLA A*0201-specific MP, infected with heat-inactivated (C) or live (D) influenza A virus and cocultured with the HLA A*0201 MP-specific T cell clone. T cells were added at a concentration of 1000/well. The APC:T cell ratio was 5:1 or 2:1. Controls included APCs and T cell clone without Ag and APCs or T cell clone alone. The number of IFN-\(\gamma\)-producing spot-forming cells (SFCs) was counted after 16 h. The number of SFCs for control samples ranged from 0 to 1 and was subtracted from the experimental values. Data are shown as the number of SFC per 1000 cells and are representative of three experiments.
cytolytic activity. At low doses of MP (1 nM), mature DCs induced IFN-γ production in higher numbers of the T cell clone than immature DCs (Fig. 4A). At higher doses of MP (1 μM; Fig. 4B) the difference was less apparent, probably due to the sensitivity and clonal nature of the line. When we evaluated presentation of nonreplicating heat-inactivated influenza virus, mature DCs were superior to immature DCs by 4- to 6-fold (Fig. 4). This indicates that at the very low levels of viral Ag expressed by nonreplicating virus, the mature DCs are presenting Ag with greater efficiency. Following infection with live influenza virus (Fig. 4D), the immature DCs demonstrated an enhanced capacity to activate IFN-γ production. Notably, monocytes were poorly stimulatory in these assays, confirming their relatively poor capacity to function as APCs. Therefore, the data obtained with the clone verify results with primary T cell populations (Fig. 2).

**Efficient expansion of influenza-specific CD8⁺ effector cells requires mature DCs**

We examined the APC requirements necessary to expand influenza-specific effector cells in longer term cultures. Immature DCs, mature DCs, and monocytes were infected with live or heat-inactivated influenza virus or pulsed with MP (1-10 μM) and were cocultured with autologous T cells at different ratios for 7 days. The expansion of Ag-specific T cells was measured by restimulating with MP pulsed mature DCs in an ELISPOT assay. The background number of SFCs following restimulation with nothing or DCs alone has been subtracted. HI Flu, heat-inactivated influenza.

![FIGURE 5.](http://www.jimmunol.org/) Mature DCs expand influenza-specific memory CD8⁺ T cells. Monocytes, immature DCs, and mature DCs from an HLA-A*0201 donor were infected with heat-inactivated influenza virus or pulsed with 10 μg/ml MP. The Ag-exposed APCs were incubated together with 2 × 10⁵ T cells at ratios of 1:30 for 7 days. The expansion of Ag-specific T cells was measured by counting the number of IFN-γ-producing cells after restimulation with MP pulsed mature DCs in an ELISPOT assay. The background number of SFCs following restimulation with nothing or DCs alone has been subtracted. HI Flu, heat-inactivated influenza.

![FIGURE 6.](http://www.jimmunol.org/) Mature DCs induce the highest expansion of HLA-A*0201-MP tetramer-binding memory CD8⁺ T cells. Monocytes, immature DCs, and mature DCs from an HLA-A*0201 donor were pulsed with 10 μg/ml MP (middle panel) or infected with live influenza virus (bottom panel). As a control for Ag-specific expansion we used APCs that were not exposed to Ag (top panel). The APCs were cocultured with bulk T cells for 7 days. The expansion of MP-specific CD8⁺ T cells was measured by staining the cells with HLA-A*0201-MP tetramer complexes followed by anti-CD3 and anti-CD8 Abs. The analyses were performed by gating on CD3⁺ T cells and plotting CD8⁺ T cells (y-axis) vs MHC class I tetramers (x-axis). The percentage of CD8⁺ tetramer-stained cells is shown in the top right corner.

In general, the ability of mature DCs to expand MP-specific CD8⁺ T cells, as measured by the tetramer assay, correlated with the ELISPOT assay. As mature DCs were the most efficient APCs in expanding MP-specific IFN-γ-producing memory T cells, we tested whether they were also the most efficient ones at inducing cytotoxic T cells. Immature and mature DCs, the latter prepared by exposure to MCM, were infected with heat-inactivated influenza virus or pulsed with MP or the HLA-A*0201-restricted HIV-1 gag peptide (SLYNTVATL). The DCs were cocultured with autologous T cells for 7 days, after which they were tested for cytotoxic activity on peptide-pulsed T2 targets. The MCM-matured DCs elicited significant cytotoxic activity when infected with the heat-inactivated influenza virus or pulsed with as little as 10 nM MP (Fig. 7A). In contrast, the immature DCs were less effective. We compared the capacity of immature vs mature DCs to elicit CTL following infection with heat-inactivated or live influenza virus (Fig. 7B). As shown previously, the immature DCs failed to induce CD8⁺ effector activity when infected with heat-inactivated virus, but could do so following infection with live virus. Mature DCs induced high levels of cytolytic activity even when one mature DC
to purified CD8 T cells were also capable of presenting influenza Ags (MP or heat-inactivated virus) to autologous T cells (Fig. 7).

In a previous study we showed that CD11c+ blood DCs (isolated directly from blood mononuclear cells) infected with influenza were capable of inducing influenza-specific CTLs directly from purified CD8+ T cells (22). Mature monocyte-derived DCs were also capable of presenting influenza Ags (MP or heat-inactivated influenza) to purified CD8+ T cells (Fig. 7D) even at low doses, whereas immature DCs were ineffective (data not shown). Therefore, in three different assays, all qualitatively different, we found that mature DCs are far more efficient at presenting influenza Ags to autologous T cells, resulting in the expansion and differentiation of Ag-specific memory CD8+ T cells into effector cells with IFN-γ production and cytotoxic function.

**Discussion**

The capacity of peripheral tissue DCs to recognize, take up, process, and present pathogens/Ags is an initial key event in the efficient activation of adaptive immunity. Once Ag uptake has occurred in immature DCs, they become activated and migrate to secondary lymphoid organs, where specific T cell and B cell responses can be triggered (7). For the most part, the stages in the DC life cycle have been mapped out in vitro. Immature DCs generated by culturing human monocytes with GM-CSF and IL-4 for 5–6 days are generally considered to be comparable to those found in peripheral tissues. Mature DCs are generated from immature cells by exposure to maturation stimuli such as MCM (which contains the inflammatory cytokines, TNF-α, IL-1 IL-6, and IFN-α) (26), LPS, CD40 ligand, TNF-α. DNA with unmethylated CpG sequences, and bacteria (35, 36). More recently, infection by influenza virus and dsRNA (poly(I:C)) have been identified to do the same (31, 37). It has been suggested that by linking the uptake of organisms with maturation, the DC is primed to activate precursor T cells in draining lymph nodes (31).

As monocyte-derived DCs are being used in immunotherapy in cancer patients, it is important to define differences in these stages of DCs with respect to CD8+ T cell activation. Mature DCs have previously been shown to be more efficient than immature DCs at stimulating allogenic or autologous responses from naive CD4+ T cells and inducing proliferation of Ag-specific CD8+ T cell clones (38, 39). In a recent study no differences were found in the ability of immature and mature DCs to prime Ag-specific T cells from naive donors against HIV Ags. However, repeated stimulation and exogenous IL-2 were used to prime the T cells; therefore, it is difficult to evaluate the relative efficacies of these APCs (40). Our report is the first to fully compare immature DCs and mature DCs in their capacity to generate effector CD8+ T cells from resting memory precursors. Bullock et al. showed that T cells from mice primed with peptide-pulsed mature DCs had higher lytic activity toward relevant targets than did cells from mice primed with peptide-pulsed immature DCs (41). This finding support ours, that mature DCs are superior APCs for T cell activation.
We chose to study the influenza virus system for examining the efficacy of monocytes and two populations of DCs, as this virus has been well studied both at the Ag presentation level and in the induction of memory CD8+ effector T cells. We first compared the effects of infection in monocytes, immature DC, and mature DC. We found that at MOIs of 1–2, most cells in all three APC populations were infected, as determined by expression of hemagglutinin protein. In contrast, significant differences were noted in cell viability. Both the monocyte and immature DC populations underwent apoptosis within 10–16 h of infection, while the mature DCs were largely resistant. Recently, Cella et al. (31) showed that human immature DCs activated by influenza infection develop resistance to the cytopathic effects of this virus, a phenomenon that is mediated by the production of type 1 IFNs and the expression of MxA protein. MxA protein is known to mediate resistance to influenza A virus (32). MCM-matured DCs did not express MxA, but this factor was elicited following infection of DCs with live influenza virus. MCM contains IFN-α, albeit in low levels (<8 U/ml). It is possible that these levels are insufficient to induce MxA, or that MxA-independent mechanisms are operative. IFN-α enhances dsRNA-dependent protein kinase activation (42, 43), which as a function of an activator of gene transcription can be involved in DC maturation, e.g., via NF-κB activation. Furthermore, IFN-α may also affect the activation of genes that sustain DC viability, such as bcl-XL (44). We have found that MCM induces the nuclear transport of NF-κB (data not shown). IFNs also induce 2′,5′-oligoadenylate synthetase, which inhibits viral replication (42). A protein with 2′,5′-oligoadenylate-synthesizing activity was recently described in maturing murine DCs (45). Whether human immature DCs express 2′,5′-oligoadenylate-synthesizing activity in forms that are readily activated upon exposure to maturation stimuli such as MCM, LPS, or influenza virus remains to be determined.

Infection of immature DCs with influenza virus or, as recently reported, dsRNA induced maturation of the cells, as illustrated by the detection of DC-LAMP and CD83 and the up-regulation of MHC and costimulatory molecules (Fig. 1C) (37). This property is unique to replicating virus, as nonreplicating virus failed to induce maturation (Fig. 1D). Since nonreplicating virus will not proceed through the dsRNA stage, our data are consistent with the concept that these molecules are essential for DC maturation. As it was unclear whether maturation was secondary to direct infection vs release of dsRNA from apoptotic influenza-infected DCs, we positively selected influenza-infected immature DCs by hemagglutinin expression and stained them with Abs to CD83. We found that 30–40% of the infected immature DCs expressed CD83, confirming that direct viral infection induces DC maturation.

The comparison of the three APC populations revealed significant differences in their abilities to activate resting CD8+ influenza-specific memory T cells. Not surprisingly, we found monocytes to be poorly capable of eliciting or expanding CD8+ effector cells regardless of the assay system employed. They were effective only when high numbers (e.g., T:APC ratio = 1:1) were used and then only for the induction of IFN-γ. PBMCs contain 10–30% monocytes. Therefore, monocytes are the major cell population presenting Ags in ELISPOT assays if responses are measured in PBMCs (27). Our results indicate that the use of PBMCs vs DCs in ELISPOT assays will grossly underestimate the frequency of Ag-specific CD8+ T cells. Indeed, we have shown that DCs activate far more EBV-specific T cells than PBMCs in ELISPOT assays (46). Interesting differences emerged between the immature and mature DC populations. Whereas immature DCs were more efficient than the monocytes, they were 10- to 100-fold less efficient than mature DCs in stimulating IFN-γ production, expanding MP-specific CD8+ T cells, and inducing cytotoxic T cells. However, if immature DCs were infected with influenza virus, which also induced their maturation, the efficiency of CD8+ T cell activation was increased up to 5-fold. Mature DCs were striking in their ability to not only induce IFN-γ production, but also to induce influenza-specific cytotoxic T cell proliferation. Binding with MHC class I tetramer complexes revealed expansions of up to 7- to 50-fold when MP was the stimulating Ag and of 32- to 110-fold when live influenza was used.

T cell activation is determined by several different factors: the duration of antigenic stimulation, the dose of Ag, and the presence of costimulation. Costimulation facilitates T cell activation by stabilizing the T cell-APC interaction and shortening the time required for T cell activation. It also serves to increase the magnitude of effector responses through sustained TCR signaling and protection of T cells from activation-induced cell death (47–49). Memory cells require a shorter duration of Ag stimulation and are less dependent on costimulation, e.g., CD28-CD86 interactions. However, even at high doses of peptide (10 μM) or long exposures to Ag in 7-day cultures, monocytes and immature DCs failed to induce sustained proliferative responses and CTL formation, indicating an inability to commit memory T cells to enter effector pathways. Immature DCs, at least in vitro, express low to moderate levels of both MHC and costimulatory molecules, but lose stimulatory activity with time after Ag exposure, probably because of rapid MHC turnover and failure to sustain the immunological synapse (50–52). These factors probably account for the observation that immature DCs and monocytes can elicit early responses, e.g., IFN-γ secretion from memory cells, because memory cells are more readily committed to respond to stimulation due to higher levels of second messengers and ready accessibility to transcription factors (47, 53, 54).

Only mature DCs were able to induce the full complement of T cell activation, i.e., IFN-γ production, clonal expansion (as measured by MHC class I tetramer complex binding), and development of cytotoxic T cells. Following maturation from standard stimuli such as LPS or influenza infection, mature DCs express 5 times more MHC class I molecules, and their half-life is increased (31). Thus, presentation to T cells can be sustained over a long period of time. Mature DCs require only low levels of Ags to induce CD8+ effector cells, probably because costimulation allows memory cells to respond to lower levels of Ags and TCR occupancy (47). We show here that mature DCs can elicit CTLs directly from purified CD8+ T cells. Presumably, maturation bypasses the need for help, perhaps by increasing costimulation, facilitating Th1 responses through IL-12 production, and increasing the lifespan of the DCs (35, 38, 55).

Recently we have shown that healthy volunteers immunized with a single injection of mature DCs pulsed with keyhole limpet hemocyanin, tetanus toxoid, and MP rapidly elicit CD4+ and CD8+ T cell immunity in humans (13). Volunteers were reinfected with mature DCs pulsed with MP alone and demonstrated a rapid reactivation of MP-specific T cells, which was stronger than the initial injection (56). Thus, mature DCs have the capacity to elicit lytic effector cells both in vitro and in vivo in the absence of CD4+ T cell help. Our data suggest that these cells will be more effective inducers of T cell immunity in vivo. Studies to compare immature and mature DCs in their capacity to elicit effector cells in humans are in progress.

References


