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Fast Dendritic Cells Stimulated with Alternative Maturation Mixtures Induce Polyfunctional and Long-Lasting Activation of Innate and Adaptive Effector Cells with Tumor-Killing Capabilities

Chiara Massa and Barbara Seliger

The clinical usage of dendritic cells (DC) for tumor immunotherapy still requires improvements. In this study, three alternative maturation mixtures were compared with the cytokine-based gold standard, and the overall interaction of the resulting DC with effector cells from the innate as well as the adaptive immunity was evaluated in healthy donors. Stimulation with the TLR-4 ligand monophosphoryl lipid A together with IFN-γ (alt-2 DC) resulted in DC with the highest levels of costimulatory molecule expression and IL-12p70/IL-10 ratio. Whereas all alternative DC were able to induce NK and γδ T cells to acquire cytotoxic properties and secrete type 1 and proinflammatory cytokines, after both short (20-h)- and long (5–8 d)-time coculture, secretion of IFN-γ by the innate populations was induced in response to alt-2 and alt-1 DC (TNF-α, IFN-α, IFN-γ, IL-1β, poly IC), but not to alt-3 DC (TNF-α, IFN-γ, IL-1β, CL097). Regarding CD8+ T cell–mediated Ag-specific immune responses, a heterogeneous pattern of responses was obtained among the healthy donors, suggesting rather a competition than a synergy among the different effector cells. Our data promote further evaluation of alt-2 fast DC for translatability into clinical immunotherapy trials, while also fostering the need to identify biomarkers for immune cell responsiveness and tumor susceptibility to be able to select for each patient the best possible DC-based therapy. The Journal of Immunology, 2013, 190: 000–000.

Dendritic cells (DC) are considered the most potent APCs of the immune system and have been investigated to employ their adjuvant activity in tumor immunotherapy. Clinical trials in different tumor entities have revealed safety and tolerability of such treatment. However, despite a frequent vaccine-induced expansion of precursor cells in the blood and/or the induction of delayed-type hypersensitivity, the rate of objective clinical responses obtained in tumor patients is very limited (1, 2). For this reason, an optimization of the protocol for vaccine DC production as well as their deep functional characterization is urgently needed to improve their clinical implementation.

The major protocol currently used for the production of vaccine DC is a two-step process, in which monocytes are first differentiated toward immature DC by a 5-d culture in the presence of GM-CSF and IL-4 (3), followed by their stimulation with the so-called gold standard cytokine mixture composed of TNF-α, IL-1β, IL-6, and PGE2 (4). The major disadvantages of this protocol are the labor- and time-consuming procedures together with an impaired production as well as their deep functional characterization is urgently needed to improve their clinical implementation.

The novel protocol currently used for the production of vaccine DC is a two-step process, in which monocytes are first differentiated toward immature DC by a 5-d culture in the presence of GM-CSF and IL-4 (3), followed by their stimulation with the so-called gold standard cytokine mixture composed of TNF-α, IL-1β, IL-6, and PGE2 (4). The major disadvantages of this protocol are the labor- and time-consuming procedures together with an impaired production as well as their deep functional characterization is urgently needed to improve their clinical implementation.

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Abbreviations used in this article: BD, Becton Dickinson; DC, dendritic cell; HSD, honestly significant difference; KIR, killer cell Ig-like receptor; MPLA, monophosphoryl lipid A; polyIC, polyinosinic:polycytidylic acid.

Monophosphoryl lipid A (MPLA) and the imidazoquinoline compound CL097 were purchased from InvivoGen (San Diego, CA), IL-1β from BioSource International (Camarillo, CA), IFN-α2b from ProSpec (Reho-
voltage (volt, Israel), IFN-γ from Immunotools (Friesoythe, Germany), polyclinics: polycytic acid (polyC) and PGE2; from Sigma-Aldrich (St. Louis, MO), TNF-α from PeproTech (Hamburg, Germany), whereas IL-6 was a gift of S. Rose-John (Institute of Biochemistry, University of Kiel, Kiel, Germany).

The following Abs were used: Alexa Fluor700 anti-CD3, eFluor450 anti- CD8a, PE-Cy7 anti-CD14, FITC anti-CD80, FITC anti-CD83, PE anti-CD86 (from eBioscience/Nat Tec, Frankfurt, Germany), PE anti-CD40, FITC anti-CD68, PE-Cy7 anti-CD68, aliphophycocyanin Alexa Fluor750 anti-CD16 (from Beckman Coulter, Brea, CA), FITC anti-granzyme B, FITC and PerCP anti-V62, aliphophycocyanin anti-CD107a (from BioLegend, San Diego, CA), PE anti-CD69, and aliphophycocyanin anti-CD25 (from Becton Dickinson [BD] Biosciences, Franklin Lakes, NJ).

A minimum of 10,000 events gated on live, propidium iodide-negative cells was acquired either on a FACScan (BD Biosciences) or a Navios (Beckman Coulter) flow cytometer and evaluated using CellQuest (BD Biosciences) or Kaluza (Beckman Coulter) software. The general gating strategy for the flow cytometric acquisition is shown in Fig. 1.

Concentration of IL-12p70 and IL-10 in DC supernatants was evaluated using commercially available ELISA kits (BioLegend and Immunotools, Friesoythe, Germany, respectively). For lymphocyte characterization, IFN-γ and TNF-α secretion was evaluated using the secretion assay kit from Miltenyi Biotec (Bergisch Gladbach, Germany), whereas the cytokine content of the culture supernatants was evaluated by means of the flow-cytomix Th1/Th2/Tfh/Th17/Th22 kit from eBioscience/Nat Tec following manufacturer’s instructions.

**DC differentiation and maturation**

Buffcoats from HLA-A–typed healthy donors were obtained from the blood bank of the University Hospital in Halle upon written informed consent. PBMCs were purified by a density gradient centrifugation on a Percoll layer (Pau-Biotec, Aidenbach, Germany), and then monocytes were isolated using anti-CD14–conjugated microbeads (Miltenyi Biotec). Differentiation was performed by seeding 10^6 cells/ml in serum-free CellGro medium (CellGenix, Freiburg, Germany) with 200 ng/ml GM-CSF (Leukine; Bayer HealthCare, Seattle, WA) and 1000 U/ml IL-4 (Immunotools) for 24–36 h to obtain immature fast DC. Maturation was induced by 18-h incubation in the presence of the gold standard (TNF-α + IL-1β + IL-6 + PGE2) and the three alternative mixtures, alt-1 (also known as α-type one polarizing; TNF-α + IL-1β + IFN-α + IFN-γ + polyC10) (10), alt-2 (MPLA + IFN-γ) (11), and alt-3 (TNF-α + IL-1β + IFN-γ + CL097; adapted from Ref. 12). The concentrations used in the maturation mixtures were as follows: CL097, 3 μg/ml; IFN-α, 3000 U/ml; IFN-γ, 500 U/ml; IL-1β, 5 ng/ml; IL-6, 5 U/ml; MPLA, 4 μg/ml; PGE2, 1 μg/ml; polyC10, 5 μM; TNF-α, 100 ng/ml.

At the end of the maturation period, supernatants were frozen for cytokine evaluation by ELISA, whereas DC were thoroughly washed and used for phenotypical (flow cytometry) and functional characterization (coculture assays).

**DC lymphocyte coculture**

All cocultures were performed in serum-free X-vivo15 medium (Lonza, Basel, Switzerland), using as effector cells the autologous PBLS corresponding to the fraction of PBMCs remaining after CD14– monocyte purification. When specified, CD8+ T cells were purified and CD56+ cells were depleted using microbeads following manufacturer’s instructions (Miltenyi Biotec).

For short-term cultures (4–21 h), 4 × 10^6 DC/well were seeded in U-bottom 96-well plates with 10^5 PBL in 200 μl, whereas for long-time culture (5–10 d), 10^5 DC were incubated with 10^6 PBL in 1 ml in 24-well plate.

For mechanisms evaluation, DC were precubinated with blocking Abs directed against IL-12p(p35+p70) (Gen-probe, San Diego, CA) for 30 min at 37°C before lymphocytes were added; the mAb concentration in the final volume was 10 μg/ml.

For evaluation of Ag–specific immune responses, mature DC were pulsed with synthetic peptides for 2 h at 37°C before incubation with effector cells for 10 d. To evaluate the expansion of naive CTL, the MelanA/Mart-1 peptide (AAGIGILTV; Peptides & Elephants, Potsdam, Germany) was used due to the high frequency of MelanA/Mart-1 precursors in healthy donors (18). In such cases, purified CD8+ T cells were purified, total, or NK-depleted PBL were incubated with peptide-pulsed DC, and 50 U/ml IL-2 (Proluken, Kloten, Switzerland) for 24–36 h (Halle) and 10 ng/ml IL-7 (Immunotech) were added on days +3, +5, and +7. On day 10, cultures were stained with PE MelanA/Mart1–specific tetramers provided by Beckman Coulter following manufacturer’s instructions. Cultures containing expanded MelanA/Mart1–specific CD8+ T cells were restimulated with irradiated, peptide-pulsed feeder PBL for 10 d in the presence of IL-2 and IL-7 before the Ag-specific degranulation was evaluated using a CD107a assay.

The expansion of memory, antiviral responses was evaluated by pulsing the stimulating mature DC with the CEFl-peptide pool HLA-A2 (PANTeacs, Tuebingen, Germany) or a 1:1 mixture of the HLA-A3–restricted epitopes from the influenza A virus nucleoprotein p265–273 (ILRGSVSAHK) and the EBV nuclear Ag-3 p471–479 (RLRAEAQVK; both synthesized from Peptides & Elephants).

**Proliferation assay**

Autologous PBL were labeled with 5 μM CFSE (Molecular Probes, Invitrogen, Paisley, U.K.) or cell proliferation dye eFluor670 (eBioscience/Nat Tec) for 10 min at 37°C in PBS supplemented with 10% FCS (Invitrogen, Karlsruhe, Germany) and then thoroughly washed. A total of 10^6 PBL was added at a 10:1 ratio to mature DC for 5 d before the proliferation was evaluated by flow cytometry as dye dilution in gated lymphocyte subpopulations. One replicate well was used for determination of the absolute cell count upon the addition of Flow-Count Fluorospheres (Beckman Coulter). The absolute counts of the different immune populations were obtained by combining the absolute total cell count from the evaluation with the beads and the percentages of the different subsets obtained upon counter staining with the different Abs.

**Evaluation of cytotoxicity**

Development of cytotoxic activity was evaluated both in a standard [51Cr] release assay (day 5–10 cocultures) and by means of a CD107a degranulation assay (overnight and day 5–20 cocultures). Target cells to evaluate innate effector killing were Daudi cells, whereas the HLA-A2+ and A3+ melanoma cell line Mel1379 was used to evaluate Ag-specific killing upon peptide pulsing. As further controls for the Ag specificity in the melanA/Mart1–setting peptide-pulsed T2 cells and the undifferentiated HLA-A2+ melanoma cell line NaB that lack the expression of MelanA/Mart1-1 were employed. The HLA-A2–restricted tyrosinase epitope p351 (YMNGMTSQV) was used as an unrelated Ag to pulse T2 cells.

For the [51Cr] release assay, labeled cells were incubated with titrated amounts of effector cells for 4 h before supernatants were collected for measurement in a gamma counter. Lysis was calculated as 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release), where the maximum and spontaneous release were obtained by culturing the target cells in 1% Triton X-100 (Sigma-Aldrich) and X-vivo15 medium, respectively.

For the CD107a degranulation assay, 10^5 PBL were incubated 1:1 with target cells for a total of 4 h and the CD107a mAb was added after the first hour of culture. At the end of the incubation time, the various immune cell populations were stained using Ab specific for CD3, CD8a, CD16, CD56, and V62 and counter stained with propidium iodide (Sigma-Aldrich).

To evaluate the content of granzyme B, PBL were first stained with the live/dead fixable dead cell stain kit (Molecular Probes, Invitrogen, Paisley, U.K.) and the Ab for surface marker, then fixed in 4% paraformaldehyde, permeabilized in 0.1% saponin, and stained with a FITC-conjugated anti- granzyme B mAb or isotype control Ab.

**Statistical analysis**

Differences between treatments were evaluated using the one-way ANOVA test for correlated samples and the Tukey honestly significant difference (HSD) test performed with the web-based software Vassarstat (http://vassarstats.net). Significance upon Ab treatment and between CD8+ T cells and total PBL was evaluated using two-tailed paired t test. Probabilities below 0.05 (*) or 0.01 (**) were considered significant. Data from different experiments are shown as mean ± SE, whereas SD among duplicate/triplicate wells are shown within single experiments.

**Results**

**Characterization of mature fast DC**

Monocytes were differentiated toward DC with the so-called fast protocol and stimulated for 18 h with the gold standard and three alternative maturation mixtures, named alt-1, alt-2, and alt-3 (see Materials and Methods for composition). In response to all maturation mixtures, fast DC upregulated the expression of the costimulatory and adhesion molecules CD40, CD54, CD80, CD83, and CD86 (Figs. 1, 2A), but to a different extent. Among the alternative mixtures, alt-2 induced the highest expression levels.
of these molecules, whereas alt-3 induced the lowest levels, which were for CD83 and CD86 even lower than that of the gold standard.

In line with published results, all alternative mixtures employed were able to induce the secretion of the bioactive IL-12p70 during DC maturation, whereas the gold standard induced no or very low levels of this cytokine (Fig. 2B, left). Furthermore, alt-1 and alt-3 induced high levels of IL-10 that were absent in the supernatants of gold standard and alt-2 DC (Fig. 2B, right). Upon reseeding in the absence of exogenous stimuli, the alt-1 and alt-2 DC were still able to secrete significant, but much lower levels of IL-12, whereas alt-1 DC also maintained a significant IL-10 secretion (Fig. 2C). Interaction with autologous PBL was able to induce IL-
12 secretion by alt-1 and alt-2 DC both when occurring immediately after maturation and after 24 h, during which the DC were left untreated (Fig. 2D), a setting mimicking the time required by vaccine DC for migration to the lymph node upon in vivo injection.

Alternative DC strongly stimulate innate cells in short-time culture

To analyze the functional properties of the various preparations, DC were incubated overnight (16–21 h) with autologous lymphocytes, and the culture supernatants were evaluated for the presence of cytokines corresponding to the different types of immune polarization, namely proinflammatory (IL-6 and TNF-α), type 1 (IL-1β, IL-2, and IFN-γ), type 2 (IL-4, IL-5, IL-10, and IL-13), type 9 (IL-9), type 17 (IL-17A), and type 22 (IL-22) cytokines. A potent secretion of IL-1β, IL-6, IFN-γ, and TNF-α was found in response to the coculture with the alternative DC, with alt-1 and alt-2 inducing higher levels than alt-3 DC, whereas almost no cytokines were detected in response to stimulation with gold standard DC. Regarding the other tested cytokines, only IL-10 was consistently detected among different donors and significantly produced uniquely in response to alt-1 DC (Fig. 3A). To dissect the responsible immune cell population(s), a cytokine secretion assay was performed and immune cells were then counterstained with CD3, CD56, and V82 Abs to identify NK (CD3-CD56+) and γδ T cells (CD3- Vδ2+) following the gating strategy illustrated in Fig. 1. Despite the highly variable frequencies in the different donors, NK and γδ T cells responded to the overnight incubation with the alternative DC by secreting IFN-γ and TNF-α (Fig. 3B). A consistent secretion of IFN-γ by the innate populations was observed already after 4-h incubation with the alt-1 and alt-2 DC (Fig. 3C). Further staining with the CD16 Ab to dissect NK cell subpopulations revealed that both the CD56dimCD16+ and CD56dimCD16- subpopulations secreted IFN-γ after 4 (Fig. 3D) as well as after 16 h (data not shown).

Overnight stimulation with the alternative DC also resulted in the upregulation of activation markers like CD25 and CD69 (Supplemental Fig. 1) as well as in the induction of cytolytic functions. Indeed, innate cells stimulated with the alternative DC displayed an enhanced degranulation in response to Daudi cells in comparison with gold standard–stimulated effectors (Fig. 4A). Concerning the NK cell subpopulations, not only the CD56dimCD16+ population, but also the CD56dimCD16- cells were induced by alt-1 (data not shown) and alt-2 DC to degranulate upon incubation with Daudi cells (Fig. 4B, top and center). Intracellular staining revealed a dramatic induction of granzyme B in the CD56dimCD16- NK population by alt-1 (data not shown) and alt-2 DC (Fig. 4C, top), suggesting that their degranulation could also be associated with target killing. In contrast, in CD56dimCD16+ NK and γδ T cells, the already high expression of granzyme B was not significantly augmented by stimulation with the alternative DC (Fig. 4C, center and bottom).

Regarding the γδ T cell population, a highly donor-dependent expression of the CD56 marker, ranging from 0–50% of positive cells, was found (data not shown). Its expression did not strictly correlate with the effector function, because the frequency of degranulation was comparable in both CD56-positive and -negative cells, or was even slightly more pronounced in the negative subset (Fig. 4C, bottom).

Alternative DC have long-time effects on innate cells

Interaction between alternative DC and innate cells also had long-term consequences, as follows: an enhanced proliferation of innate cells was demonstrated by staining of lymphocytes with a prolif-
This was accompanied by an enhanced degranulation and also effective killing of the target cells as measured in a classical \([^{51}Cr]\) release assay (Fig. 6B). Effector cells also acquired the ability to kill tumor cells, as representatively shown for the melanoma cell line Mel1379 (Fig. 6C).

**Different requirements for cytokine secretion and development of cytotoxicity**

To dissect the mechanisms of the enhanced innate activation, DC were incubated with PBL in the presence of the isotype control or blocking Abs directed against IL-12p70 prior to their functional
evaluation. Upon 16-h incubation with alt-2 DC in the presence of the anti–IL-12p70 Ab, NK and γδ T cells almost completely lost their ability to secrete IFN-γ (Fig. 7A), whereas no statistically significant alteration in their degranulation in response to Daudi cells (Fig. 7B) was found. In addition to the loss of IFN-γ secretion, a slight and statistically not significant increase of IL-10 (p = 0.068) and TNF-α (p = 0.12) was found in the coculture supernatants, whereas IL-1β and IL-6 secretion remained unaffected (Fig. 7C) and cytokines corresponding to other immune polarizations were still not detected (data not shown). Blockade of IL-12p70 in the long-term coculture confirmed the independence from IL-12p70 for the degranulation in response to Daudi cells (Fig. 7D, left), but highlighted a partial requirement for the responsiveness of NK and even more γδ T cells to the melanoma Mel1379 (Fig. 7D, right).

Excessive innate activity is counterproductive for Ag-specific CTL expansion
To determine the consequences of the enhanced activation of innate effector cells on the adaptive immunity, DC were pulsed with synthetic peptides and used to stimulate CD8+ T cells either purified or within total PBL. To investigate a cancer-related setting, HLA-A2+ DC were pulsed with the MelanA/Mart-1 peptide and cocultured with effector cells for 10 d in the presence of IL-2 and IL-7. When the DC were used to stimulate purified CD8+ T cells, alt-1 and alt-2 DC were able to strongly expand MelanA/Mart-1–specific T cells, as evaluated by staining with specific tetramers (Fig. 8A). In contrast, in the presence of the other immune cell populations of total PBL, a reduced expansion of MelanA/Mart-1–specific CD8+ T cells by alternative DC was obtained, whereas there was an improvement in gold standard DC performance (Fig. 8A).
To dissect a possible negative role of NK cells on the expansion of Ag-specific T cells, which might be either due to the adaptation of CD56\textsuperscript{+} NK cell to lyse MHC class I–positive T cells and mature DC (19, 20) or due to a NKGD2 ligand-dependent killing of recently activated CD8\textsuperscript{+} T (21, 22), the same coculture experiment was performed with PBL depleted of NK cells. Because in some donors the MelanA/Mart-1 tetramer-positive CD8\textsuperscript{+} T lymphocytes were CD56\textsuperscript{+} (data not shown), the following strategy was used: CD8\textsuperscript{α+} cells were first purified from the total PBL and the remaining negative fraction either was left untreated or underwent depletion of CD56\textsuperscript{+} cells before being added to the DC–CD8\textsuperscript{+} T cell coculture. The absence of CD56\textsuperscript{+} NK cells was not enough to restore the expansion of MelanA/Mart-1–specific T lymphocytes to the levels of the purified setting, but there was a trend, even if not reaching statistical significance toward an increased expansion (Fig. 8B).

The CD8\textsuperscript{+} T cell cultures containing expanded amounts of MelanA/Mart-1–specific T cells were restimulated with peptide-pulsed, irradiated HLA-A2\textsuperscript{+} PBL for 10 d and tested in a degranulation assay. Ag-specific degranulation was detected not only in response to peptide-pulsed T2 cells (Fig. 8C, top left), but also to endogenous Ag presentation from the Mel1379 cell line (Fig. 8C, top right). The low levels of degranulation in response to T2 cells pulsed with an unrelated peptide as well as to the MelanA/Mart-1–negative melanoma cell line Na\textsuperscript{8} (Fig. 8C, bottom) respectively confirm and support the Ag specificity of the induced degranulation.

In the next step, pools of viral peptides were used to pulse mature DC to stimulate memory CD8\textsuperscript{+} T cells within total PBL. \[^{31}Cr\] release (data not shown) and CD107a degranulation assays revealed a high level of variability in the Ag-specific immune response among different donors, not only in its intensity, but particularly in the behavior of the different DC, as representatively shown for two donors with opposite situations (Fig. 9). Higher Ag-specific CD8\textsuperscript{+} T cell responses were obtained in donors with a low reactivity of innate effector cells, as determined by the killing of Mel1379 as well as of Daudi cells. In such donors, alternative DC induced the highest levels of Ag-specific CD8\textsuperscript{+} T cell–mediated responses (Fig. 9, top panels). In contrast, donors with a higher responsiveness of innate effectors to Daudi cells exerted lower CD8\textsuperscript{+} T cell–mediated responses with alt-2 DC inducing the lowest levels of Ag-specific responses, whereas the gold standard induced the highest (Fig. 9, bottom panels).

**Discussion**

The limited clinical objective responses obtained in tumor immunotherapy in general and also in the DC-based vaccination indicate the urgent need to optimize the currently used protocols and to increase the knowledge of the induced immune responses to select for each patient the most effective vaccine schedule.

In this study, different alternative maturation mixtures were compared with the clinical gold standard for their effect on the ability of monocytes differentiated with a fast protocol toward DC to interact with effector cells of the innate and adaptive immune system. The experiments employed fast DC because in preliminary experiments comparison with standard day 5 monocyte-derived DC highlighted not only an increased cell yield, but also a more potent induction of allogeneic MLR and higher secretion of IL-12p70 (data not shown).

Concerning the phenotype of the fast DC, as determined by the expression of costimulatory molecules and by the IL-12p70/IL-10 ratio, and their ability to activate the immune system, the alt-3 DC (TNF-\(\alpha\) + IL-1\(\beta\) + IFN-\(\gamma\) + CL097) were almost comparable to the gold standard DC (TNF-\(\alpha\) + IL-1\(\beta\) + IL-6 + PGE\(_2\)). In contrast, alt-2 DC (MPLA + IFN-\(\gamma\)) exerted a similar and, particularly
in respect to γ6 T cells, even better activity than alt-1 DC, the α-type 1–polarizing DC initially proposed by Mailliard et al. (10) (TNF-α + IL-1β + IFN-α + IFN-γ + polyIC). A possible explanation is that, despite secreting similar levels of IL-12 with a donor-dependent prevalence of one DC type over the other, alt-1 DC secrete throughout all donors significantly higher amounts of IL-10 that might dampen the type 1–oriented, IL-12–induced immune response.

Regarding the functional activation of the immune system, two different processes could be identified. The first one, induced to a different extent by all alternative DC, consists in the acquisition of the cytotoxic activity and secretion of proinflammatory and type 1 cytokines via a mainly IL-12–independent signaling. Indeed, only γ6 T cell cytotoxicity revealed an IL-12 dependency and only in response to difficult targets like melanoma, but not Daudi cells. In the second process, IFN-γ secretion is induced by a highly IL-12–dependent mechanism that is performed only by alt-1 and alt-2 DC.

Despite the highly variable frequencies in the different donors, it is noteworthy that both NK and γ6 T cells were activated by the alternative DC, thus leading to the activation of multiple effector mechanisms that can be useful in the coordinated action against the tumor.

The important role of NK cells for the treatment of cancer has been initially highlighted by their involvement in the graft-versus-leukemia effects obtained in acute myeloid leukemia patients undergoing haplодendent stem cell transplantation (23). More recently, solid tumors like melanoma and renal cell carcinoma also have been evaluated in vitro for their susceptibility to matched and mismatched NK cells, confirming an increased cytotoxicity upon usage of NK cells with at least one killer cell Ig-like receptor (KIR) mismatch (24). Because in our setting a KIR typing of the PBL was not performed, the intensity of the obtained tumor killing might be biased by a mismatched situation that would not exist in vaccinated patients, in which DC-activated autologous NK would interact with the autologous tumor. In our study, alt-1 and alt-2 DC induced a potent activation of CD56dim and CD56br NK subsets regarding their cytokine production and cytotoxic activity, a finding that is in line with other reports describing IFN-γ secretion of the CD56dim subset (25, 26), as well as cytotoxic activity of the CD56dim population (20). In contrast to Jiang et al. (20) demonstrating a granzyme K–mediated killing in the absence of granzyme B in the CD56dim population, a significant induction of granzyme B upon alt-1 and alt-2 DC stimulation was detected. Due to the limited amount of CD56dim cells in the peripheral blood, this subset could not be purified to validate target killing by standard [51Cr] release assay and to assess the involved effector molecule(s). In line with the increasing reports of memory-like NK cells (27), the enhanced activity of NK was preserved 5–10 d after the stimulation with DC. Whether this correlates with the induction of memory-like NK cells, or represents a result of an expanded memory population in some of our healthy donors is currently under investigation.

In contrast to the more intraepithelial V61+ cells, the V82+ cells are normally regarded as the blood-born subset of γ6 T cells. Despite that, they have been found within the infiltrating lymphocytes of different tumor histotype (28–30), and their ability to infiltrate human skin and perform endothelial transmigration has been documented (31, 32). For these reasons, their role in the antitumor therapy is currently being investigated in phase I/II clinical trials of adoptive transfer therapy (33, 34). In our setting, alt-2 DC were more efficiently activating the V82+ γ6 T cells, particularly regarding proliferation and degranulation to the melanoma cell line. Although the presentation of the lipid A moiety of LPS in association with CD1b or CD1c to γ6 T cells has been proposed (35), a direct stimulation of γ6 T cells by MPLA could be excluded because classical monocyte-derived DC or Langerhans-like DC stimulated with the alt-2 mixture was not able to induce IFN-γ secretion in γ6 T cells despite their higher expression of CD1b and/or CD1c in comparison with fast DC (data not shown). The results from the long-term blockade of IL-12 highlighted a statistically significant inhibition of the degranulation in response to Mel1379, but only upon stimulation with alt-2 DC. Whereas the response to alt-1 DC by γ6 T cells was in general low, and thus the effect of inhibition less evident, the reduction of alt-2 DC-induced killing to levels similar to the basal alt-1 DC suggested a synergy between IL-12 and another to date undefined factor/molecule for the enhanced stimulation of γ6 T cells by alt-2 DC. Among the molecules already involved in the interaction of γ6 T cells with phosphoantigen-pulsed tumor cells (36–38), CD86 is the only marker significantly stronger expressed in alt-2 DC compared with the other DC populations investigated, and might thus be a possible candidate for the synergic partner. Whereas the constitutive and short-time incubation with DC–induced CD56 expression was highly donor variable and not correlating with function, a consistent CD56 upregulation was found in all donors upon long-term incubation with alternative DC and correlated with enhanced proliferation/degranulation, as previously reported for in vitro expanded γ6 T cells from neoplastic and infectious setting (39, 40). Thus, CD56 might represent a marker for immunomonitoring γ6 T cell vaccine-induced immune responses.

Evaluation of the consequences of the potent innate activation on the induction of Ag-specific immune response highlights a complex immune context and underscores the importance of evaluating the

**FIGURE 9.** Different levels of innate activity affect the intensity of the memory response of CD8+ T cells. HLA-A2* or HLA-A3* mature fast DC were pulsed with corresponding viral peptide pools and incubated with autologous PBL for 8–10 d in the absence of additional cytokines. The functional activity of effector cells within the PBL was evaluated by means of a CD107a degranulation assay. Target cells were the HLA-A2* and A3* Met1379 melanoma cells pulsed with the viral peptides of left unpulsed (left) and Daudi cells (right). The results are represented as the percentage of CD107a+ cells upon removal of background staining (i.e., unpulsed for CD8+ T cells and absence of target for innate effector cells) from two donors representative of the extreme situations (top versus bottom panels).
potency of new vaccine formulations within total blood lymphocytes and not with purified cell populations. Whereas alt-2 DC significantly improved the expansion of MelanA/Mart-1–specific T cells within purified CD8+ lymphocytes over gold standard DC, this advantage was almost completely lost within total PBL. Usage of viral Ags to evaluate the functionality of the expanded CD8+ T cells within total PBL highlighted a broad variability among different donors regarding the potency of the memory responses, ranging from <1% to >30% of responding cells, and the relative efficiency of the various DC formulations. Donors with a reduced activation of innate cells exerted higher CD8+ T cell–mediated immune responses, which were stronger upon alternative DC stimulation. In contrast, in donors with higher innate activity, a much lower frequency of degranulating CD8+ T cells existed, with the best Ag-specific responses detected with gold standard or alt-3 DC. However, the killing induced by alt-2 DC either via CD8+ T cells or innate cells was consistently higher when compared with the other DC preparations, as highlighted by the [51Cr] release assay.

The mechanisms responsible for this dichotomy between an Ag-specific and an innate mediated tumor killing might be due to the following: 1) Competition of effector cells for target during the assay. Despite the 1:1 ratio used in the degradation assay, it is possible that in donors with higher levels of HLA and/or KIR mismatching with the used target cells, the enhanced innate killing possible that in donors with higher levels of HLA and/or KIR specificity and an innate mediated tumor killing might be due to the assay.

Disclosures
The authors have no financial conflicts of interest.

References


