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Generation of Th1-Polarizing Dendritic Cells Using the TLR7/8 Agonist CL075

Stefani Spranger,*1 Miran Javorovic,*†,1 Maja Bürdek,* Susanne Wilde,* Barbara Mosetter,* Stefanie Tippmer,*† Iris Bigalke,*† Christiane Geiger,* Dolores J. Schendel,*†,1 and Bernhard Frankenberger*1

In this paper, we describe a new method for preparation of human dendritic cells (DCs) that secrete bioactive IL-12(p70) using synthetic immunostimulatory compounds as TLR7/8 agonists. Monocyte-derived DCs were generated using a procedure that provided mature cells within 3 d. Several maturation mixtures that contained various cytokines, IFN-γ, different TLR agonists, and PGE2 were compared for impact on cell recovery, phenotype, cytokine secretion, migration, and lymphocyte activation. Mixtures that included the TLR7/8 agonists R848 or CL075, combined with the TLR3 agonist polyinosinic:polycytidylic acid, yielded 3-d mature DCs that secreted high levels of IL-12(p70), showed strong chemotaxis to CCR7 ligands, and had a positive costimulatory potential. They also had excellent capacity to activate NK cells, effectively polarized CD4+ and CD8+ T cells to secrete bioactive IL-12(p70), which is a critical cytokine for the induction of Th1 cells and activation of NK cells.

In elegant studies in mice demonstrated that activation of TLR signaling cascades could produce mDCs with Th1-polarizing capacity (14). Human DC subsets express all known human TLRs, with the most diverse expression pattern in monocyte-derived DCs and myeloid DCs (15). On the basis of these observations, a number of studies explored the use of different TLR agonists to modulate DC function, particularly with respect to cytokine secretion profiles (14, 16–25).

We previously described DCs that were matured in a 7-d protocol using a mixture that included the imidazoquinoline-like molecule R848 as a TLR7/8 agonist (26). The resultant 7-d mDCs produced substantial amounts of IL-12(p70) and displayed phenotypic and functional characteristics desired for clinical application, including high cell recoveries. However, the 7-d culture period in a good manufacturing practice facility is a strong hindrance for clinical development. Therefore, we investigated approaches to rapidly produce monocyte-derived mDCs that would display suitable characteristics for efficient use in vaccine development. The studies reported in this paper show that maturation mixtures containing the synthetic thiazoloquinoline immunostimulatory compound CL075, or R848, as a TLR7/8 agonist, when combined with polyinosinic:polycytidylic acid [poly(I:C)] as a TLR3 ligand, yielded mDCs within 3 d that showed excellent profiles with respect to recovery, phenotype, cytokine secretion, and migration, as well as capacity to activate NK cells and CD8+ CTLs and to cultured in vitro with GM-CSF and IL-4 or IL-13 to produce immature DCs (iDCs) (11–13). Thereafter, several alternatives can be used to obtain mDCs, each of which yields cells with somewhat different properties that must be clearly specified for DC-based vaccine development. Jonuleit et al. (7) described a maturation mixture containing TNF-α, IL-1β, IL-6, and PGE2 (hereafter designated as the four-component mixture [4C]) that produced 7-d DCs with surface markers characteristic for mDCs, and these cells could be easily recovered in sufficient numbers for clinical application. This type of mDC has been used frequently in early-phase clinical trials of DC vaccination in patients with various forms of malignancies. However, these mDCs do not produce bioactive IL-12(p70), which is a critical cytokine for the induction of Th1 cells and activation of NK cells.

Abbreviations used in this paper: 4C, four-component mixture; DC, dendritic cell; iDC, immature DC; mDC, mature dendritic cell; MFI, mean fluorescence intensity; poly(I:C), polyinosinic:polycytidylic acid; pSTAT, phospho-STAT; RPMI-VLE, RPMI 1640 medium with very low endotoxin.
effectively polarize CD4+ and CD8+ T cells to secrete IFN-γ. Thereby, these 3-d mDCs are highly suited for development of DC-based antipathogen or antitumor vaccines.

**Materials and Methods**

**Leukapheresis and elutriation**

We used a closed system of elutriation (ELUTRA; Gambro BCT, Lake-wood, CO) to obtain monocytes as progenitor cells for generation of iDCs, as described previously (26). In accordance with the Declaration of Helsinki and after approval by the Institutional Review Board of the Technical University (Munich, Germany), healthy donors underwent 180 min of leukapheresis with the COBE Spectra cell separator (Gambro BCT, Hechingen, Germany) with a modified MNC program (V6.1); separation factor was set to 700 with a collection rate of 0.5 ml/min and a target hematocrit of only 1–2%. Resulting blood cells were analyzed by automatic blood counter (ACT Dif; Beckman Coulter, Krefeld, Germany) to set up conditions for the ELUTRA system. Leukapheresis products were processed according to the manufacturer’s instructions by a method of counterflow centrifugal elutriation using a fixed rotor speed (2400 rpm) and computer-controlled stepwise adjustment of media flow rate, followed by rotor-off harvesting. Five liters of running buffer consisting of RPMI 1640 medium with very low endotoxin (hereafter RPMI-VLE) (Biochrom, Berlin, Germany) with 1.5% human serum (pool of AB-positive adult males) (Institute of Transfusion Medicine, Suhl, Germany) was used for cell separation. This process resulted in five fractions with enriched monocytes present in the rotor-off fraction. The cellular composition of individual fractions was characterized by automatic cell counting and flow cytometry.

**Generation of mDCs from elutriated monocytes**

Cells from the rotor-off fraction, subsequently designated as fraction 5, were frozen in aliquots of 5 × 10^7 monocytes in freezing medium consisting of human serum albumin (20% human serum albumin solution; Octalbine, Octapharma, Langen, Germany), 20% DMSO (Merck, Darmstadt, Germany), and 10% glucose (Braun, Melsungen, Germany). Monocytes were thawed and washed with endotoxin-free PBS (Biochrom) at 1500 rpm for 10 min. The cells were resuspended and seeded at 0.5 × 10^7 per "nuncleon-3" flask (80 cm^2) (Nunc, Wiesbaden, Germany) in 15 ml DC medium containing RPMI-VLE and 1.5% human serum and cultivated for 50 min at 37°C and 5% CO2 in a humidified atmosphere. Afterward, cells were washed twice with RPMI-VLE, and 15 ml DC medium was added. On day 0, cultures were supplemented with 100 ng/ml GM-CSF (Leukine; Berlex, Richmond, VA) and 20 ng/ml recombinant human IL-4 (R&D Systems, Wiesbaden, Germany) in 3 ml fresh DC medium per flask. Full DC maturation was achieved by addition of various mixtures on day 2 (see instructions by a method of counterflow centrifugal elutriation using a fixed rotor speed (2400 rpm) and computer-controlled stepwise adjustment of media flow rate, followed by rotor-off harvesting. Five liters of running buffer consisting of RPMI 1640 medium containing RPMI-VLE and 1.5% human serum and cultivated for 50 min at 37°C and 5% CO2 in a humidified atmosphere. Afterward, cells were washed twice with RPMI-VLE, and 15 ml DC medium was added. On day 0, cultures were supplemented with 100 ng/ml GM-CSF (Leukine; Berlex, Richmond, VA) and 20 ng/ml recombinant human IL-4 (R&D Systems, Wiesbaden, Germany) in 3 ml fresh DC medium per flask. Full DC maturation was achieved by addition of various mixtures on day 2 (see Table 1). The components for the maturation mixtures were as follows: TNF-α, IL-1β, and IL-6 (R&D Systems); poly(I:C), R848, and CL075 (InvivoGen, San Diego, CA); IFN-γ (Boehringer Ingelheim, Ingelheim, Germany); and PGE2 (Sigma-Aldrich, Deisenhofen, Germany). The induction of mDCs with a tolerogenic phenotype, rIL-10 (R&D Systems), was added with a final concentration of 83.33 ng/ml. After incubation of DCs with maturation mixtures for 24 h, cells were harvested by washing twice with PBS plus 0.5% human serum with light shaking and assessed directly or cryopreserved as described above for fraction 5 monocytes.

**Surface phenotyping of DCs**

DCs were labeled with the following fluorescence-conjugated mAbs: CD14 (FITC, clone M499), CD40 (FITC, clone 2331 FUN-1), CD80 (PE, clone L307.4), CD274 (B7-H1, FITC, clone MIH1) (all BD Biosciences, Heidelberg, Germany), HLA-DR (PE, clone B8.12.2), and CD83 (PE, clone HB15a) (all BD Biosciences, Heidelberg, Germany); and PGE2 (Sigma-Aldrich, Deisenhofen, Germany). For the detection of surface molecules, mDCs were harvested and pulsed with 1 μg/ml peptide for 120 min at 37°C followed by rotor-off harvesting. Five liters of running buffer consisting of RPMI 1640 medium, supplemented with 200 mM t-glutamine, 100 mM sodium pyruvate, 10% U/ml penicillin and streptomycin (all Invitrogen), and 10% pooled human serum. NK cells were stimulated with 1 × 10^7 autologous mDCs. After 24 h, supernatant was collected and analyzed using an IFN-γ ELISA. Cytokine-stimulated mDCs were harvested afterward with CD3 (FITC,clone UCHT1; BD Biosciences), CD56 (allophycocyanin, clone n901; Immunotech), and CD69 (PE, clone TPI.55.3; Immunotech) Abs to depict the activated NK cell populations. After washing, cells were analyzed by flow cytometry using an LSR II instrument (BD Biosciences). Postacquisition data analysis was done with FlowJo 8 software (Tree Star). Aliquots of activated NK cells were also cryopreserved and later evaluated for cytotoxic function.

**Activation of allogeneic T lymphocytes**

Cryopreserved PBMCs isolated from HLA-A2+ donors were cocultured with allogeneic mDCs prepared from HLA-A2+ donors using 1 × 10^6 PBMCs and 1 × 10^5 mDCs in T cell medium (RPMI 1640 medium, 12.5 mM HEPES, 4 mM t-glutamine, and 100 U/ml penicillin and streptomycin, supplemented with 10% pooled human serum). Following 7 d of coculture, recovered lymphocytes were analyzed by flow cytometry for intracellular cytokine staining.

**Activation of Ag-specific T lymphocytes**

mDCs were harvested and pulsed with 1 μg/ml peptide for 120 min at 37°C and 5% CO2 in a humidified atmosphere. For Ag-pulsing of DCs, a MART1/Melan-A nonamer (ELAGIGILTV; Metabion, Martinsried, Germany) as well as a peptide pool (CEF) comprising peptides of human CMV, EBV, and human cytomegalovirus (CMV) (PANATECs, Tuebingen, Germany) were used. Cryopreserved PBMCs isolated from HLA-A2+ donors were cocultured with autologous, peptide-pulsed mDCs using 1 × 10^6 PBMCs and 1 × 10^5 mDCs in T cell medium (RPMI 1640 medium, 12.5 mM HEPES, 4 mM t-glutamine, and 100 U/ml penicillin and streptomycin, supplemented with 10% pooled human serum). Following 7 d of coculture, recovered lymphocytes were tested directly for cytokotoxic activity. Recovered cells were also restimulated using the same cryopreserved batch of peptide-pulsed DCs for 24 h, at which time supernatants were collected for determination of IFN-γ content using a standard ELISA. Background activity in cytotoxicity and secretion assays was determined using non–peptide-pulsed mDCs.

**Multimer staining**

PBLS obtained after 7 d of coculture with 3-d peptide-pulsed mDCs were harvested, washed, and stained with MART1/Melan-A25-34 (ELAGIGILTV)–specific multimer. Control stainings were performed using a CMV pp65-peptide 45-53 multimer (both provided by Prof. Dr. D. Busch, Technical University of Munich). Afterward, staining of surface molecules was performed as described above using the following Abs: CD3 (PerCP, clone SK7; BD Biosciences); CD4 (FITC, clone 13B2; Immunotech); and CD8 (allophycocyanin, clone SK1; BD Biosciences). Postacquisition data analysis was done using FlowJo 8 software (Tree Star).

**Intracellular cytokine staining**

PBLS activated for 7 d with mDCs were harvested, washed, and stimulated for 1 h at 37°C with 1 ng/ml PMA and 250 ng/ml ionomycin (Sigma-Aldrich). Afterward, brefeldin A (10 μg/ml) and monensin (50 μM) were added, and the cells were incubated for an additional 4 h. Staining of

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surface molecules were performed as described above using the following Abs: CD3 (PerCP, clone SK7; BD Biosciences); CD4 (PE, clone 13B8.2; Immunotech); CD8 (allophycocyanin, clone SK1; BD Biosciences); CD16 (APC, clone 3G4; CallTag Laboratories, Buckingham, U.K.); and CD56 (PE, clone N901; Immunotech). Afterward, cells were fixed with 1% paraformaldehyde, washed twice, and permeabilized with 0.1% saponin solution (Sigma-Aldrich). Cells were incubated with IFN-γ-specific (FITC, clone 25723.11; BD Biosciences) and IL-4-specific (PE, clone 3010.211; BD Biosciences) Abs in 0.35% saponin solution in PBS for 20 min at 4°C. After washing, cells were analyzed by flow cytometry as described above. Postacquisition data analysis was performed using FlowJo 8 software (Tree Star).

Cytokine secretion measurement by ELISA and multiplex technology
Secretion of IL-12(p70) and IL-10 by mDCs in signal 3 assays as well as secretion of IL-5, IL-13, and IFN-γ by activated PBLs were detected by standard ELISA, using pretested Ab duo sets for detection of IL-5, IL-10, IL-12(p70), or IFN-γ (BD Systems, Heidelberg, Germany) and IL-13 (R&D DuoSet, Wiesbaden, Germany), according to the manufacturer’s instructions. Colorimetric substrate reaction with tetramethylbenzidine and H2O2 was measured, after stopping the reaction with H3PO4, at 450 nm and wavelength correction at 620 nm and analyzed with “easy fit” software (SLT, Craliinheim, Germany). Multiple cytokine and growth factor secreted by cell populations of mDCs were quantified by multiplex protein arrays (Human Gp 1 cytokine 17-Plex Panel; Bio-Rad, Hercules, CA), according to the manufacturer’s instructions. In brief, microspheres coated with cytokine-specific capture mAbs were incubated for 30 min at room temperature with 50 µl supernatant medium. After three washing steps, biotinylated detection mAbs were added and incubated for 30 min at room temperature, followed by 10 min of incubation with streptavidin-PE. A two-laser array reader (BioPlex System, Hercules, CA) simultaneously quantified 17 different cytokines, chemokines, and growth factors. Standard curves and concentrations were calculated with BioPlex Manager 4.1.1 on basis of the five-parameter logistic plot regression formula.

Cytotoxicity assay
Cytotoxicity activity of NK cells and T cells that were precultivated in DC cocultures was analyzed in a standard 4-h chromium release assay. K562 cells were used as target cells in the NK assays combining 1.5 × 104 target cells and NK cells at E:T cell ratios of 10:1, 5:1, 2.5:1, and 1.25:1. Melanoma cell lines Mel624.38 (HLA-A2+, MART-1/Melan-A+) and MelA375 (HLA-A2+, MART-1/Melan-A+) (28) were used as target cells for activated T cells at E:T of 20:1, 10:1, 5:1, or 2.5:1. Specific lysis was determined as described previously (29). The percentage of specific lysis was calculated as 100 × (experimental release – spontaneous release)/maximum release – spontaneous release). Spontaneous release was assessed by incubating target cells in the absence of effector cells and was generally <15%.

Western blotting
mDCs were stimulated with the maturation mixture for an additional 2 h, harvested and washed with PBS, and lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl [pH 7.4], 150 mM CHAPS, 1 mM PMSF, 1 mM Na3VO4, 1 mM NaF, and 1× Complete). Protein concentration was detected using the BCA Protein Assay Kit (Pierce Chemical Co., Rockford, IL), according to the manufacturer’s instructions. Western blotting was performed following the instructions of the NuPAGE System (Invitrogen). For specific phospho-STAT (pSTAT) staining, polyclonal anti-pSTAT Abs were used (STAT1-pTyr701 and STAT6-pTyr641; Cell Signaling Technology, Danvers, MA), β-actin was stained using a polyclonal Ab from Sigma-Aldrich (A5400), and detection was performed using a polyclonal anti-IgG Ab (Cell Signaling Technologies).

Results
mDCs can be efficiently generated in 3 d
Monocytes were obtained by elutriation of leukapheresis products of healthy donors. These cells were used for the in vitro generation of iDCs using GM-CSF and IL-4 in a fast protocol lasting 2 d (8). DC maturation was induced on day 2, creating different populations of 3-d mDCs, using mixtures summarized in Table I. 4C represented the well-characterized 4C that did not include any TLR ligands (7). 5C+R848 was used previously to prepare 7-d mDCs (26). It included the synthetic ligand R848 (resiquimod), which is an imidazoquinoline-like molecule that interacts with TLR7/8 receptors. It also included the TLR3 agonist poly(I:C). Poly(I:C) is known also to bind to the MDA-5 receptor that belongs to the family of cytosolic helicase pattern recognition receptors (30). It is accepted that TLR3 detects extracellular viral dsRNA internalized into the endosomes, whereas RIG-I/MDA-5 detects intracellular viral dsRNA (31, 32). The synthetic thiazoloquinolinodervative type I immune response modifying compound CL075 can also induce signals via TLR7/8 in some cells, but its impact on DC maturation and function has not been reported to date (16, 17). Therefore, we also assessed this substance for its impact on DC maturation. However, a few experiments used mixture 5C alone, which did not include a TLR7/8 agonist but did contain poly(I:C) as a TLR3 agonist. Mixtures 5C, 5C+R848, and 5C+CL075 also differed from 4C in the inclusion of IFN-γ, the exclusion of IL-6, and a 4-fold reduction in the concentration of PGE2.

Mixtures induce mature surface marker phenotypes on iDCs
mDCs were prepared from three or four healthy donors, applying the various maturation mixtures for 24 h. After a total 3-d culture period, mDCs were recovered from the culture vessels, and percentages of cell recovery were compared. The mDC recoveries ranged from 7.9 to 9%, and there were no significant differences noted among the three groups (Supplemental Table I). The three mDC populations, as well as iDCs from two donors, were then analyzed by flow cytometry for expression of surface markers that are characteristic for iDCs and mDCs. In addition to determining the percentages of positive cells, the levels of surface marker expression were also compared (Table II). The mDCs displayed the expected shift to high percentages of cells, expressing substantial levels of CD80, CD83, and CCR7. There were no substantial differences noted among the different mixtures with respect to the various surface markers that were upregulated on the mDCs.

Production of IL-12(p70) is superior by DCs matured using TLR7/8 agonists and poly(I:C)
Our goal in creating TLR-containing maturation mixtures was to generate a population of optimized DCs for clinical studies that could secrete bioactive IL-12(p70) while producing no or only low levels of IL-10 to obtain cells that would polarize Th cells in a Th1 direction (14). Furthermore, for vaccine development, it is important to assure that mDCs retain the capacity to secrete IL-12(p70) upon stimulation via CD40, which were significantly different from those of DC4C. In contrast, the DC populations secreted only very low amounts of IL-10, with no significant differences noted among the three groups.

mDCs show chemotaxis to CCL19 signals
If mDCs are considered for use in vaccine development it is important to demonstrate that they have an adequate potential to migrate to lymph node sites. This is primarily governed by
expression of the chemokine receptor CCR7, which mediates a chemotactic response to the chemokines CCL19 and CCL21 that are expressed in the lymph nodes (33, 34). On the basis of the assessment of CCR7 phenotype, it was apparent that mDCs produced with mixtures 5C+R848 and 5C+CL075 were comparable to those matured with 4C, both with respect to percentages of positive cells and levels of expression. Although substantial numbers of cells displayed this critical chemokine receptor, this does not necessarily equate directly with migratory responses to CCR7 chemokine signals. Therefore, Transwell migration assays were used to assess the spontaneous migration of the mDC populations, as well as their migratory responses to CCL19 as a chemotactant. As illustrated in Fig. 2, low percentages of mDC populations showed spontaneous migration in the absence of chemotactant, but substantially higher percentages of mDCs showed Transwell chemotaxis to CCL19 chemokine, with an overall significant difference to spontaneous migration in each group ($p = 0.03$). However, there were no significant differences among the three groups of mDCs.

**mDCs prepared using TLR7/8 agonists strongly activate NK cells**

According to phenotype, cytokine secretion, and migratory capacity, it was clear that CL075 and R848 had comparable impacts on the development of mDCs. We next addressed the capacity of DC5C+R848 and DC5C+CL075 to activate various lymphocyte populations, as well as their migratory responses to CCL19 as a chemotactant. As illustrated in Fig. 2, low percentages of mDC populations showed spontaneous migration in the absence of chemotactant, but substantially higher percentages of mDCs showed Transwell chemotaxis to CCL19 chemokine, with an overall significant difference to spontaneous migration in each group ($p = 0.03$). However, there were no significant differences among the three groups of mDCs.

### Table I. Composition of mixtures used for DC maturation

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Population</th>
<th>Inflammatory Cytokines/IFNs</th>
<th>Other Additives</th>
<th>TLR Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>4C</td>
<td>DC4C</td>
<td>TNF-α, IL-1β, IL-6</td>
<td>PGE2</td>
<td>Poly(I:C)</td>
</tr>
<tr>
<td>5C</td>
<td>DC5C</td>
<td>TNF-α, IL-1β, IFN-γ</td>
<td>PGE2</td>
<td>Poly(I:C), R848</td>
</tr>
<tr>
<td>5C+R848</td>
<td>DC5C+R848</td>
<td>TNF-α, IL-1β, IFN-γ</td>
<td>PGE2</td>
<td>Poly(I:C), R848</td>
</tr>
<tr>
<td>5C+CL075</td>
<td>DC5C+CL075</td>
<td>TNF-α, IL-1β, IFN-γ</td>
<td>PGE2</td>
<td>Poly(I:C), CL075</td>
</tr>
</tbody>
</table>

The following concentrations were used in the individual mixtures (bold): 4C, 10 ng/ml TNF-α, 10 ng/ml IL-1β, 1000 ng/ml PGE2, and 15 ng/ml IL-6; 5C, 10 ng/ml TNF-α, 10 ng/ml IL-1β, 250 ng/ml PGE2, 5000 U/ml IFN-γ, and 20 ng/ml poly(I:C); 5C+R848, 10 ng/ml TNF-α, 10 ng/ml IL-1β, 250 ng/ml PGE2, 5000 U/ml IFN-γ, 20 ng/ml poly(I:C), and 1 μg/ml R848; 5C+CL075, 10 ng/ml TNF-α, 10 ng/ml IL-1β, 250 ng/ml PGE2, 5000 U/ml IFN-γ, 20 ng/ml poly(I:C), and 1 μg/ml CL075.

As a second parameter for NK cell activation, we measured upregulation of the activation marker CD69 on NK cells after 24-h activation with mDCs. Results of NK cells from one representative donor are shown in Fig. 3B. NK cells expressing this marker after exposure to DC5C cells did not differ substantially from NK cells cultured in medium alone. Strong changes were seen for NK cells activated with DC5C+R848 or DC5C+CL075, showing substantially increased expression of CD69. Notably, DC5C+R848 and DC5C+CL075 stimulation had a strong impact on CD69 expression by both the CD56dim and CD56bright NK cells.

As a third parameter, the killing capacity of activated NK cells was determined after activation with mDCs for 24 h. Effector cells were tested in a standard 4-h chromium release assay using the K562 cell line as the target cell. NK cells cultured with DC5C displayed only slightly enhanced killing activity at high E:T when compared with NK cells not exposed to DC stimulation (Fig. 3C). In contrast, NK cells activated by either DC5C+R848 or DC5C+CL075 showed greater killing at all E:T compared with NK cells activated with DC4C.

### Table II. Expression of typical DC surface markers

<table>
<thead>
<tr>
<th>% Positive cells</th>
<th>CD14</th>
<th>CD80</th>
<th>CD83</th>
<th>CD86</th>
<th>HLA-DR</th>
<th>CCR7</th>
</tr>
</thead>
<tbody>
<tr>
<td>iDCs</td>
<td>25 ± 5</td>
<td>29 ± 20</td>
<td>22 ± 3</td>
<td>99 ± 0</td>
<td>100 ± 0</td>
<td>8 ± 0</td>
</tr>
<tr>
<td>4C</td>
<td>3 ± 0</td>
<td>98 ± 1</td>
<td>95 ± 4</td>
<td>100 ± 0</td>
<td>98 ± 4</td>
<td>75 ± 2</td>
</tr>
<tr>
<td>5C+R848</td>
<td>3 ± 1</td>
<td>99 ± 0</td>
<td>97 ± 1</td>
<td>98 ± 3</td>
<td>77 ± 24</td>
<td>72 ± 6</td>
</tr>
<tr>
<td>5C+CL075</td>
<td>3 ± 1</td>
<td>99 ± 0</td>
<td>97 ± 1</td>
<td>98 ± 3</td>
<td>95 ± 8</td>
<td>83 ± 2</td>
</tr>
</tbody>
</table>

**MFIs**

<table>
<thead>
<tr>
<th>% Positive cells</th>
<th>CD14</th>
<th>CD80</th>
<th>CD83</th>
<th>CD86</th>
<th>HLA-DR</th>
<th>CCR7</th>
</tr>
</thead>
<tbody>
<tr>
<td>iDCs</td>
<td>19 ± 9</td>
<td>16 ± 2</td>
<td>14 ± 1</td>
<td>144 ± 31</td>
<td>497 ± 124</td>
<td>25 ± 7</td>
</tr>
<tr>
<td>4C</td>
<td>20 ± 6</td>
<td>76 ± 12</td>
<td>86 ± 28</td>
<td>760 ± 107</td>
<td>732 ± 203</td>
<td>56 ± 8</td>
</tr>
<tr>
<td>5C+R848</td>
<td>14 ± 5</td>
<td>143 ± 33</td>
<td>61 ± 19</td>
<td>946 ± 286</td>
<td>542 ± 151</td>
<td>48 ± 10</td>
</tr>
<tr>
<td>5C+CL075</td>
<td>12 ± 2</td>
<td>144 ± 25</td>
<td>48 ± 5</td>
<td>842 ± 369</td>
<td>496 ± 158</td>
<td>58 ± 6</td>
</tr>
</tbody>
</table>

*Data represent mean percentages of positive-stained cells ± SEs for three to four independent donors.

*DCs matured in mixtures shown in Table I.

*Significant differences between SC+R848 and 5C+CL075.

*Data represent MFIs ± SEs for three to four independent donors.
In a representative example, ∼6% of CD4+ T cells cultured in medium alone were positive for IFN-γ, and 1.5% expressed IL-4 (Fig. 4A). These values increased to ∼25% of CD4+ T cells with IFN-γ and 6.5% with IL-4 after activation for 1 wk with DC4C cells. In contrast, around twice as many CD4+ T cells expressed IFN-γ after coculture with DC5C+R848 or DC5C+CL075, whereas CD4+ T cells producing IL-4 remained at the background levels of unstimulated PBMCs. Similar effects were seen on polarization of CD8+ T cells, with higher percentages of cells producing IFN-γ (Fig. 4B), without alterations in percentages of IL-4–stained cells (data not shown), following coculture with DC5C+R848 or DC5C+CL075, as compared with DC4C.

IL-5 and IL-13 represent additional Th2 cytokines of importance. To determine the amount of IL-5 and IL-13 secreted by cells cultured for 7 d with allogeneic mDCs, we restimulated CD4–enriched T cells or PBLs for 24 h with allogeneic PBMCs. The levels of cytokines secreted into the supernatant medium were analyzed using standard ELISA. Neither T cells activated with DC4C nor T cells stimulated with DC5C+R848 or DC5C+CL075 secreted appreciable amounts of IL-5 and IL-13 (data not shown).

**DCs matured with TLR7/8 agonists prime better Ag-specific T cell responses**

The capacity of DCs to prime naïve CD8+ cells to specific Ags is of particular importance for development of efficient antitumor immune responses. To address this capacity of mDC populations, we cocultured PBLs from HLA-A*0201 donors and incubated for 24 h with washed DCs that were matured in mixtures 4C, 5C+R848, and 5C+CL075. A, Secretion of IFN-γ was quantified by a standard ELISA and shown as individual values on a log₁₀ scale (4C, 5C+CL075: n = 7; 5C+R848: n = 4; line represents mean value; Mann-Whitney U test was applied for statistical analyses). B, Cocultured cells were stained for CD3, CD56, and CD69 expression. Depicted is a representative specific staining of CD56 versus CD69 of the CD3⁺ population (n = 4). C, Cytotoxic activity of NK cells assessed in a standard 4-h chromium release assay against K562 target cells at the varying effector to target cell ratios indicated on the x-axis. Data are derived using cells from two independent donors and shown as mean with SEM.

**FIGURE 3.** Activation of NK cells by mDCs. NK cells were prepared from several unrelated donors and incubated for 24 h with washed DCs that were matured in mixtures 4C, 5C+R848, and 5C+CL075. A, Secretion of IFN-γ was quantified by a standard ELISA and shown as individual values on a log₁₀ scale (4C, 5C+CL075: n = 7; 5C+R848: n = 4; line represents mean value; Mann-Whitney U test was applied for statistical analyses). B, Cocultured cells were stained for CD3, CD56, and CD69 expression. Depicted is a representative specific staining of CD56 versus CD69 of the CD3⁺ population (n = 4). C, Cytotoxic activity of NK cells assessed in a standard 4-h chromium release assay against K562 target cells at the varying effector to target cell ratios indicated on the x-axis. Data are derived using cells from two independent donors and shown as mean with SEM.
Expression of CD80 was highest on DC5C+CL075 and significantly more than mixtures 4C or 5C+CL075, with or without addition of IL-10. The intensity (MFI) values of CD80 and CD274 on DCs matured using a representative example of gated CD4+ cells were found with respect to expression of the positive costimulatory molecule CD80 (B7.1) versus the negative costimulatory molecule CD274 (B7-H1). A preponderance of CD80 compared with CD274 seems to be important for transmission of positive costimulatory signals to T cells. For example, differences were found between DC4C and DC5C+R848 or DC5C+CL075 for STAT1, STAT3, and STAT6 that was only marginal in DC4C. Increased STAT1 phosphorylation that was only marginal in DC4C.

In addition to IL-12(p70) secretion, we observed several other differences between DC4C and DC5C+CL075 that may impact on lymphocyte activation and function. For example, differences were found with respect to expression of the positive costimulatory molecule CD80 (B7.1) versus the negative costimulatory molecule CD274 (B7-H1). A preponderance of CD80 compared with CD274 seems to be important for transmission of positive costimulatory signals to T cells. In contrast, the multimer-positive fraction increased through coculture with DC5C+CL075 displayed strongly different to DC4C. DC5C+CL075+IL-10 was generated by addition of IL-10 to the maturation mixture as a tolerogenic control. Treatment with IL-10 has been shown to reduce the stimulatory capacity of DCs, resulting in anergized T cells (35, 36). IL-10–stimulated DCs showed a reciprocal relationship of CD80 to CD274, with lower CD80 and higher CD274 expression. Similar observations were made with DC5C+R848 (data not shown).

On a molecular level, STAT molecules are important transcription factors in the downstream signaling pathways of TLRs and IFN-γRs. DCs matured with 4C and 5C+CL075 were assessed for the activation status of the STAT proteins 1 (pTyr701), 2 (pTyr694), 3 (pSer727 and pTyr705), 4 (pTyr701), 5 (pTyr694), and 6 (pTyr641). STAT1, STAT3, and STAT6 were found to be activated in the mDCs based on their phosphorylation status (data not shown), but differences between DC4C and DC5C+CL075 were only found for STAT1 and STAT6. DC5C+CL075 displayed strongly increased STAT1 phosphorylation that was only marginal in DC4C. Activation of STAT1 has been demonstrated previously for DCs matured in mixtures containing R848 (24).

DCs matured with a TLR3 agonist are inferior to those matured with TLR3 and TLR7/8 signals

Clearly mDCs prepared using a combination of TLR3 and TLR7/8 agonists were superior to DCs matured with mixture 4C that lacked TLR signaling capacity. However, the impact of the TLR3 signal alone was not clear. Therefore, comparisons of DC5C and DC5C+CL075 were made to ascertain the role of TLR3 stimulation in the absence of TLR7/8 activation (Fig. 6). Mixture 5C induced a maturation phenotype similar to 5C+CL075, albeit with somewhat lower levels of CD80, CD83, and CCR7 (Fig. 6A). DC5C secreted high levels of bioactive IL-12(p70) in signal 3 assays that were comparable to DC5C+CL075 and substantially greater than DC4C (Fig. 6B). Coculture of NK cells with DC4C and DC5C for 24 h yielded comparable percentages of activated NK cells expressing CD69, but these were less than half the value obtained through activation by DC5C+CL075 (Fig. 6C). Furthermore, secretion of IFN-γ and IL-4 were secreted noticeably higher amounts of IFN-γ compared with PBLs cocultured with peptide-pulsed mDC4C (data not shown).

To assess the killing capacity of T cells primed with different mDC populations, PBLs were cocultured with ELA-pulsed mDCs for 7–10 d. Staining with a MART-1/Melan-A peptide multimer and CD8-specific Ab on day 10. Killing was assessed on day 7 in a standard 4-h chromium-release assay using Mel624.38 (HLA-A2+, MART-1/Melan-A+) and MelA375 (HLA-A2+, MART-1/Melan-A+) tumor cell lines as positive and negative target cells, respectively. Shown are two independent donors as mean values with SEM.

In addition to IL-12(p70) secretion, we observed several other differences between DC4C and DC5C+CL075 that may impact on lymphocyte activation and function. For example, differences were found with respect to expression of the positive costimulatory molecule CD80 (B7.1) versus the negative costimulatory molecule CD274 (B7-H1). A preponderance of CD80 compared with CD274 cells (gray) and CD8+ cells (black), respectively. Shown are mean values with SEM; significance was analyzed using an ANOVA test in combination with a Bonferroni posttest (p < 0.01; **p < 0.001; each compared with unstimulated control or compared with CD8+ T cells stimulated with DC4C). C, Autologous PBLs of HLA-A2+ donors were stimulated with MART-1/Melan-A (ELA-) peptide-pulsed mDCs for 7–10 d, followed by a staining with a MART-1/Melan-A multimer and CD8-specific Ab on day 10. D, Killing was assessed on day 7 in a standard 4-h chromium-release assay using Mel624.38 (HLA-A2+, MART-1/Melan-A+) and MelA375 (HLA-A2+, MART-1/Melan-A+) tumor cell lines as positive and negative target cells, respectively. Shown are two independent donors as mean values with SEM.
T cells stimulated with peptide-pulsed DC5C+CL075 displayed much greater than T cells cultured in the absence of mDC. In contrast, exposure times were 3 min, 30 s, and 3 s for 4C, 5C+CL075, and 5C+CL075, and was stained as a control. Exposure times were 3 min, 30 s, and 3 s for 4C, 5C+CL075, and 5C+CL075+IL-10.

FIGURE 5. Costimulator profile and STAT activation in mDCs. mDCs were generated with the 3-d-protocol. A. Shown is the expression level of the costimulatory molecules CD80 and CD274 (B7-H1) detected by flow cytometry as mean values of six individual experiments (three independent donors) with SEM. As a tolerogenic control, mDCs were prepared using 10 ng/ml IL-10 incorporated into mixture 5C+CL075. Statistical analysis was performed using an ANOVA test in combination with a Bonferroni post-test. B. Shown is a Western blot stained for pSTAT1 and pSTAT6; β-actin was stained as a control. Exposure times were 3 min, 30 s, and 3 s for 4C, 5C+CL075, and β-actin staining, respectively.

IFN-γ by T cells primed and restimulated with mDCs was much less for DC4C and DC5C compared with DC5C+CL075 (Fig. 6D). Killing capacity of MART-1/Melan-A–specific CTL primed over 7 d was comparable for peptide-pulsed DC4C and DC5C and somewhat greater than T cells cultured in the absence of mDC. In contrast, T cells stimulated with peptide-pulsed DC4C+CL075 displayed much higher levels of killing of tumor cells coexpressing MART-1/Melan-A and HLA-A2 (Fig. 6E). These results demonstrated that DCS matured using only TLR3 signals were functionally inferior in activation of both NK cells and T cells to DCS matured using the combination of TLR3 and TLR7/8 agonists.

Discussion

In DC-based vaccination, mDCs are essential to induce effective immune responses; therefore, the first generation vaccine trials that applied iDCs may not have achieved adequate in vivo maturation to allow induction of optimal immune responses. The second generation of vaccines using mDCs provided cells that were more proficient at delivering signal 1 to T cells through abundant MHC class I and class II expression and were capable of delivering signal 2 through an improved expression of costimulatory molecules, but they did not express a cytokine profile that would foster induction of optimal antitumor responses. The discovery that TLR signaling in murine DCs could activate the NF-κB pathway opened the door for inclusion of synthetic TLR agonists in maturation mixtures to modulate the cytokine profile of mDCs (37).

A variety of TLRs are expressed by human monocyte-derived DCs (38). Signaling through different TLRs, including TLR3, TLR7, and TLR8, also causes activation of the NF-κB pathway in human cells, with subsequent induction of several immuno-modulatory genes (14, 24, 25). This leads to production of cytokines and chemokines, such as TNF-α, IFN-γ, IL-6, IL-10, IL-12, and MIP-1α, in various cell types (16, 17). The availability of synthetic ligands for several TLRs, including imiquimod (R837), resiquimod (R848), S-27609, CL097, CL075 (3M-002), CL087, or loxoribine, has enabled the impact of TLR activation to be assessed in various cell types (18, 22). On the basis of these observations, mixtures containing various synthetic TLR agonists have been used to prepare human mDCs. Mailliard et al. (23) described a maturation mixture composed of TNF-α, IL-1β, IFN-γ, IFN-α, and the TLR3 agonist poly(I:C), which enhanced the production of bioactive IL-12(p70) in 7-d mDCs but gave only low cell yields. This could be a limiting factor in obtaining sufficient numbers of mDCs for clinical studies if vaccination protocols do not entail direct intralymphatic injection (clinical trial ID UPIC 03-118; http://clinicaltrials.gov/ct2/show/NCT00390338) but rely instead on migration of a small proportion of mDCs to neighboring lymph nodes after s.c. or intradermal injection (39, 40). An alternative approach included only the TLR7/8 ligand resiquimod (R848) for stimulation of iDCs; however, this yielded DCs lacking several phenotypic characteristics of fully mature DCs desired for clinical use (41). Dauer et al. (10) recently incorporated TLR4 and TLR7/8 ligands in their maturation mixture to obtain 2-d mDCs secreting bioactive IL-12(p70). This study revealed that TLR ligands enhanced IL-12(p70) secretion in mDCs as well as stimulatory capacity; however, the maturation status and migratory capacity were lower than mDCs produced with mixture 4C.

We previously described DC maturation mixtures including R848 and poly(I:C) as respective agonists for TLR7/8 and TLR3, in combination with additional cytokines within a 7-d-protocol (26). CL075 is a synthetic small molecule thiazoloquinoline immunostimulatory compound that is a preferential activator of TLR8, resulting in downstream activation of NK-kb and other transcription factors. It is also involved in transcriptional activation of numerous genes encoding cytokines, chemokines, and costimulatory molecules (17). So far, the impact of CL075 on DC maturation has not been reported. We found in this study that mDCs prepared with maturation mixtures using CL075 displayed phenotypes and functions suitable for antitumor vaccine development.

Comparison of mDCs prepared without TLR agonists versus those stimulated with TLR3 [poly(I:C)], alone or in combination with TLR7/8 agonists (R848/CL075), revealed that all populations of mDCs were similar with respect to percentages of positive cells expressing costimulatory molecules. Some variations in HLADR and CCR7 were noted and mixture 5C+CL075 provided the highest percentages of mDCs expressing these two molecules. The good expression of CCR7 was paralleled by a strong spontaneous migratory capacity of the mDCs as well as positive chemotactic responses to CCL19 chemokine signals.

As expected, TLR signaling altered the cytokine secretion profile of mDCs, leading to high production of bioactive IL-12(p70). DCs prepared with mixtures including only a TLR3 signal or in combination with a TLR7/8 agonist induced high levels of IL-12(p70) secretion in signal 3 assays that mimic DC encounters with T cells. These findings are supported by a study of Larange` et al. (42), which demonstrated that signaling through TLR8 had a high impact on secretion of IL-12(p70).

The modulated cytokine profile of mDCs achieved through combined TLR3 and TLR7/8 signaling had a strong impact on functional activation of NK cells, as seen by upregulation of CD69 on both CD56dim and CD56bright NK cell subpopulations, as well as on the levels of IFN-γ secretion and killing capacity by NK cells after a 24-h exposure to mDCs. A recent study also showed that IL-12(p70) secreted by DCs influences NK activation, in
particular IFN-\(\gamma\) secretion, in a cell-contact–dependent manner (6), as also found in our studies. Thus, DCs matured with 5C+CL075 or R848 should support innate responses that can lead to direct killing of tumor cells by activated CD56dim NK cells as well as through the capacity of CD56bright NK cells to support development of adaptive immune responses by cytokine and chemokine secretion (43).

TLR7/8 signaling with CL075 or R848 had a positive impact on the ability of CD4+ and CD8+ T cells to produce IFN-\(\gamma\). Although some CD4+ and CD8+ allogeneic T cells were polarized to Th1 and Th2 cells using DC4C cells, substantially higher percentages of IFN-\(\gamma\)-polarized T cells were obtained using TLR-activated mDCs. In addition, these mDCs were able to induce greater numbers of CTLs that displayed specific killing capacity for tumor cells. We also observed that the levels of CD274 were not significantly different. Therefore, it would be expected that such TLR-activated mDCs would have a superior capacity for T cell activation. This is in accordance with recent findings by Selenko-Gebauer et al. (44), who nicely showed that the expression levels of positive costimulatory molecules prevailed over low expression of inhibitory molecules in T cell activation, whereas a reverse profile resulted in poor T cell stimulation. In addition, they postulated that inhibitory molecules had a greater influence when the overall expression of costimulatory molecules was low.

The molecular mechanisms responsible for these phenotypic and functional differences in DCs matured by 4C and 5C+CL075 were indicated by the strong differences in STAT1 activation. In particular, pSTAT1-Tyr701 was strongly increased in DC5C+CL075 compared with DC4C. This activation is known to result in an altered expression profile of downstream STAT1-dependent genes, including cytokines and costimulatory molecules (42, 45). Phosphorylated STAT1 can bind to an IFN-\(\gamma\)–activated sequence or an IFN-stimulated response element in DNA. In both cases, this leads to increased expression of IFN-stimulated genes (46). Moreover, IFN-stimulated response element binding sites are present in the promoter regions of CD80 and CD274 (47, 48), likely explaining...
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the significantly enhanced expression of these molecules on DCs matured with 5C+CL075. Others found that phosphorylation of STAT1 was induced in DCs by the TLR7/8 agonist R848 and that decreased IL-12p70, CD40, and CD83 expression was seen following inhibition of the STAT1 signaling pathway in studies using 7-d mDCs (24, 42).

Altogether, maturation mixtures including CL075 or R848 as TLR7/8 agonists, together with a TLR3 agonist, allowed mDCs to be produced in 3 d that had superior characteristics, when compared with DCs matured in the absence of TLR signals or activated by only a TLR3 signal. The production of mDCs in 3 d rather than 7 d provides a great cost reduction for DC vaccine generation, because cell cultures must not be resupplemented with cytokines during the short culture period. Furthermore, occupancy time of clean room facilities is greatly reduced. This allows rapid generation of mDCs that can be used for development of antipathogen and antitumor vaccines.

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Disclosures

S.S., M.J., D.J.S., and B.F. declare a competing interest through pending Disclosures support.

References


Table SI. Recovery of mature DC

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<th>iDC&lt;sup&gt;i&lt;/sup&gt;</th>
<th>4C&lt;sup&gt;i&lt;/sup&gt;</th>
<th>5C + R848</th>
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<tr>
<td>Recovery (% seeded cells)</td>
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<tr>
<td></td>
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<td>0.8</td>
<td>1.7</td>
<td>2.1</td>
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<sup>i</sup> immature DC  
<sup>i</sup> DC matured in cocktails shown in Table 1  
<sup>‡</sup> Number of independent donors;  
<sup>§</sup> mean, given as percentage of cells determined as quotient of recovered to seeded cells  
<sup>¶</sup> standard deviation