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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. 

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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 secretion of the bioactive IL-12p70 that is critical for the induction of a type 1–oriented, cell-mediated immune response (5, 6). Therefore, alternative protocols for a faster differentiation of monocytes (7, 8) and a better maturation toward vaccine DC (9–12) have been developed during the last decade. Despite the fact that the major aim of vaccination is the induction of a CD8+ T cell–mediated, Ag-specific immune response, innate effector cells have recently rekindled interest, NK, NK-T, and γδ T cells have been frequently defined as a first line of response, exhibiting cytotoxic capabilities that do neither require previous stimulation nor develop into memory cells. During the last years, this minimalistic view was altered due to the increased knowledge about the cross-talk between cells from the innate and adaptive immunity, indicating that innate effectors can also provide a helper role influencing the outcome of the adaptive immunity (13). For example, NK cells have been functionally and phenotypically dissected into two different subsets, the cytotoxic NK cells characterized by a dim expression of the CD56 marker and positivity for CD16 (CD56dimCD16+) and a CD16-negative CD56bright (CD56brCD16−) cytokine producer subset (14). Moreover, murine models of viral infection as well as immunomonitoring of infected humans suggest that NK cells might develop memory-like properties and can thus provide stronger and quicker responses at a second encounter (15–17).

Based on these data, the potency of newly proposed protocols for vaccine DC should be evaluated regarding the adaptive and innate immunity. Therefore, a comparative analysis of the effects of different maturation mixtures on the interactions of fast DC with the different immune cell populations of the human blood was performed.

Materials and Methods

Maturation stimuli, Abs, and ELISA kits

Monophosphoryl lipid A (MLPA) and the imidazoquinoline compound CL097 were purchased from InvivoGen (San Diego, CA), IL-1β from BioSource International (Camarillo, CA), IFN-α2b from ProSpec (Reho-
DC OPTIMIZATION FOR INNATE AND ADAPTIVE ACTIVATION

volt., Israel), IFN-γ from Immunotools (Friesoythe, Germany), polyosinic: polycytidylic acid (polyIC) 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, eFluoro450 anti-CD8a, PE-Cy7 anti-CD14, FITC anti-CD80, FITC anti-CD83, PE anti-CD86 (from eBioscience/NatuTec, Frankfurt, Germany), PE anti-CD40, FITC anti-CD4, PE anti-CD60, PE-Cy7 anti-CD56, allophycocyanin, Alexa Fluor750 anti-CD16 (from Beckman Coulter, Brea, CA), FITC anti-granzyme B, FITC and PerCP anti-V62, allopolyocyanin anti-CD107a (from BioLegend, San Diego, CA), PE anti-CD69, and allopolyocyanin 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) software. The general gating strategy for the flow cytometric acquisition is shown in Fig. 1.

Concentration of IL-12 p70 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 were 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/Th9/Th17/Th22 kit from eBioscience/NatuTec following manufacturer’s instructions.

DC differentiation and maturation

Buffy coats 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 10⁶ 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 (ImmunoBiosciences) for 24–36 h to obtain 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 + polyIC) (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; polyIC, 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 (cytolysis assays).

DC lymphocyte coculture

All cocultures were performed in serum-free X-vivo15 medium (Lonza, Basel, Switzerland), using as effector cells the autologous PBMCs 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 × 10⁴ DC/well were seeded in U-bottom 96-well plates with 10⁵ PBL in 200 μl, whereas for long-time culture (5–10 d), 10⁵ DC were incubated with 10⁶ PBL in 1 ml in 24-well plate.

For mechanisms evaluation, DC were preincubated with blocking Abs directed against IL-12(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 (AAGGILTV; 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 (Pulexim immunotherapy, 24 h, or 10 ng/ml IL-7) (Immunoconcepts) were added on days +3, +5, +7. On day 10, cultures were stained with PE MelanA/Mart-1–specific tetramers provided by Beckman Coulter following manufacturer’s instructions. Cultures containing expanded MelanA/Mart-1–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 CEF-peptide pool HLA-A2 (PANtech, Tuebingen, Germany) or a 1:1 mixture of the HLA-A3–restricted epitopes from the influenza A virus nucleoprotein p265–273 (ILRGSVAKH) and the EBV nuclear Ag-3 p471–479 (RLRRAEQQVK; 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 eFluoro700 (eBioscience/NatuTec) for 10 min at 37°C in PBS supplemented with 10% FCS (Invitrogen, Karlsruhe, Germany) and then thoroughly washed. A total of 10⁶ PBL was added at a 1:10 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 Fluospheres (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 cytoxicity

Development of cytoxic 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/Mart-1 setting peptide-pulsed T2 cells and the undifferentiated HLA-A2* melanoma cell line NaB that lack the expression of MelanA/Mart-1 were employed. The HLA-A2-restricted tyrosinase epitope p35 (YMNQM/SQNV) was used as an unrelated Ag to pulse T2 cells.

For the [3H]Cr 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, 10⁶ 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, CD8α, 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 0.01% 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 Table 1). fast DC maturation 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.

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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-

**FIGURE 1.** Flow cytometry gating strategy for effector cells. First, PBL were gated on the basis of the physical parameters, forward and side light scatter (A), before selecting single (B) and live cells (C). The different subpopulations were determined upon counterstaining with different combinations of the mAb specific for CD3, CD8α, CD16, CD56, and V62. After identification of the CD3+ V62+ γδ T cells (D), the remaining population was used for the identification of the conventional CD3+ αβ T lymphocytes and of CD3− CD56+ NK cells (E) that were further subdivided into the CD56dimCD16− and CD16+CD56dim subsets (F). For evaluation of Ag-specific immune responses, CD8α+ T lymphocytes were also identified as CD3+ CD8α+ cells (G). An example of staining with the MelanA/Mart-1–specific tetramer is shown (H).

**FIGURE 2.** Functional characterization of mature fast DC. Monocytes were differentiated toward DC with the fast protocol and stimulated for 18 h with the various maturation mixtures. (A) Mature DC were evaluated for expression of costimulatory and adhesion molecules by flow cytometry. The mean fluorescence intensity of mature DC is normalized to immature DC. Reported are the mean ± SE of 10 donors. (B and C) Supernatants obtained at the end of maturation (B) or 48 h after reseeding mature DC in the absence of stimuli (C) were analyzed for IL-12p70 and IL-10 content by ELISA. Represented are the mean ± SE of at least 7 different donors. (D) Mature DC were reseeded with autologous PBL immediately after maturation (0–24 h) or after 24 h, in which they were left untreated (24–48 h). Represented are the mean ± SE of 3 different donors. *p < 0.05, **p < 0.01, n.s. > 0.05 in the Tukey HSD test.
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 poten 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+) 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 discern NK cell subsets 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-

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This was accompanied by an enhanced degranulation and also effective killing of the target cells as measured in a classical [51Cr] 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

FIGURE 4. Alternative DC enhance cytotoxic potential of innate lymphocytes. (A and B) PBL were incubated for 16–21 h with mature DC in X-vivo 15, thoroughly washed, and then tested in a CD107a degranulation assay upon 4-h incubation at a 1:1 ratio with Daudi cells. The different effector populations were identified upon counterstaining with mAb directed against CD3, CD56, CD16, and V62, as described in legend to Fig. 1. (A) The mean ± SE of at least three different experiments of target-induced degranulation (i.e., upon subtraction of the background CD107a exposition obtained with PBL incubated alone) are shown. *p < 0.05, **p < 0.01 in the Tukey HSD test. (B) Representative plot from alt-2 DC-stimulated PBL is shown for CD56br and CD16+CD56dim NK as well as γδ T cells. Gates were set based on the background CD107a staining of PBL incubated alone. (C) PBL stimulated 16 h with mature DC were fixed, permeabilized, and stained for granzyme B. Representative histogram overlays of one of three donors are shown for CD56br and CD16+CD56dim NK as well as γδ T cells upon stimulation with gold standard and alt-2 DC.

FIGURE 5. Sustained expansion of innate cells by alternative DC. PBL were labeled with cell proliferation dyes (CFSE or eFluor670) and incubated with autologous DC (10:1 ratio) in X-vivo 15 in the absence of exogenous Ags or cytokines. After 5 d, proliferation was evaluated by flow cytometry as dye fluorescence dilution in the differently gated lymphocyte populations (A–C), whereas absolute cell counts were calculated using counting beads and reported upon normalization to PBL in the absence of any DC stimulation (D). The results are represented as the mean ± SE of 10 (A) and four different experiments (D), respectively. Representative dot plots of gold standard and alt-2 DC-stimulated NK (B) and γδ T cells (C) are also shown. *p < 0.05, **p < 0.01 in the Tukey HSD test.
Upon 16-h incubation with alt-2 DC in the presence of the anti–IL-12p70 Ab, NK and gd 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 was found (Fig. 7B). 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 gd 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).
The Journal of Immunology

were pulsed with MelanA/Mart-1 peptide and incubated with purified CD8+ T cells of one of three experiments are shown. 

Table 8. Alternative DC ability to expand naive CD8+ T cells is hindered by the presence of other immune populations. (A) Mature fast DC were pulsed with MelanA/Mart-1 peptide and incubated with purified CD8+ T cells or total PBL for 10 d in the presence of IL-2 and IL-7. Expansion of MelanA/Mart-1-specific CTL was evaluated by tetramer staining via flow cytometry. Represented are the mean ± SE of six different experiments. *p < 0.05 in the Tukey HSD test (within purified CD8+ T cells) or Student t test (for CD8+ T cells versus total PBL). (B) Stimulation of purified CD8+ T cells by MelanA/Mart-1 peptide-pulsed Alt-1 and Alt-2 DC was evaluated in the absence (CD8) or presence of other immune cell populations, namely the whole negative fraction of the PBL remaining after CD8+ T cell purification [CD8 + PBL(−)], or the fraction left after further depletion of CD56+ cells [CD8 + PBL(−, g, b)]. Reported are the mean ± SE of four different experiments. *p < 0.05 in the Tukey HSD test. (C) CD8+ T lymphocytes stimulated for 10 d with MelanA/Mart-1–pulsed DC were restimulated with irradiated MelanA/Mart-1–pulsed PBL before being evaluated in a CD107a assay against T2 cells pulsed with MelanA/Mart-1 or irrelevant peptide (left) as well as against the HLA-A2+ melanoma cell lines Mel1379 (top, right) and Na8 (bottom, right) that are MelanA/Mart-1–positive and –negative, respectively. Representative dot plots from Alt-2 DC-expanded CD8+ T cells of one of three experiments are shown.

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-α + IL-1β + IFN-γ + CL097) were almost comparable to the gold standard DC (TNF-α + IL-1β + IL-6 + PGE2). In contrast, alt-2 DC (MPLA + IFN-γ) exerted a similar and, particularly
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 CD56<sup>bn</sup> and CD56<sup>dim</sup> NK subsets regarding their cytokine production and cytotoxic activity, a finding that is in line with other reports describing IFN-γ secretion of the CD56<sup>dim</sup> subset (25, 26), as well as cytotoxic activity of the CD56<sup>bn</sup> population (20). In contrast to Jiang et al. (20) demonstrating a granzyme K-mediated killing in the absence of granzyme B in the CD56<sup>bn</sup> population, a significant induction of granzyme B upon alt-1 and alt-2 DC stimulation was detected. Due to the limited amount of CD56<sup>bn</sup> 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<sup+b</sup> cells, the V82<sup+b</sup> cells are normally regarded as the blood-born subset of γδ 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<sup+b</sup> γδ 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 γδ T cells has been proposed (35), a direct stimulation of γδ 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 γδ 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 γδ 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 γδ T cells by alt-2 DC. Among the molecules already involved in the interaction of γδ 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 γδ T cells from neoplastic and infectious setting (39, 40). Thus, CD56 might represent a marker for immunomonitoring γδ 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
potency of new vaccine formulations within total blood lymphocytes and not with purified cell populations. Whereas all-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 effectors exhibited 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 all-3 DC. However, the killing induced by all-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 degranulation 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 might lead to the lack of targets for the adaptive response (2). The different outcome might be also mediated by different processes during the DC priming. The only slight recovery of the expansion upon NK depletion from the cultures confirms the existence of a negative role of NK cells on CD8+ T cell priming, although this appears to be of marginal importance. With regard to the γδ T cells as another candidate, there exist discrepant results regarding the induction of Ag-specific CD8+ T cell responses. Whereas Taka-hara et al. (41) demonstrated a positive effect, Traxlmyar et al. (42) described an expansion of suppressive γδ T cells reducing the expansion of CD4+ and CD8+ T cells upon stimulation with DC matured with LPS and IFN-γ. The possible development of a γδ T cell–based negative feedback loop upon PBL stimulation with all-3 DC is currently under investigation. However, it has to be underlined that MPLA shares much of the stimulatory activity of type 1 cytokines, proliferation, and release of cytotoxic granules upon interaction with melanoma cell lines. Additionally, the absence of IL-12 might not induce the toxic cytokine storm (43), suggesting that the all-2 DC could lack this negative effect.

Overall, monocytes differentiated with the fast protocol and matured with a combination of MPLA and IFN-γ provide the strongest activation of the immune system, evaluated as secretion of type I cytokines, proliferation, and release of cytotoxic granules upon interaction with melanoma cell lines. Due to the dichotomy between the induction of a CD8+ T cell–mediated or innate-mediated killing of tumor cells, the identification of markers able to predict the response of the innate and adaptive immune system of patients is required to focus the vaccine DC formulation toward the best responses. This might be mediated, for example, by choosing between the addition of antigenic peptides versus ligands for a sustained activation of innate effector cells like γδ T or also invariant NKT–T cells.

Disclosures

The authors have no financial conflicts of interest.

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as rapid producers of abundant IFN-gamma on activation. 


Online Material

Figure S1. Alternative DC induce activation markers on innate lymphocytes

PBL were incubated for 16 h with mature DC in X-vivo15 and thoroughly washed before the expression levels of the activation markers CD25 and CD69 were evaluated by flow cytometry. The different effector populations were identified upon counter staining with mAb directed against CD3, CD56, CD16 and V62 as described in legend to Figure 1. The mean ± SE of four different donors are shown for CD25 (A) and CD69 (B). C) Representative dot plots of “gold-standard” and “alt-2” DC stimulated CD56br (top) and CD16+56dim NK (center) as well as γδ T cell (bottom) from one representative donor are shown.
Figure S1

![Graphs and histograms showing data comparison]

Legend:
- gold st DC
- alt-1 DC
- alt-2 DC
- alt-3 DC

A and B: Comparison of % CD38+ cells in NK and γδ T cells across different conditions.
C: Flow cytometry plots comparing CD25 and CD69 expression between gold st DC and alt-2 DC.