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Independent Regulation of Chemokine Responsiveness and Cytolytic Function versus CD8+ T Cell Expansion by Dendritic Cells

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The ability of cancer vaccines to induce tumor-specific CD8+ T cells in the circulation of cancer patients has been shown to poorly correlate with their clinical effectiveness. In this study, we report that although Ags presented by different types of mature dendritic cells (DCs) are similarly effective in inducing CD8+ T cell expansion, the acquisition of CTL function and peripheral-type chemokine receptors, CCR5 and CXCR3, requires Ag presentation by a select type of DCs. Both “standard” DCs (matured in the presence of PGE2) and type 1-polarized DCs (DC1s) (matured in the presence of IFNs and TLR ligands, which prevent DCs “exhaustion”) are similarly effective in inducing CD8+ T cell expansion and acquisition of CD45RO+IL-7R+IL-15R+ phenotype. However, granzyme B expression, acquisition of CTL activity, and peripheral tissue-type chemokine responsiveness are features exclusively exhibited by CD8+ T cells activated by DC1s. This advantage of DC1s was observed in polyclonally activated naive and memory CD8+ T cells and in blood-isolated melanoma-specific CTL precursors. Our data help to explain the dissociation between the ability of cancer vaccines to induce high numbers of tumor-specific CD8+ T cells in the blood of cancer patients and their ability to promote clinical responses, providing for new strategies of cancer immunotherapy. The Journal of Immunology, 2010, 184: 591–597.

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Materials and Methods

Cell lines, media, and reagents

Serum-free AIM-V medium (Invitrogen, Carlsbad, CA) was used to to generate DCs and IMDM (Invitrogen) with 5% human serum (Atlanta Biologicals, Norcross, GA) was used for in vitro sensitization (IVS) experiments. The following factors were used to generate mature DCs: recombinant human (rhu) GM-CSF and IL-4 (gifts from Schering-Plough, Kenilworth, NJ), IFN-α (intron A), rhuTNF-α, rhuIL-1β (all from Strathmann Biotech, Hannover, Germany), rhuIL-6 (Genzyme, Cambridge, MA), LPS (Sigma-Aldrich, St. Louis, MO), PGE2 (Sigma-Aldrich), and poly-IC (Sigma-Aldrich). IL-2 (Chiron, Emeryville, CA) and rhuIL-7 (Strathmann Biotech) were used to support the CD8+ T cell expansion.
Generation and maturation of DCs
PBMCs were obtained from the blood of healthy donors or melanoma patients using lymphocyte separation medium (Cellgro, Mediatech, Herndon, VA). Monocytes were isolated on density gradients, with Percoll (Sigma-Aldrich), followed by plastic adherence. Monocytes were cultured for 6 d in 24-well plates (BD Falcon, Franklin Lakes, NJ) at 5 × 10^5 cells/well in rhuGM-CSF and IL-4 (both 1000 U/ml). At day 6, maturation was induced by exposing the DCs to the following combinations of maturation stimuli: LPS (250 ng/ml) and IFN-γ (1000 U/ml), LPS and PGE2 (10−6 M), TNF-α (100 ng/ml) and IFN-γ, and TNF and PGE2 for 48 h (apart from Fig 1B, when 24–96-h maturation was used, as indicated). In addition, as representatives of clinically applicable polarized and nonpolarized DCs currently used as cancer vaccines, DCs were induced for 48 h in the presence of TNF-α (50 ng/ml), IL-1β (25 ng/ml), PGE2 (10−6 M), and IL-6 (1000 U/ml) (15), and αDC1 matured using the cytokine mixture composed of TNF-α (100 ng/ml), IL-1β (25 ng/ml), IFN-γ (1000 U/ml), poly-I-C (20 μg/ml), and IFN-α (3000 U/ml) (14).

Isolation of peripheral blood CD8+ T cell populations
PBMCs were obtained from the blood of healthy donors or melanoma patients using lymphocyte separation medium (Mediatech). Naive CD8+ CD45RA+CD45RO− T cells were isolated from the lymphocyte fraction by negative selection with CD8 enrichment mixture with the addition of biotinylated anti-CD45RO Ab (StemCell Technologies, Vancouver, British Columbia, Canada) as a uniform population of CD8+CCR7+CD45RA− CD45RO− cells (16, 17). CD8+CCR7+CD45RA+ (CD45RO+) memory T cell population was flow-sorted using MoFlo high-speed cell sorter (DakoCytomation, Carpinteria, CA), after labeling with appropriate Abs.

Flow cytometry
Two- and three-color cell surface and intracellular immunostaining analysis was performed using Beckman Coulter Epics XL flow cytometer, after staining with the Abs against human granzyne B (GrB) (BD Pharmingen and CellSciences), CCR7 (R&D Systems, Minneapolis, MN), CCR5 (BD Pharmingen, San Diego, CA), or the corresponding isotypes IgG2a and IgG1. HLA-A2/MART-112-35 tetramer staining (Beckman Coulter, Immunomics, Fullerton, CA) was performed according to the manufacturer’s instructions.

IVS (polyclonal)
Naive CD8+CD45RA−CCR7high T cells (5 × 10^5 cells/well) were activated with staphylococcal enterotoxin B (SEB)-pulsed monocyte-derived DCs (5 × 10^3 cells/well), as described previously (16, 17). Autologous or allogeneic DCs were used with similar results. On days 5–6, expanded CD8+ T cells were counted and analyzed for the expression of chemokine receptors and chemokine responsiveness and for CTL phenotype and function (see Supplemental Fig. 1 for the kinetics of acquisition of CTL functions in the differentially primed CD8+ T cells). Alternatively, the cultures were fed with low-dose IL-2 and IL-7 (10 ng/ml) every 2 d and analyzed for cell surface and intracellular markers on days 16–20. When indicated, neutralizing IL-12 Ab (clone 29410; R&D Systems) was added at the beginning of the IVS culture. In preliminary experiments, we compared the outcome of naive CD8+ T cell priming by polarized and nonpolarized DCs in the additional presence of CD40L-expressing J558 cells. Because the presence of CD40L did not abolish the differences in the phenotype and function of the resulting T cells, all subsequent experiments were performed in the absence of CD40L.

IVS (melanoma specific)
Bulk CD8+ T cells (5 × 10^5 cells/well) were activated with the HLA-A2–restricted peptide MART-112–35-pulsed autologous DCs (5 × 10^3 cells/well). A total of 3000 rad-irradiated CD40L-J558 cells (5 × 10^5 cells) were added as surrogates of CD40L-expressing CD4+ Th cells, as described previously (14). On day 4, rhuIL-2 (50 U/ml) and IL-7 (10 ng/ml) were added. CD8+ T cell cultures were expanded by an additional stimulation (day 14) with irradiated peptide-pulsed autologous PBMCs. At day 24, the differentially induced CD8+ T cell lines were stained for CCR5, GrB, and MART-1. CTL activity was determined by 51Cr release assays against HLA-A2+ melanoma (Fem X), with HLA-A2*08:39 melanoma cells serving as negative specificity control.

Chemotaxis assay
Chemotaxis assays were performed in 96-well Transwell plates with a 3-μm pore-size polycarbonate filter (Corning, Corning, NY). The lower chamber was filled with 200 μl of rhuCCL19 (100–1000 ng/ml) or rhuCCL5 (100–1000 ng/ml) in RPMI 1640 plus 0.5% FBS (chemotaxis media), and 50 μl (5 × 10^5 cells) of differentially activated CD8+ T cells was added in the upper chamber, and migration chambers were incubated for 3 h at 37°C. After 3 h, the cells from lower wells were harvested and counted. The number of cells that migrated in media alone was subtracted to normalize for background migration.

CTL assay
Cytolytic activity against HLA-A2+ melanoma cells (Fem X) was determined by standard 4-h 51Cr release assays, with HLA-A2*08:39 397 melanoma cell line serving as negative control of specificity. The results were calculated and recorded as percent target killing at individual E:T ratios, or percentage of cytolyis was converted to LUs (U/L)103 as described previously (18).

Statistical analysis
The data were analyzed using Student’s t test (with paired tests being used for comparisons including αDC1s- versus sDCs-induced responses from multiple donors). Values of p < 0.05 were considered significant.

Results
Independent regulation of CD8+ T cell expansion and acquisition of CTL functions by polarized and nonpolarized DCs
In order to delineate the requirements for the effective expansion of CD8+ T cells and their acquisition of effector functions, we compared the outcome of CD8+ T cell priming by DCs induced to mature by mediators of acute inflammation (combination of IFNs and TLR ligands) or by mediators of chronic inflammation (presence of PGE2) (19–21). Although the DC maturation in the presence of PGE2 is associated with an irreversible process of DC “exhaustion” manifested by reduced ability to produce IL-12, the key mediator of inflammatory-type responses (22), and reduced ability to induce Th1 responses of CD4+ T cells (10, 23, 24), DCs1 induced in the conditions of early inflammation avoid the maturation-associated DC “exhaustion”, retaining their ability to produce IL-12 and to induce Th1 responses of CD4+ T cells (7, 14, 23, 24).

As shown in Fig. 1A, left, both polarized and nonpolarized DCs induced similar rates of expansion of naive CD8+ T cells. However, only naive CD8+ T cells primed by the polarized DCs1 in our previously established model of priming of naive CD8+ T cells (16, 17) demonstrated an effective induction of GrB (Fig. 1A, right), a marker of effector T cell differentiation (25). In sharp contrast, the low IL-12–producing nonpolarized DCs (14, 23, 24) did not prime naive CD8+ T cells to express GrB (Fig. 1A, right), despite inducing a similar or higher T cell expansion (Fig. 1A, left).

Importantly for their use as therapeutic agents in vivo, DCs1 retained a significant (although reduced) ability to induce GrB expression in expanding CD8+ T cells, even at later times (96 h) after the induction of their maturation (Fig. 1B). These latter observations indicate that the maturation of DCs in the conditions mimicking early inflammation allows them to at least partially avoid or delay the acquisition of an “exhausted” status (10, 24), previously shown to be associated with abrogated ability to induce functional Th1 responses in the population of CD4+ T cells (10).

Using the clinically-relevant TNF-α/IL-1β/poly-I:C/IFN-γ/IFN-α–matured αDC1s (14) and TNF-α/IL-1β/IL-6/PGE2–matured sDCs (15) as representatives of type 1-polarized versus non-polarized DCs, we observed that the induction of GrB correlated with the superior cytolytic function of CD8+ T cells primed by the polarized DCs (Fig. 2A). In contrast, priming of CD8+ T cells by the PGE2–matured sDCs led to low levels of GrB and poor ability to kill Ag-pulsed target cells (Fig. 2A–C), despite effective proliferation of T cells in these cultures and induction of CD45RO (Figs. 1, 2B). In accordance with the central role of IL-12 in the development of CTL activity in CD8+ T cells, neutralization of that factor abrogated GrB induction by DCs1 (Fig. 2D).
Because certain conditions of effector T cell induction can be associated with their irreversible differentiation into short-lived, terminally differentiated effector cells (26), we tested the ability of the DC1-induced effector cells to respond to secondary activation and undergo secondary CTL differentiation. As shown in Fig. 3, after completing the effector phase of activation (>2 wk after priming), the αDC1-primed CD8+ T cells downregulated the levels of GrB expression and their cytolytic activity. Consistent with the ability of polarized αDC1s to induce long-lived CD8+ T cells (14), such resting αDC1-primed CD8+ T cells expressed high levels of IL-15Ra and IL-7Ra (CD127) (Fig. 3A; see Supplemental Fig. 2 for the levels of both receptors in naive CD8+ T cells), the memory cell-associated receptors for the homeostatic cytokines mediating long-term survival of CD8+ T cells (27, 28) and were fully capable of rapidly reacquiring high levels of CTL activity upon restimulation with polarized αDC1s (Fig. 3B).

Polarized DCs induce a switch in chemokine receptor expression and peripheral tissue-associated chemokine responsiveness in expanding CD8+ T cells: key role of IL-12

Because polarized αDC1s and sDCs both promoted the expansion of naïve T cells but had a differential impact on the induction of their CTL function, we tested their influence on the CD8+ T cell expression of CCR7 and CCR5, the respective lymphoid versus peripheral effector-type chemokine receptors, and the migratory responsiveness to their respective ligands. Lymph node-associated CCL19/MIP3β (29–31) and CCL5/RANTES, a ubiquitous peripheral tissue-produced chemokine (29, 30) known to be overexpressed in cancer tissues (30, 32).

As shown in Fig. 4A and 4B, αDC1s effectively induced the expression of CCR5, the chemokine receptor typical for effector (and effector-memory) CD8+ T cells (33–35), with a concomitant loss of CCR7 on 50–70% of CD8+ T cells. In contrast, CD8+ T cells stimulated by sDCs retained high levels of CCR7 expression and did not acquire CCR5.

In accordance with their differential expression of CCR7 and CCR5, the differentially activated CD8+ T cells showed reciprocal patterns of migratory responsiveness to the lymph node-associated versus peripheral tissue-associated chemokines (CCL19 and CCL5, respectively [29, 30, 32, 35]) with αDC1-primed CTLs preferentially migrating toward the peripheral tissue chemokine CCL5 (RANTES), whereas the sDC-primed T cells preferentially responded to the lymphoid chemokine CCL19 (Fig. 4C).

Because in the CD4+ T cell system, the levels of DC-produced IL-12 were shown to be the key to the differential ability of DCs to induce a Th1 or Th2 pattern of differentiation in naïve CD4+ T cells (7, 8, 10) and rIL-12 was shown to directly affect the expression of Th1- and Th2-associated chemokine receptors (36, 37), we tested the role of IL-12 in the DC-induced switch in chemokine receptor expression of CD8+ T cells. As shown in Fig. 4D, the neutralization of IL-12 during T cell priming abrogated the above differences, preventing the downregulation of CCR7 and elevation of CCR5 on CD8+ T cells activated by the polarized DCs. These data indicate that IL-12, originally identified as a factor supporting killer activities of CD8+ T cells and NK cells (reviewed in Ref. 22), is also a key DC-produced factor responsible for the switch from central to peripheral chemokine receptor pattern in the differentiating naïve CD8+ T cells.

Polarized and nonpolarized DCs differentially regulate CTL activity and chemokine receptor expression on tumor Ag-specific CD8+ T cells

Prompted by the results of the experiments with polyclonally activated naïve CD8+ T cells (Fig 2A) and similar data obtained using memory cells (Supplemental Fig. 3), we have compared the outcome of IVS of HLA-A2–restricted melanoma-specific CD8+ T cells using MART-127–35–loaded autologous αDC1s or sDCs, currently applied as cancer vaccines.

In contrast to the short-term experiments performed in the polyclonal system, the generation of high numbers of MART-1–specific
T cells required prolonged cultures of the differentially sensitized CD8+ T cells. Although in these long-term cultures we could not detect the differences in CCR7 expression between the differentially-sensitized CD8+ T cells (CCR7 was low on both populations; data not shown), exclusively the MART-1–specific (tetramer positive) CD8+ T cells sensitized with polarized aDC1s showed high GrB expression and high CTL activity against MART-1–expressing HLA-A2+ melanoma cells (but not against HLA-A2– melanoma cells; Fig. 5A–C). Although in contrast to their inability to induce CTL activity in naive CD8+ T cell population (see Figs. 1 and 2), nonpolarized DCs showed significant ability to induce CTL function in tumor-specific T cells from melanoma patients, though DC1s were clearly more efficient (Fig. 5C), with the level of advantage comparable to that observed in the polyclonal model of (re)activation of “bulk” (memory and naive) CD8+ T cells (Supplemental Fig. 4). In accordance with the data obtained in the polyclonal models (Fig 4), MART-1–specific CD8+ T cells sensitized by polarized aDC1s also showed elevated levels of CCR5 (Fig. 5D).

In addition to CCR5, which shows high effectiveness in attracting mouse effector cells to melanoma lesions (38) and was recently implicated in the responsiveness of melanoma patients to immunotherapy (39), another CTL-associated chemokine receptor, CXCR3, has been recently implicated in melanoma regression (40) and prolonged survival of patients with advanced disease (41). Therefore, we compared the expression of CXCR3 on MART-1–specific CD8+ T cells presensitized with polarized aDC1s and sDCs. As shown in Fig. 5D, polarized aDC1s induced strongly elevated levels of CXCR3 in MART-1–specific CD8+ T cells from melanoma patients.

**Discussion**

Our data demonstrate that the ability of DCs to activate T cells and to efficiently induce their expansion does not predict their ability to induce CTL activity and the ability to respond to peripheral-type chemokines. In contrast, we observed that although the expansion of CD8+ T cells can be driven efficiently by the DCs matured in
FIGURE 4. Polarized DCs induce a switch in chemokine receptor expression and chemokine responsiveness. Naïve CD8+ T cells were primed by αDCs or sDCs. Differentially primed CD8+ T cells were harvested on day 5 and analyzed for the expression of chemokine receptors. A, Data from a representative donor: Levels of expression of CCR7 and CCR5 (black lines), compared with isotope controls (gray lines). B, Cumulative data from three donors. Fold increase in MFI of CCR7 and CCR5 were calculated as in Fig. 1. Data are shown as mean ± SEM of three independent experiments that all showed advantage of polarized αDCs in promoting the loss of CCR7 expression (p < 0.0005) and induction of CCR5 (p < 0.005). C, Differentially primed CD8+ T cells were analyzed for their responsiveness to chemokine receptor ligands CCL19 and CCL5 by chemotaxis assay (mean ± SEM of three independent experiments). In the three donors tested, at the maximal concentrations of the two chemokines, the migration of αDCs was 3.4- to 5.2-fold lower than the migration of sDCs to CCL19, whereas the migration of αDCs in response to CCL5 was 3.6- to 11.8-fold higher that the migration of sDCs. *, Undetectable. D, IL-12-blocking Ab was added during the priming of naïve CD8+ T cells by polarized αDCs. CCR7 and CCR5 expression (black lines) was assessed by flow cytometry on day 5. Gray line indicates isotype control in all histograms. Similar data were observed in two additional experiments.

a wide spectrum of inflammatory conditions, the induction of the CD8+ T cell effector functions in naïve CD8+ T cells and a switch in their chemokine responsiveness was a sole property of the “nonexhausted” IL-12-producing DCs matured in the conditions that mimic acute inflammation (presence of IFNs and TLR ligands). This “inflammatory” pathway of activation of CD8+ T cells, associated with the IL-12–dependent induction of GrBhigh CTLs, eventually results in a resting population of memory-type (CD8+CD45RO+GrBlow) cells. In accordance with the previously reported long-lived character of cells activated by the high IL-12–producing DCs (14) and with the ability of rIL-12 to promote CTL survival (42, 43), the CD8+ T cells undergoing such “inflammatory” pathway of differentiation expressed high levels of IL-7R and IL-15R (Fig. 3), known to be essential for the homeostatic proliferation and long-term survival of CD8+ T cells in vivo (27, 28), and could effectively reacquire CTL function following restimulation with polarized DCs.

The effectiveness of polarized DCs in inducing functional CCR5 (and CXCR3)-expressing CTLs suggest that these cells can be useful tools to direct the vaccination-induced T cells to tumors in therapeutic conditions. Because melanomas are known to overexpress CCL5/RANTES (44, 45), on which they rely as an autocrine growth factor (45, 46), CCL5-responsive, αDC1-induced T cells are likely to show improved therapeutic activity, not only because of their higher per-cell killer activity, but also because of their ability to preferentially home to tumor tissues. In support of the opposite roles of tumor-expressed CCR5 versus T cell-expressed CCR5 in melanoma progression (respectively, tumor promoting versus tumoricidal), it was recently shown that although overall populations of melanoma patients lacking functional CCR5 (CCR5Delta32+ individuals) and CCR5-competent melanoma patients have similar course of disease, functional CCR5 is needed for positive response to immunotherapy (39). Similarly, in accordance with high expression of CXCR3 ligands (CXCL9/MIG and CXCL10/Ip10) in melanoma tissues (47) and the presence of CXCR3 on tumor-infiltrating lymphocytes in regressing melanoma lesions (40), high levels of CXCR3 on circulating CD8+ T cells has been recently implicated in effective control of advanced melanoma (41).

In contrast to such “proinflammatory/effector” pathway of differentiation driven by polarized DCs, naïve CD8+ T cells activated by standard nonpolarized DCs did not acquire CTL functions and remained responsive to lymph node-associated chemokines, even though they vigorously expanded. Although our preliminary data indicate that such cells can be effectively reactivated by polarized DCs (data not shown) to undergo secondary CTL differentiation, the identity and functional role of such “non-effector” CD8+ T cells induced by standard “exhausted” DCs remains a subject of our follow up studies. Interestingly, although nonpolarized DCs were unable to induce the de novo effector function in naïve CD8+ T cells, they showed a significant (although lesser than polarized DCs) ability to induce CTL function in (expectedly previously primed) tumor-specific T cells from melanoma patients, type (Fig. 5A–C), and in the polyclonal model of (re)activation of “bulk” (memory and naïve) CD8+ T cells (Supplemental Fig. 4).

The current demonstration that the ability of DCs to induce proliferation and expansion of tumor-specific CD8+ T cells is independent from their ability to induce their tumor-relevant homing properties and tumoricidal effector functions helps to interpret the limited effectiveness of cancer vaccines observed in recent clinical trials (3–6) and aids in designing corrective measures to enhance the efficacy of cancer immunotherapies. Several recently tested cancer vaccines involving
antigenic peptides or tumor Ag-expressing viral vectors were shown to promote massive increase of blood-circulating tumor-specific CD8⁺ T cells but not clinical responses (1, 2, 4–6). Interestingly, at least one study indicated that such split effectiveness of cancer vaccines can be corrected by a follow up treatment of the vaccinated patients with IFN-α (1). Although our current data (Fig. 2D) demonstrate the key role of IL-12 in the induction of functional CTLs by DC1s, it remains to be tested whether other factors may supplement or replace the function of IL-12 in differentially matured DCs.

Our current data suggest that the limitations of current cancer vaccines, including “standard” DC-based vaccines (48), may result from their selective deficit in inducing the effector functions in tumor-specific T cells and may be corrected by the modification of the current therapeutic vaccines, or their combination with proinflammatory factors, capable of inducing tumoricidal function and tumor-homing ability in tumor-specific T cells.

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Disclosures
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References
Supplemental Figure 1. Kinetics of induction and disappearance of killing capacity of differentially primed CD8+ T cells. The cytolytic activity of DC1- and standard DC-primed CD8+ T cells against SEB pulsed JY-1 cells was determined at specified time points after priming, using a standard 4 hour 51Cr release assay.
Supplemental Figure 2. Expression of CD127 (IL-7Rα) and IL-15Rα on naïve CD8+ T cells. The expression of CD127 and IL-15Rα (solid lines) on CD45RO-CD8+ T cells was determined by flow cytometry. Dotted lines are mouse IgG1 isotype controls.
Supplemental Figure 3: Blood isolated memory CD8⁺ T cells require stimulation by polarized DC1s for secondary CTL differentiation. Circulating memory CD8⁺CD45RO⁺CCR7⁺ T cells were flow sorted from peripheral blood and stimulated with polarized DC1s or standard DCs. After 3 days, the cells were analyzed for cytolytic potential, using a standard 4 hour ⁵¹Cr release assay. Similar data was obtained in 3 independent experiments.
Supplemental Figure 4: Activation of bulk (previously-primed and naïve) CD8⁺ T cells by differentially matured DCs results in the induction of killing capacity. Bulk CD8⁺ T cells, normally consisting of 60% CD45RA⁺ CD8⁺ T cells and 40% CD45RO⁺ CD8⁺ T cells, were isolated from blood using negative selection. Cultures of dendritic cells and T cells were supplemented with 50 U/ml IL-2. The cytolytic activity of DC-primed and standard DC-primed bulk CD8⁺ T cells against SEB pulsed JY-1 cells was determined 5 days later using a standard 4 hour ⁵¹Cr release assay. Data from 1 of 2 experiments that yielded similar results.