Dendritic Cells Generated Either from CD34⁺ Progenitor Cells or from Monocytes Differ in Their Ability to Activate Antigen-Specific CD8⁺ T Cells

Guido Ferlazzo, Amy Wesa, Wei-Zen Wei and Anne Galy

*J Immunol* 1999; 163:3597-3604; ;
http://www.jimmunol.org/content/163/7/3597

**References**  This article cites 23 articles, 15 of which you can access for free at: http://www.jimmunol.org/content/163/7/3597.full#ref-list-1

**Subscription**  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Dendritic Cells Generated Either from CD34⁺ Progenitor Cells or from Monocytes Differ in Their Ability to Activate Antigen-Specific CD8⁺ T Cells

Guido Ferlazzo, Amy Wesa, Wei-Zen Wei, and Anne Galy

Dendritic cells (DC) can be generated in vitro from monocytes (M-DC) or from CD34⁺ hemopoietic progenitor cells (CD34-DC) but their precursors are not equivalent cells, prompting a comparison of the functional capacities of these APC. Both types of DCs established from the same individuals using the same cytokines displayed a comparable phenotype of mature DC (CD1a⁺, CD83⁺, CD86⁺, CD4⁺, HLA-DR⁺⁺, CD14⁺, CD15⁻) and were equally potent stimulators of allogeneic T cell proliferation, being both more powerful than immature M-DCs. An autologous panel of APCs produced in HLA-A2⁺ individuals, including CD34-DC, M-DC, monocytes, and EBV-lymphoid cell line was comparatively evaluated for presentation of the Erb-B2 peptide E75 to a CTL line. After short exposures (5 h) to E75-loaded APCs, similar levels of intracellular IFN-γ were induced in Ag-specific CD8⁺ T cells regardless of APC type. In sustained cultures (4–14 days), more Ag-specific T cells were obtained when peptide was presented on CD34-DC (p < 0.05) rather than on M-DC, EBV-lymphoid cell lines, or monocytes, and these effects were dose-dependent. Activated T cells expressed 4-1BB, and the presence of 4-1BB-Ig fusion protein partially blocked Ag-specific CD8⁺ cell activation after CD34-DC or M-DC presentation. Our results show that 34-DC have a preferential capacity to activate CD8⁺ T cells and that this property is not strictly correlated to their ability to induce allogeneic T cell proliferation but due to mechanisms that remain to be defined. The Journal of Immunology, 1999, 163: 3597–3604.

Dendritic cells (DC) are found at trace levels in tissues or in circulation and control many lymphocyte functions (1). DC are powerful APC able to process and to present Ags to CD8⁺ and CD8⁻ T cells while delivering the costimulatory signals necessary for T cell activation. The identification of culture conditions enabling the generation of DC in vitro are useful to study their biology and to prepare large amounts of APC for immunotherapeutic purposes. A promising field of application for DC is cancer immunotherapy because DC presenting tumor-associated Ags induce protective and therapeutic T cell-mediated immunity (2–4). In humans, clinical studies suggest that DC presenting MHC class I-restricted tumor peptides may be effective (5). DC can be readily generated in vitro from the mobilized peripheral blood of patients undergoing treatment for cancer using either CD34⁺ hemopoietic progenitor cells or monocytes (6, 7). These two types of DC precursor cells are found in vastly different amounts in G-CSF-mobilized apheresed blood, constituting respectively 1.5 ± 1.5% and 42.2 ± 14.3% (n = 14) of the mononuclear cell (MNC) fraction in our breast cancer patient population. It is not clear whether DC generated from CD34⁺ cells (CD34-DC) are equivalent to those generated from monocytes (M-DC). Developmentally, monocytes appear to constitute a relatively homogeneous DC precursor cell, while CD34⁺ cells contain different subsets of DC progenitor cells (8) and employ at least two developmental pathways to generate DC that differ in Th-related functions (9). Very few studies have been conducted to evaluate functional differences between CD34-DC and M-DC, particularly in their capacity to activate CD8⁺ T cells directly. We recently reported that fli3 ligand, c-kit ligand, GM-CSF, IL-4, and TNF-α could be used to generate DC from monocytes or from CD34⁺ cells and that either type of DC had the ability to activate an HLA-B44-restricted HIV gag peptide-specific CTL clone inducing similar levels of IFN-γ secretion (10). A recent study made similar observations showing that both CD34-DC and M-DC were equally able to present the Melan-A/Mart-1 peptide to CTL clones and to induce comparable secretion of GM-CSF and IFN-γ in short-term assays (18 h) (11). This study also reported that Melan-A/Mart-1-specific CTLs were better propagated after an initial in vitro stimulation with CD34-DC rather than M-DC, suggesting that these two APCs differed in some aspects of CD8⁺ T cell stimulation. It is important to assess whether functional differences between M-DC and CD34-DC may result from the experimental conditions used to generate the APCs. Indeed, M-DC such as those used to expand Mart-1-specific CTLs were generated in GM-CSF plus IL-4, conditions known to generate immature DC better specialized to process Ag than to activate T cells. In contrast, CD34-DC are often generated in the presence of multiple cytokines, including TNF-α known to induce DC maturation and T cell activation capacity (12, 13). This prompted us to evaluate whether mature DC generated from CD34⁺ cells or monocytes under the same conditions have distinct T cell stimulatory properties. To specifically test this aspect of Ag presentation independently of Ag processing capacity, we analyzed presentation of allogeneic Ags and of exogenous MHC class I-binding peptides. We used purified populations...
of APCs to exclude indirect effects of other cells on Ag presentation. A model of MHC class I-restricted presentation was established with the Erb-B2 peptide E75. This model tumor Ag was chosen because E75 is presented by HLA-A2, a common allele, and E75 is immunodominant, able to elicit immune responses in vitro and in vivo (14–16). We report that mature DCs with strong and comparable allogeneic T cell stimulatory capacity were generated from CD34+ cells or from monocytes, but these DC differed in their capacity to generate Ag-specific CD8+ T cells.

Materials and Methods
Source of cells
Chemotherapy and G-CSF-mobilized peripheral blood (MPB) samples were obtained from metastatic stage IV breast cancer patients undergoing collection of MPB for hematopoietic rescue following high dose chemotherapy (HyClone, Logan, UT). Patients signed a separate informed consent for the collection of additional MPB cells to be used for these studies. MPB was collected by leukapheresis following administration of taxol (170 mg/m2/24 h), cytoxan (2 g/m2/1 h), and G-CSF (Neupogen, Amgen, Thousand Oaks, CA) to isolate CD34+ cells. Purity of the cell population was >85% as ascertained after extensive blocking with normal mouse serum and direct staining with FITC-conjugated HPCA2 anti-CD34 (Becton Dickinson, San Jose, CA) recognizing a different epitope than Qbend10.

Isolation of CD34+ cells
MNC (density < 107/mL) were prepared by centrifugation through Ficoll (Pharmacia, Piscataway, NJ) and cells were cryopreserved with 10% DMSO. MNC were thawed in the presence of DNase (100 U/mL) and heparin (10 U/mL) (Sigma, St. Louis, MO) to minimize clumping and optimize recovery (the procedure does not affect cell-surface phenotype). Cells were incubated with Qbend10 anti-CD34 mAb (Immunotech, Westborough, MA) and goat anti-mouse colloidal paramagnetic beads (Miltenyi Biotec, Sunnyvale, CA) to isolate CD34+ cells. Purity of the cell population was >85% as ascertained after extensive blocking with normal mouse serum and direct staining with FITC-conjugated HPCA2 anti-CD34 (Becton Dickinson, San Jose, CA) recognizing a different epitope than Qbend10.

Isolation of monocytes
Monocytes were obtained by plastic adherence of MNC. MNC were plated at about 4 x 105 cells in 75-cm2 tissue culture flasks (Corning Costar, Oneonta, NY) in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (HyClone, Logan, UT). After 2 h, nonadherent cells were removed, and, unless otherwise indicated, adherent cells were further incubated overnight in the same medium before being washed and detached by incubation in ice-cold Ca2+-Mg2+-free PBS for 10–20 min and tapping.

DC cultures
CD34+ cells were plated at 104 cells/ml in 24-well plates (Corning) in RPMI 1640 medium (Life Technologies), containing 10% FCS, penicillin, streptomycin (100 U/ml and 100 μg/ml, respectively), L-glutamine (2 mM), 2-ME (2 mM), and 2-ME at 37°C, 5% CO2 with variable numbers of irradiated APC (4000 cGy with a Cs 137 source; J. L. Shepherd, San Fernando, CA) in 96-well round-bottom microtiter plates (Corning). During the last 18 h of cultures, cells were incubated with autologous CD8+ T cells (prepared by negative depletion of B cells and phagocytes after incubation with G28-5 and IV.3 Abs (American Type Culture Collection) and panning on goat anti-mouse Ig-coated plates (Sigma). Further purification was achieved by eliminating cells reacting with HLA-DR, CD19, CD14, and CD15, using sheep anti-mouse IgG-coated beads and immunomagnetic depletion (Dynal, Lake Success, NY). The resulting cell population contained routinely >98.5% CD3+ T cells.

Allo-stimulation assays
Purified allogeneic T cells (1 x 106 cells per well) were incubated for 6 days in RPMI 1640 medium with 10% FBS, antibiotics, glutamine, and 2-ME at 37°C, 5% CO2, with variable numbers of irradiated APC (4000 cGy with a Cs137 source; J. L. Shepherd, San Fernando, CA) in 96-well round-bottom microtiter plates (Corning). During the last 18 h of cultures, 1 μCi of [3H]thymidine (DuPont NEN, Boston, MA) was added to each well, and cellular incorporation was determined after harvesting cells on glass fibers and liquid scintillation counting on a Tracer Analytic Mark III scintillation counter. Results are expressed as average cpm of triplicate wells ± SD.

E75-specific CTL line
An HLA-A2-restricted CTL line specific for the E75 immunodominant epitope of the tumor-associated Ag Erb-B2, was established using a slight modification of a described procedure (17). Briefly, DC were prepared from a normal HLA-A2+ volunteer by culture of blood monocytes in flt3 ligand, c-kit ligand, GM-CSF, TNF-α, and IL-4 for 6 days in 10% autologous serum with AIMV medium (Life Technologies). Such DC were loaded with 40 μg/ml E75 peptide (369-377; KIFGSLFL, Quality Controlled Biochemicals, Hopkinton, MA) in the presence of 3 μg/ml B2 mioglobulin (Sigma, St. Louis, MO) for 2 h at 37°C. After three washes, DC were incubated with autologous CD8+ T cells (prepared by negative depletion of blood MNC removing cells expressing CD20, CD14, and CD132 with magnetic beads) in the presence of 10 ng/ml IL-7 (R&D Systems). After 24 h, 10 ng/ml IL-10 (a kind gift from Dr. Yssel) was added to the T cell culture, and cells were incubated for 6 days at 37°C, 5% CO2 in humidified atmosphere. The antigenic stimulus was repeated twice, using monocytes as APC at weekly intervals and low doses of IL-2 (4 ng/ml). The CTL line established under these conditions was fed twice weekly with IL-2 (4 ng/ml) and 10% T-Stim (T stim without PHA; Becton Dickinson, Bedford, MA). T stim is a conditioned medium obtained from cultures of normal human blood MNC stimulated with PHA subsequently removed by affinity adsorption. According to the manufacturer, 10% T stim solution contains ~20 U/ml IL-2.

Peptide stimulation
For short-term stimulation experiments, T cells were incubated with peptide-loaded APC at an APC:T cell ratio of 1:10 in AIMV plus 10% T stim in the presence of 2 μM E75 monomer (Sigma) for 5 h. For long-term stim experiments, T cells were cultured at the concentration of 1 x 106 to 3 x 106 cells per well in 24- or 96-well plates (Corning) with irradiated APCs (4000 cGy) (Cs137 irradiator; J. L. Shepherd, San Fernando, CA).

Flow cytometric detection of cell-surface Ags and cell sorting
Incubations were performed on ice using PBS plus 0.2% BSA plus 0.02% sodium azide as staining buffer (azide was omitted when cells were pre-pared for culture). Nonspecific binding was blocked by the addition of 10 μg human γ globulin/106 cells (Gammune, Miles, Elkhart, IL). Negative controls included directly labeled IgG1 and IgG2a irrelevant mAbs. Cells were incubated with mAbs for 30–40 min, washed twice in staining buffer, and resuspended in a solution of 5 μg/ml propidium iodide (PI) before being analyzed on a FACSscan (Becton Dickinson). Analysis was performed on live cells using the PC Lysis software (Becton Dickinson). Compensation controls and negative controls were used to determine the bound-aries of regions in two-color dot plots such that >98% of the cells would be comprised in the appropriate regions. Purified populations of DC were isolated on a Vantage cell sorter (Becton Dickinson) by flow cytometry sorting gating of live PI− cells with the specified CD characteristics.

Intracellular cytokine detection
After being stained for cell-surface markers as described above, cells were washed once in PBS, then fixed in 4% formaldehyde for 10 min on ice, washed in PBS, and permeabilized by incubation in staining buffer containing 0.1% saponin. Nonspecific mAb binding was blocked after satura-tion with Gammune diluted in saponin for 10 min, and anti-IFN mAbs were added (0.1 μg/106 cells) for 30 min. Cells were washed twice in permeabilization buffer and staining was analyzed on the FACSscan by electronically gating on lymphocytes, excluding debris and large aggregates.

Isolation of highly purified T cells
Purified allogeneic T cells were prepared from buffy coats by eliminating B cells and phagocytes after incubation with G28-5 and IV.3 Abs (American Type Culture Collection) and panning on goat anti-mouse Ig-coated plates (Sigma). Further purification was achieved by eliminating cells re-acting with HLA-DR, CD19, CD14, and CD15, using sheep anti-mouse IgG-coated beads and immunomagnetic depletion (Dynal, Lake Success, NY). The resulting cell population contained routinely >98.5% CD3+ T cells.

3598 CD34-DERIVED DC ARE SUPERIOR APC FOR CD8+ T CELLS

Downloaded from http://www.jimmunol.org/ by guest on April 15, 2017
Monocytes are commonly used to generate DCs. Two cytokines, GM-CSF plus IL-4, are sufficient to induce the differentiation of monocytes into immature DCs that effectively process Ag but are not optimal for T cell stimulation (12). In a recent study, we found that monocytes cultured with GM-CSF plus IL-4 acquired DC markers such as CD1a, CD83, costimulatory molecules, and allo-stimulatory capacity in a time-dependent manner peaking at day 14 of culture. However, at the peak of their development these M-DCs were less allo-stimulatory than autologous CD34-DCs generated with flt3 ligand plus c-kit ligand plus GM-CSF plus IL-4 (FKGmT4) (10). Consequently, we stimulated monocytes with FKGmT4 to test whether their T cell stimulatory properties could be increased. The positive effects of FKGmT4 on M-DC were evident because more M-DC survived (Fig. 1A) with up-regulated CD86 costimulatory molecules in FKGmT4 as opposed to GM-CSF plus IL-4 (Fig. 1B). The capacity to present alloantigens was measured by T cell proliferation. Unstimulated monocytes were poor allo-DC stimulators, whereas M-DC were able to induce this activity, and those generated in FKGmT4 were more active than those generated in GM-CSF plus IL-4 (Fig. 2). Cells in cultures of M-DC prepared with FKGmT4 for 2 wk were homogeneously large, expressing CD1a and high levels of HLA-DR (Fig. 3A) as well as the typical DC cell-surface phenotype CD1a+ CD14−, CD83+, CD15− CD4+ CD86+ (Fig. 3B). In contrast and as described previously (10), cultures of CD34+ cells in FKGmT4 for 2 wk were heterogeneous, containing about 40% of cells with the phenotypic characteristics of DC (Fig. 3). There was an almost complete correlation between the large size of the cells (above 5 \(\mu\)m monensin) and expression of IFN-\(\gamma\) production on CD8+ cells by multicolor flow cytometry.

**Statistical analysis**

Statistical analysis was performed with the Kruskal-Wallis nonparametric ANOVA and Dunn’s method to compare groups using the SigmaStat version 1.0 software (Jandel Scientific, San Rafael, CA).

**Results**

**Generation of DC with powerful allo-stimulatory capacity**

To study the Ag-specific activation of CD8+ T cells by tumor-associated Ags, we established a CTL line specific for E75 (KIFGSLFL), an HLA-A2+-binding immunodominant peptide of the tumor-associated Ag Erb-B2 (14, 15). E75-specific T cells were obtained from an HLA-A2+ blood donor by in vitro immunization in a protocol adapted from Kawashima et al. (17) using autologous M-DC generated in FKGmT4. After four rounds of Ag-specific stimulation and subsequent T cell expansion, a bulk T cell culture was generated that was enriched in CD8+ cytolytic T cells.

**Measurement of E75-specific CD8+ T cell activation**

To study the Ag-specific activation of CD8+ T cells by tumor-associated Ags, we established a CTL line specific for E75 (KIFGSLFL), an HLA-A2+-binding immunodominant peptide of the tumor-associated Ag Erb-B2 (14, 15). E75-specific T cells were obtained from an HLA-A2+ blood donor by in vitro immunization in a protocol adapted from Kawashima et al. (17) using autologous M-DC generated in FKGmT4. After four rounds of Ag-specific stimulation and subsequent T cell expansion, a bulk T cell culture was generated that was enriched in CD8+ cytolytic T cells.
A

M-DC

CD34-DC

CD1a

FSC

HLA-DR

FSC

B

M-DC

CD34-DC

CD1a

CD14

CD83

CD84

CD86

FIGURE 3. Cell-surface phenotype and size (FSC) characteristics of CD34-DC and M-DC. Cells were derived in parallel from the same individual using FKGmT4. Live (PI<sup>-</sup>) cells were analyzed by two-color flow cytometry at day 14. A, The correlation between size (FSC) and CD1a or HLA-DR is shown. B, The expression of markers used to characterize DC is shown.

cells able to kill the HLA-A2<sup>+</sup> T2 cell line coated with E75 peptide (specific killing: 86 ± 8% of peptide-coated T2 cells vs 31 ± 2% of T2 cells alone at an E:T ratio of 3:1). Two sublines enriched in CD8<sup>+</sup> T cells but with slightly different composition were selected after the fifth Ag-specific stimulation (Table I). Multicolor flow cytometry was used to quantify Ag-specific activation of the CD8<sup>+</sup> T cells by detection of intracellular levels of IFN-γ. Autologous EBV-LCL, as well as HLA-A2<sup>+</sup> APC such as EBV-LCL or CD34-DC, presented E75 inducing the accumulation of detectable levels of intracellular IFN-γ in 5 h (Table II). This induction was peptide specific, genetically restricted, and APC dose-dependent and -sensitive because the addition of only 2% peptide-loaded DC (1 DC for 50 T cells) induced detectable IFN-γ production above background.

Presentation of E75 peptides to CD8<sup>+</sup> T cells by in vitro-generated APCs

DC are pivotal for the initiation of cytolytic T cell responses but it is not clear that DC produced in vitro can directly present Ags to activate CD8<sup>+</sup> T cells and it has not been determined how their activity compares to that of EBV-LCL, an APC that is commonly used for CTL expansion. To address these questions we compared the Ag-presenting activity of homogeneous populations of APC, M-DC, CD34-DC, unstimulated monocytes (M) and EBV-LCL that have been generated from the same HLA-A2<sup>+</sup> individual. The results were normalized to the activation induced by the presentation of E75 on CD34-DC, to reduce the interexperimental variability. In all experiments, and with all APC, the presentation of E75 induced detectable IFN-γ production above the background stimulation by the irrelevant C85 peptide (Fig. 4). Significantly greater Ag-specific activation was induced by CD34-DC than by monocytes (p < 0.05), but there were no other statistically significant difference between the APC. These data demonstrate that purified M-DC or CD34-DC generated in vitro from breast cancer patients can directly present peptides to CD8<sup>+</sup> T cells, causing a specific and rapid induction of IFN-γ production. In a short-term read-out, DC or EBV-LCL appear to elicit the activation of the same proportion of Ag-specific T cells.

Repeat stimulation experiments

There is evidence that Ag-specific CD8<sup>+</sup> T cells can fail to grow upon inappropriate antigenic restimulation and when there are limiting amounts of IL-2 (19). Following presentation of E75, IFN-γ was transiently increased, returning to background levels after 4–7 days because at that time there was little to undetectable IFN-γ produced in the absence of specific stimulation (Table III). However, IFN-γ production was specifically reinduced in 5 h by adding the E75 peptide with EBV-LCL. To test whether the initial presentation of Ag by any of the APCs affected the growth of E75-specific T cells, we incubated T cells with different E75 peptide-loaded APCs for 4 to 14 days in the presence of low levels of IL-2 (provided by 10% T stim conditioned medium). The numbers of Ag-specific T cells were measured by restimulating with E75 peptide plus EBV-LCL for 5 h and counting CD8<sup>+</sup> T cells with detectable intracellular IFN-γ as shown in Fig. 5. Cultures initially stimulated with CD34-DC had higher percentages of CD8<sup>+</sup> T cells producing IFN-γ than cultures initially stimulated with EBV-LCL or M-DC (p < 0.05) (Table IV). In each experiment, total numbers of cells tended to be higher in cultures stimulated by CD34-DC but statistical analysis of aggregated results showed no significant difference (p > 0.05) among APCs for total cell numbers. There was little T cell expansion generated in these experiments because little exogenous cytokine was used to supplement the effects of APC and Ag presentation. Despite these limiting conditions, cultures initiated with CD34-DC sometimes expanded modestly and always contained the most total number of cells of all conditions tested. Importantly, cultures induced with CD34-DC contained significantly more reinduced CD8<sup>+</sup> IFN-γ<sup>+</sup> T cells than cultures

Table 1. Composition of two E75-specific T cell sublines

<table>
<thead>
<tr>
<th>Subline</th>
<th>CD3&lt;sup&gt;+&lt;/sup&gt;</th>
<th>CD4&lt;sup&gt;+&lt;/sup&gt;</th>
<th>CD8&lt;sup&gt;+&lt;/sup&gt;</th>
<th>CD3&lt;sup&gt;-&lt;/sup&gt;CD56&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>99.5</td>
<td>2</td>
<td>78</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
<td>97.8</td>
<td>1.3</td>
<td>25</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Percentage of Cells in Culture Having the Cell-Surface Markers
We prepared CD34-DC after 13 days in culture and we prepared M-DC after only 9 days of culture using frozen aliquots of the same tissue. DC were purified from both cultures by flow cytometry sorting, coated with peptide, washed, and added at different ratios to E75-specific T cells. After 7 days, there was greater viability and greater cell numbers with CD34-DC in a dose-dependent manner. Cells were restimulated to measure Ag-specific T cells (as described in Fig. 5), confirming that more Ag-specific T cells were obtained in cultures of day 13-CD34-DC than in cultures of day 9 M-DC (Fig. 6). These data demonstrate that on a cell per cell basis, CD34-DC have a greater ability to activate and expand Ag-specific CD8$^+$ T cells than other APC such as monocytes, M-DC, and EBV-LCL.

Activation of T cells by CD34-DC

To characterize the level of T cell activation induced by Ag presentation on CD34-DC, we examined the expression of 4-1BB (CD137), an important stimulatory molecule for CD8$^+$ T cell stimulation and of the pan T cell activation marker p55 IL-2R $\alpha$-chain (CD25). CD137 is an inducible receptor, absent on resting lymphocytes but rapidly up-regulated by Ags or mitogens (20). Cells of Expt. 1 in Table IV were analyzed at restimulation to compare the effects of CD34-DC (the best APC) to those of monocytes (the worst APC in this system). While CD25 was expressed on CD8$^+$ T cells regardless of initial Ag exposure, 4-1BB levels were specifically induced in response to the initial antigenic stimulation (Fig. 7). Approximately the same proportion of CD8$^+$ T cells could be reinduced to produce IFN-\(\gamma\) by CD34-DC or by monocytes (Table IV and Fig. 5); however, after exposure to CD34-DC, CD8$^+$ IFN-\(\gamma\)^- cells were slightly more activated because 63% (48.76; Fig. 7A) and 72% (50.69; Fig. 7B) of them expressed 4-1BB and CD25, respectively, as opposed to 39% and 57% in cultures of E75-loaded monocytes. Interestingly, the composition of cultures initially stimulated by CD34-DC was distinct, with greater proportions of cells expressing the phenotype CD8$^+$ IFN-\(\gamma\)^- 4-1BB$^+$ or CD8$^+$ IFN-\(\gamma\)^- CD25$^+$ (48 and 50%, respectively) compared with cultures initially stimulated by monocytes (17 and 23%, respectively). These data suggest that 4-1BB plays a role in E75-specific T cell activation. To explore this possibility, we stimulated T cells in the presence or absence of 4-1BB-Ig fusion protein because it has been described that such fusion protein blocks T cell activation by interfering with TCR-mediated induction of proliferation and survival (18). Results of two experiments (Table V) show that the presence of 4-1BB Ig partially blocks

![Image](http://www.jimmunol.org/)

**FIGURE 4.** Comparison of CD8$^+$ T cell activation by various APCs. Purified DCs were generated in FKGmT4 for 2 wk and prepared by flow cytometry sorting of large live cells from cultures of CD34$^+$ cells and from culture of M-DC. APCs were loaded with excess peptide, then washed extensively before incubation with T cells at a ratio of 10% APC:T cells. The relative percentage of CD8$^+$ T cell activation was measured by flow cytometric analysis of intracellular IFN-\(\gamma\) production 5 h after presentation of E75 or C85 peptides on the different APCs and calculated as the percentage of the CD8$^+$ T cells that produce IFN-\(\gamma\) after a given stimulation divided by the percentage of CD8$^+$ T cells that produce IFN-\(\gamma\) after stimulation with E75-loaded CD34-DC $\times$ 100.

**Table II. Specificity of the T cell lines**

<table>
<thead>
<tr>
<th>Expts. 1–3</th>
<th>Stimuli</th>
<th>HLA</th>
<th>Peptide</th>
<th>Relative Percentage of CD8$^+$ T Cell Activation$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O</td>
<td>N/A</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>PMA + Ca ionophore</td>
<td>N/A</td>
<td>N/A</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>Autologous EBV-LCL</td>
<td>A2+</td>
<td>E75</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Heterologous EBV-LCL</td>
<td>A2+</td>
<td>E75</td>
<td>65.8</td>
</tr>
<tr>
<td></td>
<td>Heterologous CD34-DC 10%</td>
<td>A2+</td>
<td>E75</td>
<td>0</td>
</tr>
<tr>
<td>Expt. 4</td>
<td>Heterologous CD34-DC 10%</td>
<td>A2+</td>
<td>C85</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Heterologous CD34-DC 2%</td>
<td>A2+</td>
<td>E75</td>
<td>4.4</td>
</tr>
<tr>
<td></td>
<td>Heterologous CD34-DC 2%</td>
<td>A2+</td>
<td>C85</td>
<td>0.6</td>
</tr>
</tbody>
</table>

$^a$ T cell activation is calculated as the mean percentage of CD8$^+$ IFN-\(\gamma\)^+ T cells in a given condition divided by the percentage of CD8$^+$ IFN-\(\gamma\)^+ T cells induced by autologous EBV-LCL plus E75 (in Expts. 1–3) or by CD34-DC plus E75 (in Expt. 4) $\times$ 100.

$^b$ N/A. Not applicable.

initiated by M-DC, monocytes, or EBV-LCL ($p < 0.05$), indicating that they contained more Ag-specific T cells. The length of the primary stimulation was varied from 4 to 14 days and the same trends were observed. Because M-DC were less active than CD34-DC, we considered the possibility that M-DC were not being tested at their optimal peak of activity. Indeed, we observed that M-DC lose expression of CD1a and revert to monocytes after prolonged periods of culture (10). In two experiments, M-DC were generated only after 9 days and compared with autologous CD34-DC generated after 20 days of culture. In both cases, CD34-DC generated more Ag-specific T cells than M-DC (Table IV, Expts. 2 and 3), suggesting that the difference is unlikely to be caused by suboptimal DC differentiation. To further ascertain that this was the case,


T cell activation by E75-coated CD34-DC because only 65–49% of Ag-specific CD8+ T cells are produced compared with controls. To determine whether there were differences in costimulation requirements between CD34-DC and M-DC, we compared the effects of 4-1BB-Ig on autologous flow-cytometry-purified CD34-DC and M-DC. In this experiment, we confirmed that CD34-DC generated more Ag-specific T cells than M-DC but that 4-1BB-Ig blocked the effects of both APCs to a similar extent (about 50%). The induction of 4-1BB levels on the surface of T cells at day 1 of stimulation were found to be similar in cultures presented with CD34-DC or M-DC (not shown). We conclude that 4-1BB-mediated interactions are involved in the process of Ag presentation by CD34-DC and M-DC to the E75-specific T cells.

Discussion

Altogether, our data show that differences exist among APCs in their capacity to activate Ag-specific CD8+ T cells that are not strictly correlated to their ability to induce T cell proliferation in allogeneic MLR. In a dose-dependent manner, we show that greater numbers of Ag-specific T cells are obtained after presentation by CD34-DC rather than with other APCs. The effects of CD34-DC involve 4-1BB-mediated interactions, but the mechanisms for the difference between the different APCs remain to be defined. Our data show that homogeneous populations of in vitro-generated M-DC and CD34-DC can directly present peptides to CD8+ T cells, inducing their activation measured by IFN-γ production. Thus, we confirm that in vitro-generated DC are able to present antigenic peptides and to activate some aspects of CD8+ T cell functions as was shown earlier with induction of IFN-γ secretion in a HIV gag peptide-specific CTL clone (10) and as reported by others with induction of cytolysis in flu matrix-specific and Melan-A/Mart-1-specific T cell lines (11). The novel findings are that the nature of APC has an effect on the numbers of Ag-specific CD8+ T cells that can be obtained after Ag presentation. This was determined on homogeneous populations of APC and by comparing autologous cells. Our data provide the first direct evidence that CD34-DC and M-DC are distinct on a cell per cell basis for the activation of CD8+ T cells. The difference is quite profound because about half as many Ag-specific T cells can be obtained from cultures of M-DC compared with CD34-DC. This may explain why CD34-DC were better able to expand Mart-1-specific CTLs from the blood of melanoma patients than M-DC (11). We speculate that CD34-DC and M-DC are intrinsically different in their ability to generate Ag-specific CTLs. The difference is unlikely to be caused by suboptimal maturity of either one of these DC as seen in Table IV and Fig. 6. The difference is seen whether these two types of DC are generated in the same cytokine environment such as in our study or in the presence of different cytokines as in other studies (11). The nature of the difference between M-DC and CD34-DC for the generation of Ag-specific CD8+ T cells remains to be determined, but a reasonable explanation is that CD34-DC and M-DC are not entirely overlapping populations of DC. Whereas monocytes appear to be a relatively homogeneous population of cells, CD34+ cells have a heterogeneous developmental potential (21) and employ at least two pathways to differentiate into DC in response to GM-CSF plus c-kit ligand plus TNF-α (9). We have obtained recent evidence that a small population of CD34+ cells with lymphoid progenitor potential also generates DC with great ability to stimulate IFN-γ production by Ag-specific T cells (A. G., unpublished observations).

Table III. Transient production of IFN-γ is specifically reinducible in E75-specific T cells

<table>
<thead>
<tr>
<th>T cells</th>
<th>APC</th>
<th>Peptide</th>
<th>% IFN-γ CD8+ T cells after 5 h</th>
<th>APC</th>
<th>Peptide</th>
<th>% IFN-γ CD8+ T cells after 5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CD34-DC</td>
<td>E75</td>
<td>42</td>
<td>EBV-LCL</td>
<td>E75</td>
<td>38</td>
</tr>
<tr>
<td>1</td>
<td>CD34-DC</td>
<td>E75</td>
<td>Not tested</td>
<td>EBV-LCL</td>
<td>E75</td>
<td>0.7</td>
</tr>
<tr>
<td>3</td>
<td>CD34-DC</td>
<td>E75</td>
<td>Not tested</td>
<td>EBV-LCL</td>
<td>E75</td>
<td>7.0</td>
</tr>
</tbody>
</table>

* Second stimulation after 4 days.
* Second stimulation after 5 days.
* Second stimulation after 7 days.

![FIGURE 5. Flow cytometric determination of IFN-γ production by CD8+ T cells. T cells were incubated with APC at 1:10 ratio for 7 days, and all cultures were restimulated in parallel with EBV-LCL plus E75 peptide (top row) or control C85 peptide (bottom row) for 5 h with monensin to measure the numbers of Ag-specific T cells by flow cytometry using mAbs to CD8 and IFN-γ. Controls include isotype-control stained cells (not shown) to determine the quadrant boundaries and control cells restimulated with an irrelevant peptide C85 (bottom panel), further confirming the absence of background for the detection of IFN-γ production by CD8+ T cells. Percentages of cells stained in the respective quadrants are indicated on the dot-plots.](http://www.jimmunol.org/Downloadedfrom/ftp://www.jimmunol.org/Downloadedfrom/)

---

3602 CD34-DERIVED DC ARE SUPERIOR APC FOR CD8+ T CELLS
While CD34-DC and M-DC have comparable ability to induce allogenic T cell proliferation, they differ in CD8$^{+}$ T cell activation, suggesting that the requirements for the activation of Ag-specific CD8$^{+}$ T cells are distinct from those of CD4$^{+}$ T cells and that this activity may involve different APC. The role of CD28 was excluded because the E75-specific T cells are CD28$^{-}$ (data not shown). The lack of CD28 is not unusual on effector CTLs and has been employed by us to establish the E75-specific T cell lines. Furthermore, a critical role of CD28 for CTL activation was ruled out in CD28 null mice that have reduced Th functions but retain intact abilities to process Ag. In our system we have examined the restimulation of 4-1BB in vivo augments CTL-mediated responses during graft vs host disease and in vitro enhances CD3-mediated T cell proliferation with a preferential effect on resting CD8$^{+}$ T cells (25). In our study, we show that CD137 plays a role in T cell activation after Ag presentation by CD34-DC but that this is not an exclusive property of CD34-DC. There is a partial blocking effect by 4-1BB-Ig fusion protein that is similar after stimulation with CD34-DC or M-DC. The mechanisms underlying the differences between the two types of DC will remain to be defined.

The ability of in vitro-generated DC to present Ag and to activate CD8$^{+}$ T cells is an important functional aspect of APC generated for anti-cancer immunotherapy because MHC class I-restricted Ags play a major role in the recognition of solid tumors that do not spontaneously express MHC class II Ags. In our study, we tested the presentation of the tumor-associated Erb-B2 peptide E75 and find that CD34-DC are superior APC. Other aspects of tumor Ag presentation are important to consider, in particular the ability to process Ag. In our system we have examined the response of already-activated T cells, and further studies are needed to determine whether CD34-DC also provide better priming of naive T cells. Also, it is unclear that CD34$^{+}$ cells and peripheral blood monocytes generated from our patient population are representative of cells that can be obtained in normal individuals or after

### Table IV. CD34-DC provide the highest numbers of Ag-specific T cells

<table>
<thead>
<tr>
<th>Expt.$^{a}$</th>
<th>T Cells$^{b}$</th>
<th>Time$^{c}$(days)</th>
<th>Fold Cellular Expansion$^{d}$</th>
<th>Relative Percentage of CD8$^{+}$ T Cell Activation$^{e}$</th>
<th>Normalized Numbers of Activated T Cells$^{f}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>34-DC</td>
<td>M-DC</td>
<td>M</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>4</td>
<td>0.6</td>
<td>NT$^{g}$</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>5</td>
<td>0.5</td>
<td>0.4</td>
<td>NT</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>7</td>
<td>1.5</td>
<td>1.4</td>
<td>NT</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>7</td>
<td>1.6</td>
<td>0.7</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>7</td>
<td>0.9</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>7</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>14</td>
<td>2</td>
<td>1.2</td>
<td>0.5</td>
</tr>
</tbody>
</table>

$^{a}$ Each experiment compares APCs from the same individual. DCs were generated in contemporary parallel cultures except for Expts. 2 and 3 where 9-day-old M-DC were compared to 20-day-old CD34-DCs. Three MPB samples and two donors were used to generate the APCs.

$^{b}$ T cell subline used for activation.

$^{c}$ Length of time for T:APC first stimulation.

$^{d}$ Fold expansion was calculated by dividing the number of live cells excluding trypan blue at the end of first stimulation by the number of live cells at the start of culture.

$^{e}$ Relative percentages of activation were obtained after restimulation with EBV-LCL plus E75 for 5 h and calculated as percentage of the CD8$^{+}$ T cells producing IFN-γ in the test sample divided by the percentage of CD8$^{+}$ T cells producing IFN-γ in CD34-DC cultures from the same experiment times 100.

$^{f}$ Normalized numbers of activated T cells were calculated by multiplying the total number of cells in culture by the percentage of restimulated cells expressing both CD8$^{+}$ and IFN-γ.

$^{g}$ NT, Not tested.

### FIGURE 6. Comparative titration of CD34-DC and M-DC. Titrations were used to compare the relative potency of 13-day-old CD34-DC and autologous 9-day-old M-DC. Various numbers of these E75-loaded DCs were incubated with the same numbers of T cells for 6 days, after which restimulation by EBV-LCL plus peptide was used to count the number of CD8$^{+}$ IFN-γ$^{+}$ cells in each of these cultures.

### FIGURE 7. Cell-surface marker expression at restimulation. Triple-color flow cytometry was used to measure cell-surface expression of 4-1BB or CD25, CD8, and intracellular levels of IFN-γ. Cells were incubated with the indicated peptide and APCs for 4 days then restimulated for 5 h with EBV-LCL plus E75 before being analyzed by flow cytometry. The dot plots represent the correlated expression of IFN-γ and of 4-1BB or CD25 on the gated CD8$^{+}$ T cell population.
The effects of 4-1BB-Ig fusion protein on numbers of Ag-specific CD8+ T cells

<table>
<thead>
<tr>
<th>4-1BB-Ig</th>
<th>CD34-DC</th>
<th>M-DC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt 1</td>
<td>100</td>
<td>65</td>
</tr>
<tr>
<td>Expt 2</td>
<td>100</td>
<td>49</td>
</tr>
</tbody>
</table>

* Normalized numbers of Ag-specific CD8+ T cells were calculated after 7 days of stimulation with E75-coated APCs as described in Table IV.

** Autologous, flow-cytometry-purified CD34-DC and M-DC were tested at 10% APC:T cell ratio.

NT, Not tested.

Table V. Effects of 4-1BB-Ig fusion protein on numbers of Ag-specific CD8+ T cells

Different treatments. Immune responses against Ags such as E75, which is a nonmutated self-peptide, may not be representative of immune responses against other Ags. More studies will have to be done to determine the biological relevance of our observations. However, we speculate that the observed difference between CD34-DC and M-DC is significant and might indicate that CD34-DC are better able to serve as adjuvants to establish antigenic memory in vivo. This could have important implications for immunotherapy using in vitro-generated DC.

Acknowledgments

We thank Drs. H. Yssel (DNAX Research Institute) and B. Hill (Systemix) for providing cytokines and Abs, Dr. J. Klein and the personnel of the Apheresis Unit and Bone Marrow Transplantation Laboratory of the Karmanos Cancer Institute for help with procurement of patient samples, and Dr. L. Heilbrun for help with the statistical analysis. We also thank the technical support of the Flow Cytometry Core Facility of the Karmanos Cancer Institute. We are grateful to Dr. R. Bright for critical review of the manuscript.

References