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ORIGINAL ARTICLE

Maturation of monocyte-derived dendritic cells with Toll-like receptor 3 and 7/8 ligands combined with prostaglandin E_2 results in high interleukin-12 production and cell migration

A. C. Inge Boullart · Erik H. J. G. Aarntzen · Pauline Verdijk · Joannes F. M. Jacobs · Danita H. Schuurhuis · Daniel Benitez-Ribas · Gerty Schreibelt · Mandy W. M. M. van de Rakt · Nicole M. Scharenborg · Annemiek de Boer · Matthijs Kramer · Carl G. Figdor · Cornelis J. A. Punt · Gosse J. Adema · I. Jolanda M. de Vries

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Abstract Dendritic cells (DC) are professional antigen-presenting cells of the immune system that play a key role in regulating T cell-based immunity. In vivo, the capacity of DC to activate T cells depends on their ability to migrate to the T cell areas of lymph nodes as well as on their maturation state. Depending on their cytokine-secreting profile, DC are able to skew the immune response in a specific direction. In particular, IL-12p70 producing DC drive T cells towards a T helper 1 type response. A serious disadvantage of current clinical grade ex vivo generated monocyte-derived DC is the poor IL-12p70 production. We have investigated the effects of Toll-like receptor (TLR)-mediated maturation on ex vivo generated human monocyte-derived DC. We demonstrate that in contrast to cytokine-matured DC, DC matured with poly(I:C) (TLR3 ligand) and/or R848 (TLR7/8 ligand) are able to produce vast amounts of IL-12p70, but exhibit a reduced migratory capacity. The addition of prostaglandin E_2 (PGE_2) improved the migratory capacity of TLR-ligand matured DC while maintaining their IL-12p70 production upon T cell encounter. We propose a novel clinical grade maturation protocol in which TLR ligands poly(I:C) and R848 are combined with PGE_2 to generate DC with both high migratory capacity and IL-12p70 production upon T cell encounter.

Keywords Immunotherapy · Dendritic cells · Maturation · Cell trafficking · Tumor immunology · Toll-like receptor ligands

Both A. C. Inge Boullart and Erik H. J. G. Aarntzen contributed equally to this work.

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A. C. I. Boullart · E. H. J. G. Aarntzen · P. Verdijk · D. H. Schuurhuis · D. Benitez-Ribas · G. Schreibelt · M. W. M. M. van de Rakt · N. M. Scharenborg · A. de Boer · M. Kramer · C. G. Figdor · G. J. Adema · I. J. M. de Vries (✉)
Department of Tumor Immunology,
Nijmegen Centre for Molecular Life Science,
Radboud University Nijmegen Medical Centre,
P.O.Box 9101, 6500 HB Nijmegen, The Netherlands
e-mail: j.devries@ncmls.ru.nl

A. C. I. Boullart · E. H. J. G. Aarntzen · C. J. A. Punt
Department of Medical Oncology,
Radboud University Nijmegen Medical Centre,
Nijmegen, The Netherlands

J. F. M. Jacobs · I. J. M. de Vries
Department of Paediatric Hemato-Oncology,
Radboud University Nijmegen Medical Centre,
Nijmegen, The Netherlands

Abbreviations

CDC	Conventional cytokine matured DC (matured with IL-1β, IL-6, TNFα, PGE_2)
DC	Dendritic cells
HS	Human serum
PBL	Peripheral blood lymphocytes
PGE_2	Prostaglandin E_2
Poly(I:C)	Polyinosinic:polycytidylic acid
TLR	Toll-like receptor
TLR3-DC	TLR3 ligand (poly(I:C)) matured DC
TLR7/8-DC	TLR7/8 ligand (R848) matured DC
TLR-DC	DC matured with TLR3 ligand (poly(I:C)) and TLR7/8 ligand (R848)

Introduction

Dendritic cells (DC) are highly specialized antigen-presenting cells, acting as the sentinels of the immune system [1].

Immature DC are located in peripheral tissues, where they capture and process antigens. Pro-inflammatory cytokines and microbial products, as well as endogenous factors, can activate immature DC that successively undergo a complex maturation process. This process is characterized by a switch in cytokine and chemokine receptors and up-regulation of MHC complexes and co-stimulatory molecules [2]. Upon maturation, DC migrate via the lymphatic vessels to the secondary lymphoid tissue where they relocate to the T cell zone of the lymph node [3, 4]. Depending on the stimuli received, DC are able to skew naive T cell differentiation towards either a T helper 1 (Th1) or Th2 response, thus orchestrating specific immune responses.

Because of their central role in the induction of immunity, ex vivo generated monocyte-derived DC have been applied in cancer immunotherapy [5–8]. Previous clinical studies have demonstrated that effective induction of specific antitumor cytotoxic T cell (CTL) responses require a mature status of the DC [6, 9]. As the activation of T cells occurs in secondary lymphoid organs, the capacity of the ex vivo generated DC to migrate into lymph nodes in vivo appears to be of crucial importance [10–12]. To date, the most widely applied DC are monocyte-derived DC that are ex vivo matured with a defined cocktail of the pro-inflammatory cytokines TNFα, IL-1β, IL-6 and prostaglandin E_2 (PGE_2) [13]. This maturation cocktail leads to phenotypically mature DC with adequate migratory capacities in vitro and in vivo [14].

PGE_2 is included in this maturation cocktail for its participation in podosome dissolution and high-speed migration [15]. Although these DC have shown to be capable of inducing Th1-type tumor-specific immune responses in vivo [16], they secrete only limited amounts of the Th1 inducing cytokine interleukin-12p70 (IL-12p70). On the other hand, the presence of PGE_2 during maturation of DC inhibits the production of IL-12p70 [17, 18].

IL-12p70 production enhances the ability of DC to induce tumor specific IFNγ producing Th1 cells and CTL and, thereby, the ability to mount an adequate anti-tumor response in vivo [19]. Furthermore, in vitro studies indicated that IL-12p70 positively influences the properties of CD8+ T cells to become memory cells and to recruit natural killer (NK) cells [20, 21]. From preclinical studies it is suggested that inadequate IL-12p70 production is one of the hurdles to take in improving the clinical outcomes in cancer immunotherapy studies [22–24].

TLR are innate receptors that sense microbial and viral products and trigger DC maturation and cytokine production [25, 26]. Triggering Toll-like receptors (TLR) on DC induces high IL-12p70 production [27]. In mice, the maturation of DC by pro-inflammatory cytokines yields DC that support T cell clonal expansion, but fail to efficiently direct effector T cell differentiation [28]. Interestingly, DC matured in the presence of TLR ligands were able to induce full T cell effector function and unleashed more potent immune responses [29]. Moreover, cross-presentation of exogenous antigen in MHC class I is enhanced in the presence of TLR ligands [30].

Monocyte-derived DC express TLR3, TLR4, TLR7, TLR8 and TLR 9 [31, 32]. In 2004, Mailliard et al. [22] developed a clinical grade maturation cocktail to obtain the so-called alpha-type 1 polarized DC, by including IFNα and the TLR3 ligand polyinosinic:polycytidylic acid (poly(I:C)) in addition to IL-1β, TNFα and IFNγ. The addition of poly(I:C), a viral dsRNA mimic, to the pro-inflammatory cytokine maturation cocktail dramatically increases the IL-12p70 production and subsequently the number of tumor-specific CTL in vitro [22, 33]. The TLR7/8 ligand R848 belongs to the imidazoquinolines family and also strongly induces the production of IL-12p70 [34]. Napolitani et al. [27] showed that the combination of TLR3 and TLR7/8 ligands leads to a synergistic IL-12p70 production by DC. However, TLR-ligand matured DC have an altered expression of regulator of G protein signaling proteins, which may negatively affect the chemotaxis and migration of these cells [35].

DC used for clinical vaccination studies require both migratory and cytokine production capacities. Currently, no clinical grade maturation protocol is available that yields DC that combines these desired features. In this study we investigated the application of TLR ligands in ex vivo maturation of DC and propose a clinically applicable maturation protocol consisting of two TLR ligands and PGE_2, to generate DC that fit the desired physiological role of migration to the lymph nodes and IL-12p70 production upon T cell contact to induce tumor specific IFNγ producing Th1 cells and CTL.

Materials and methods

Antibodies and immunostaining

The phenotype of the DC populations was determined by flow cytometry. The following primary monoclonal antibodies (mAbs) or the appropriate isotype controls were used: anti-HLA-ABC (W6/32), anti-HLA DR/DP (Q5/13) and anti-CD80 (all Becton Dickinson, Mountain View, CA, USA); anti-CD83 (Beckman Coulter, Mijdrecht, the Netherlands), anti-CD86 (Pharmingen, San Diego, CA, USA); anti-CCR7 (kind gift of Martin Lipp, Max Planck Institute, Berlin, Germany), anti-CD14 (Beckman Coulter) followed by Goat-anti-Mouse FITC (Roche).

Culture media and cytokines

For DC culture, X-VIVO 15 (BioWhittaker, Walkersville, MD, USA) was supplemented with 2% human serum (HS;

serum of six blood donors type AB was pooled) (Sanquin, Bloodbank Zuid-Oost, Nijmegen, the Netherlands), IL-4 (300 U/ml) and GM-CSF (450 U/ml) (both from Strathmann, Hamburg Germany). For maturation the following products were used: recombinant TNFα (10 ng/ml; CellGenix, Freiburg, Germany), IL-1β (5 ng/ml or 25 ng/ml; Immunotools, Friesoythe, Germany), PGE_2 (10 μg/ml) (Pharmacia & Upjohn, Puurs, Belgium), IL-6 (15 ng/ml) (CellGenix), IFNα (10,000 U/ml) (Roche, Mijdrecht, the Netherlands), IFNγ (1000 U/ml) (Roche), poly(I:C) (20 μg/ml) (Sigma Chemicals Co., St Louis, MO), R848 (3 μg/ml) (PharmaTech, Shanghai, China).

Generation of mature DC

Buffy coats were obtained from healthy volunteers according to institutional guidelines. Monocytes were isolated from peripheral blood mononuclear cells (PBMC) by adherence, as described previously [36]. On day 6 or day 7, cells were harvested (immature DC) or maturation cocktail was added for 48 h, after which the cells were harvested.

Migration assay

Flat-bottomed 96-well plates (Costar, Corning Inc. Corning, NY) were coated with 50 μl/well fibronectin (20 μg/ml; Roche) for 60 min at 37°C, washed with PBS and blocked with 100 μl/well 0.01% gelatin (Sigma) for 30 min at 37°C. Totally 4,000 DC/well were seeded and recorded for 60 min at 37°C, after which migration tracks of individual DC were analyzed using an automated cell tracking system [37].

Transwell migration assay

Chemotaxis of DC in response to CCL21 (a ligand for the CCR7 chemokine receptor) was measured in 24-well plates carrying transwell permeable supports with 5 μm pore size polycarbonate membrane (Costar). Briefly, culture medium alone or supplemented with 1–100 ng/ml CCL21 (R&D Systems, Minneapolis, MN) was placed in the lower compartment in a total volume of 600 μl, and 10^5 DC were loaded into the inserts in 100 μl. Kinesis was determined by measuring the migration in the presence of chemokine in both the lower and upper compartments. Chambers were incubated for 120 min in a 5% CO_2, humidified in a incubator at 37°C. Thirty minutes before the end, 5 mM EDTA was added to the lower compartment. Cells were harvested, centrifugated and resuspended in 100 μl PBS. The number of migrated DC (lower chamber) was determined by flow cytometry by acquiring events for a fixed time period of 60 s (FACScan, Becton Dickinson). All conditions were tested in duplicate.

IL-12p70 production

The production of IL-12p70 was measured in the supernatants harvested 48h after maturation (1×10^6 immature DC (day 3) in 2 ml per well were matured at day 6 with 250 μl of the indicated maturation cocktails), or 24 h after CD40L stimulation (5×10^3cells/100 μl) and mixed lymphocyte reaction (MLR: DC:T cell ratio of 1:5 with 100.000 PBL, final volume 100 μl) using a standard sandwich ELISA (Pierce Biotechnology, Rockford). The procedure was performed according to the manufacturer's instructions.

Allogeneic MLR

The allostimulatory capacity of the DC was tested in a mixed lymphocyte reaction (MLR). Allogeneic T cells were co-cultured with differently matured DC in a 96-well tissue culture microplate (DC:T cell ratio 1:5 with 100.000 PBL). After 24 h the cytokines in the supernatant were analyzed with a cytometric bead array for human Th1/Th2 cytokines (BD Biosciences, San Diego, CA) according to the manufacturer's instructions (detecting IL-2, IL-4, IL-5, IL-10, TNFα and IFNγ).

CD40L stimulation

DC were harvested, washed and seeded in a 96-well round-bottomed plate at 50×10^3 cells in 100 μl per well. To mimic the interaction with CD40L-expressing Th-cells, CD40 L trimers (Leinco Technologies, Missouri, USA) were added at a concentration of 1 μg/ml. Twenty-four-hour supernatants were analyzed by IL-12p70 ELISA.

Antigen-specific proliferation assay

Cellular responses against the protein keyhole limpet hemocyanin (KLH) were measured in a proliferation assay. In our vaccination studies, KLH is added to the immature DC culture as a immunomonitoring tool. Peripheral blood mononuclear cells (PBMC) were isolated from blood sample from four patients taken after four biweekly vaccinations with mature DC. CD4+ T cells were isolated with a CD4+ T cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purified T cells were plated in a 96-well tissue culture microplate with autologous DC that were cultured with or without KLH and matured with the cytokine cocktail or with poly(I:C) and R848 with or without PGE_2. After 4 days of culture, 1 μCi/well of tritiated thymidine was added for 8 h, and incorporation of tritiated thymidine was measured in a beta-counter.

Statistics

Data were analyzed using unpaired Student *t*-test, *p*-values <0.05 were considered to be statistically significant.

Results and discussion

To find the optimal maturation procedure to generate DC that have both migratory and IL-12p70 producing capacities, we compared the effect of various maturation cocktails. Cytokine matured DC that are conventionally used in clinical studies were compared with DC matured with poly(I:C) or R848 or both. Pro-inflammatory cytokines such as TNFα, IFNα and IFNγ can further enhance the IL-12p70 production [22, 27, 38, 39], however, the effect on migratory capacity of DC is unknown. In an effort to improve the migration of TLR-ligand matured DC, while maintaining IL-12p70 production we tested the effect of these pro-inflammatory cytokines and PGE_2. Monocyte-derived DC were, thus, matured as follows: (1) conventional-DC (cDC), matured with the pro-inflammatory cocktail (IL-1β, IL-6, TNFα, PGE_2) as described by Jonuleit et al. [13], (2) Toll-like receptor DC (TLR-DC), matured with TLR-ligands alone (poly(I:C), R848, or both; respectively TLR3-DC, TLR7/8-DC or TLR-DC), or (3) poly(I:C) and R848 in combination with pro-inflammatory cytokines (TNFα, IFNα or IFNγ; respectively TNFα-TLR-DC, IFNα-TLR-DC and IFNγ-TLR-DC) or prostaglandin E_2 (PGE_2-TLR-DC).

TLR-ligands and pro-inflammatory cytokines induce DC with a mature phenotype

We first compared the effect of the different maturation cocktails on the morphology and phenotype of the DC. cDC had the characteristic semi-round appearance with multiple dendrites. In contrast, both TLR3-DC, TLR7/8-DC and TLR-DC showed a more elongated morphology and were more adherent to plastic than cDC (Fig. S1a in electronic supplementary material). Although they all expressed the maturation markers CD80, CD86 and MHC class II (Fig. 1a), the expression of CD83 using the TLR ligands alone for maturation was slightly decreased in comparison with the cDC cocktail and the combination of the TLR-ligands. The increased adherence was reflected in a slightly lower yield and viability of especially TLR7/8-DC and TLR-DC (Fig. S1b in ESM). Addition of a pro-inflammatory cytokines to the DC matured with TLR-ligands did not change the morphology of the DC. On the contrary, DC cultured with TLR ligands in the presence of PGE_2 were more similar to cDC (Fig. S1a in ESM).

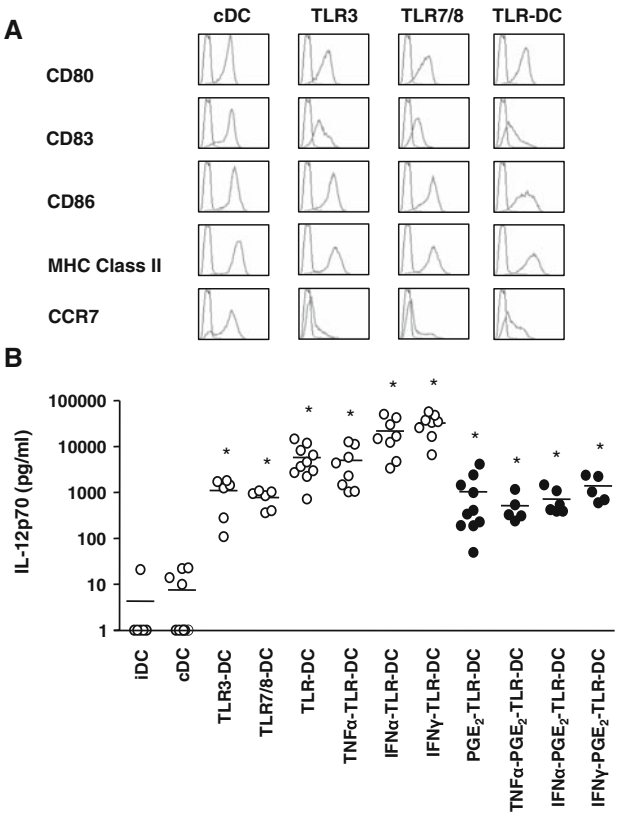


Fig. 1 Phenotype and IL-12p70 production after DC maturation with different cocktails. Immature DC were matured for 48 h with different maturation cocktails, maturation markers were evaluated by flow cytometry and IL-12p70 secreted was measured in the supernatant by ELISA. **a** DC from three different donors matured with the conventional cytokine cocktail (TNFα, IL-6, IL-1α and PGE_2), TLR3 ligand poly(I:C), TLR7/8 ligand (R848) or both. *Upper panels* show the different maturation cocktails used. Horizontally the expression of CD80, CD83, CD86, CCR7 and MHC class II from one representative donor measured by flow cytometry is shown (*bold line*). The *thin line* represents the isotype control. The *lower panel* shows the IL-12p70 production in the supernatant measured by ELISA. **b** DC from different donors were matured with the conventional cytokine cocktail (TNFα, IL-6, IL-1β and PGE_2), or poly(I:C) or R848 or poly(I:C)/R848 in the combination with TNFα, IFNα, IFNγ with and without PGE_2. IL-12p70 production was measured after 48 h. Per condition each *symbol* represents one donor. Means are shown for each maturation cocktail and were compared to cDC (* $p < 0.05$), comparison of the different cocktails with TLR-DC were significant with all the different cocktails. There is no significant difference between PGE_2-TLR-DC and the TNFα/IFNα/IFNγ-PGE_2-TLR-DC

All different maturation cocktails generate phenotypically mature DC, expressing MHC class I and II, co-stimulatory molecules (CD80, CD86), DC-maturation marker CD83 and chemokine-receptor CCR7, which is consistent with previously published data for the above-mentioned maturation cocktails [6, 22, 27]. These phenotypes were stable and did not change when the maturation cocktail was removed after 48 h and cells were cultured in fresh medium without maturation stimuli over the next 72 h (data not shown).

IL-12p70 production is dramatically increased by TLR-ligands and hampered by PGE_2

IL-12p70 secreted by mature DC skews the effector response towards Th1 type and recruits memory T cells and NK cells. Upon TLR-triggering, DC rapidly produce high levels of IL-12p70 [27]. So, we examined the IL-12p70 production 48 h after addition of the different maturation factors. As reported before [22], cDC hardly produce any IL-12p70 (Fig. 1b). DC matured with poly(I:C) or R848 produced elevated levels of IL-12p70, which was further boosted when these ligands were combined (Fig. 1b), confirming the synergistic effect described by Napolitani et al. [27], which presumably results from concurrent triggering of the intracellular signaling pathways via both MyD88 and TRIF. We found that TLR-DC produce the majority of IL-12p70 within 24 h, while little is produced in the next 24 h (data not shown). The addition of IFNα or IFNγ boosted the IL-12p70 production even further (Fig. 1b). IFNγ appeared to be the most potent inducer of stable IL-12p70 production, as it resulted in a fivefold increase in IL-12p70 production. As expected [17], the addition of PGE_2 significantly reduced the IL-12p70 production (Fig. 1b). Thus, also in clinical grade DC preparations, poly(I:C) and R848 strongly induce IL-12p70 secretion during maturation, which is enhanced by type I and II interferons and inhibited by PGE_2.

Migration of TLR-DC is poor and restored by the addition of PGE_2

cDC exhibit known high migratory capacity in vitro and in vivo [22], but do not produce IL-12p70. In contrast, DC matured with poly(I:C) and/or R848 produce high levels of IL-12p70 [22, 27, 34]. According to Luft et al. [18] mature DC differentiate in either migratory or cytokine secreting cells. For immunotherapy with DC, both qualities are of crucial importance for the induction of potent immune responses. Therefore, we compared the migratory capacity of cDC with TLR-ligand matured DC and tested the effect of different cytokines and PGE_2. Random migration of individual DC on fibronectin was analyzed using an automated cell-tracking system [37]. The migrated distance in 1 h of the TLR-DC was significantly lower compared to the cDC (Fig. 2a). Detailed analysis of individual cells indicated that the activity of the cells (percentage of time migrating) was significantly lower in all TLR-ligand matured DC (Fig. 2b). The addition of TNFα, IFNα or IFNγ to the TLR-DC had no effect (Fig. 2). The addition of PGE_2 to the different maturation cocktails markedly restored the motility of DC in all combinations (Fig. 2; Fig. S2 in ESM), indicating that PGE_2 stimulates also the motility of TLR-ligand matured DC.

PGE_2 is known for its favorable effect on the migratory capacity of DC. It has a critical role in podosome dissolution

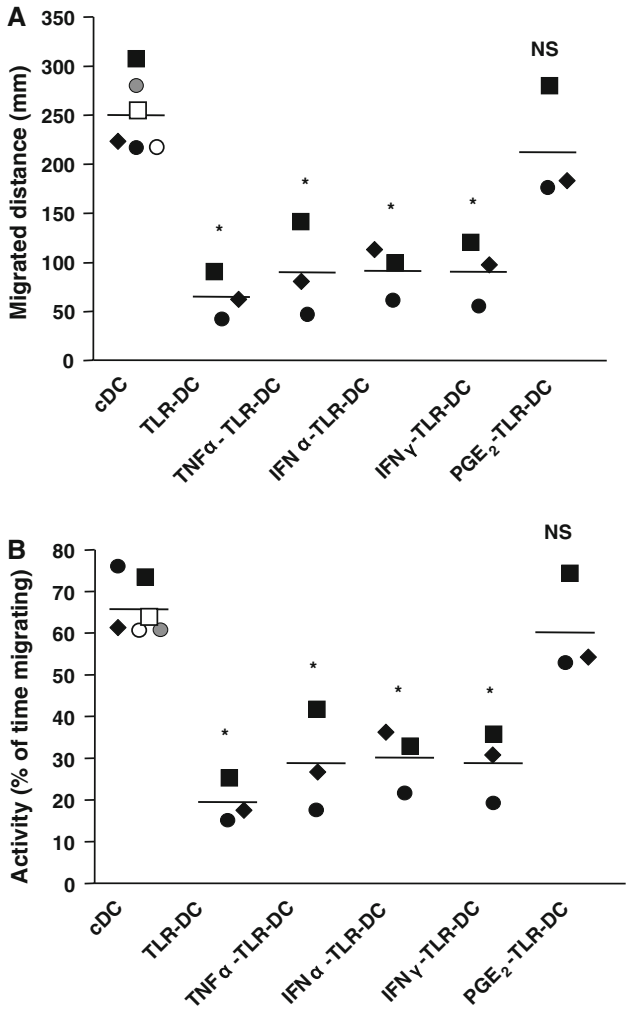


Fig. 2 Migration of the TLR-ligand DC is poor and can be restored upon addition of PGE_2. Differently matured DC from three to six different donors were added to a fibronectin-coated plate and individual cells were monitored for 60 min for their motility. The migrated distance (in μm) (**a**) and the activity (the percentage of time the cells were migrating) (**b**) is plotted for the different maturation cocktails and compared to cDC. Per condition, each *symbol* represents the mean for each donor (individual donors indicated by different *symbols*). Means are shown for each maturation cocktail and were compared to cDC (* = $p < 0.005$)

and induction of high-speed migration [15]. PGE_2 can accomplish both the expression and activation of CCR7, that allows DC to efficiently migrate towards the lymph node in response to CCL19 [23] and CCL21 [24]. The morphology of our cells upon maturation suggests that the inclusion of PGE_2 in the maturation cocktail results in less adherent cells (Fig. S1a in ESM) what may favor migration. FACS analysis showed that CCR7 expression was more upregulated on cDC and PGE2-TLR-DC compared to TLR-DC (Fig. 3a). In addition, cDC and PGE_2-TLR-DC but not TLR-DC migrated similarly towards CCL21 in a chemotaxis assay (Fig. 3b), demonstrating that CCR7 was expressed in an active form. Thus, unlike the pro-inflammatory cytokines

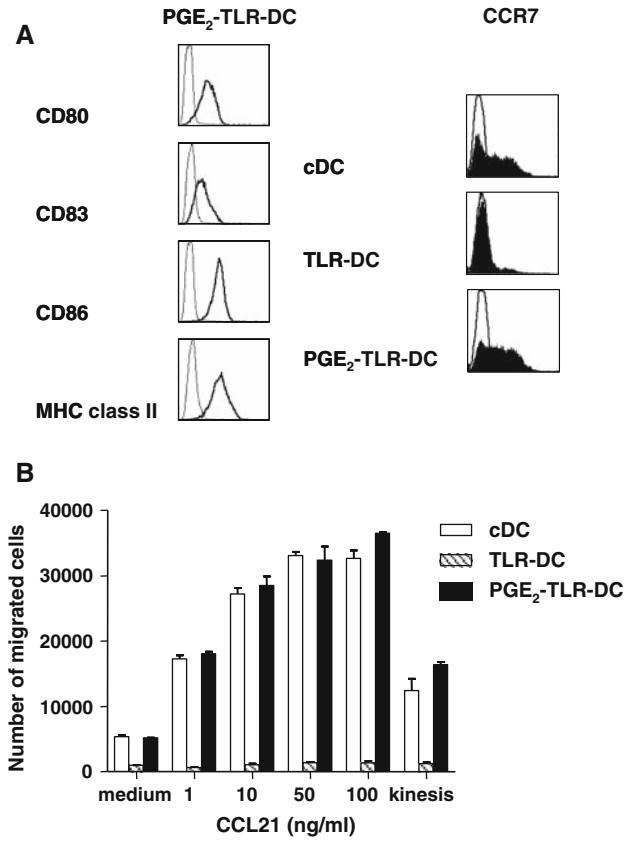


Fig. 3 CCR7 expression and CCR7-mediated chemotaxis of cDC, TLR-DC and PGE_2-TLR-DC. DC from three different donors were matured with the conventional cytokine-cocktail or with poly(I:C), R848 with or without PGE_2. **a** CCR7 expression of the different matured DC and CD80, CD83, CD86 and MHC class II expression of PGE_2-TLR-DC measured by flowcytometry is shown. **b** Chemotaxis was determined by the number of cells that had migrated into the lower compartment of a transwell system containing increasing concentrations of CCL21, counted in 1 minute by flow cytometry. Kinesis was determined by the number of cells that had migrated into the lower compartment when CCL21 is present in both compartments in the same concentration. The graph shown here is representative for three different donors. The *error bars* show the standard deviation

TNFα, IFNα and IFNγ, PGE_2 appears to be critical for dendritic cell migration, resulting in comparable motility and chemotactic capabilities of PGE_2-TLR-DC and cDC. Recently, Lehner et al. [40] showed that DC matured with only Poly(I:C) or R848 are also capable of CCR7-mediated migration, although the number of migrated cells was lower and the cells were strongly adherent. We show here that adding PGE_2 to the combination of Poly(I:C) and R848 generated mature DC that do not only show CCR7-mediated migration but are also less adherent and highly migratory.

PGE_2 does not inhibit secondary IL-12p70 in TLR-ligand matured DC

In clinical vaccination studies, DC should produce IL-12p70 after migration to the lymph nodes and upon subsequent T cell contact [40, 41]. At this time point, local and probably low levels of IL-12p70 in the contact area between DC and T cells may suffice to direct naive T cells towards Th1. Moreover, low levels of IL-12p70 will prevent aspecific activation of T cells in the microenvironment of the DC. Importantly, IL-12p70 production was not detectable when mature TLR-DC and PGE_2-TLR-DC were cultured for another 24 h in fresh medium without stimuli (Fig. 4). To test the ability to produce IL-12p70 upon T cell encounter, we cultured differently matured DC in the presence of allogeneic peripheral blood lymphocytes (PBL). cDC demonstrated a very low IL-12p70 producing capacity as shown previously by Mailliard et al. [22], whereas TLR-DC produced IL-12p70 (Fig. 4). Interestingly, the production of IL-12p70 by PGE_2-TLR-DC equaled that of TLR-DC, matured without PGE_2.

This increased production of IL-12p70 after T cell stimulation might be explained by a feedback loop: DC–T cell interaction via CD40–CD40 L triggers the DC to secrete IL-12p70, which activates and attracts T cells but also NK cells to produce IFNγ [42], which can in return stimulate the DC to produce more IL-12p70. To test the hypothesis that production of IL-12p70 upon T cell encounter depends on CD40-ligation, differently matured DC were stimulated for 24 h with CD40L-trimers. Indeed, stimulation of PGE_2-TLR-DC with CD40L-trimers increased the production of IL-12p70 (Fig. 4). Thus, upon either PBL or CD40L stimulation, PGE_2-TLR-DC and TLR-DC produce similar levels

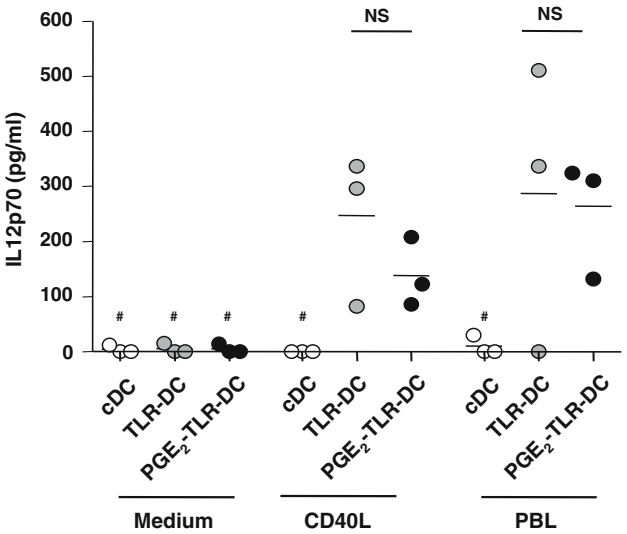
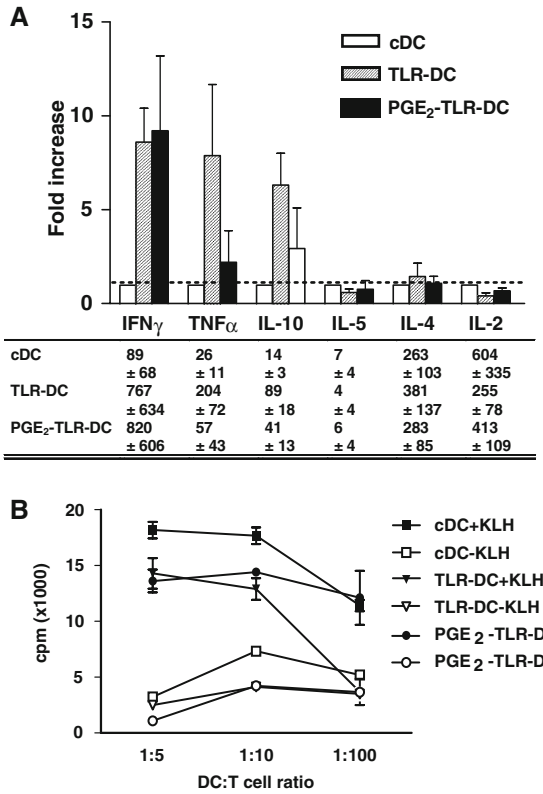


Fig. 4 IL-12p70 production upon secondary stimulation. DC were matured with the conventional cytokine cocktail (TNFα, IL-6, IL-1β and PGE_2), or TLR ligands (poly(I:C)/R848) or TLR ligands in combination with PGE_2 for 48 h and then cultured for another 24 h with or without PBL or CD40L-trimers. IL-12p70 production was measured in the supernatant by ELISA. Per condition, each *symbol* represents the mean for each donor. Means are shown for each maturation cocktail (*open symbols* cDC, *gray symbols* TLR-DC, *black symbols* PGE_2-TLR-DC, *NS* non significant)



	IFNγ	TNFα	IL-10	IL-5	IL-4	IL-2
cDC	89 ± 68	26 ± 11	14 ± 3	7 ± 4	263 ± 103	604 ± 335
TLR-DC	767 ± 634	204 ± 72	89 ± 18	4 ± 4	381 ± 137	255 ± 78
PGE_2-TLR-DC	820 ± 606	57 ± 43	41 ± 13	6 ± 4	283 ± 85	413 ± 109

Fig. 5 Cytokine production of T cells in contact with cDC, TLR-DC and PGE_2-TLR-DC. **a** The profile of cytokines secreted by PBL upon contact with cDC, TLR-DC and PGE_2-TLR-DC was measured by cytokine bead array. The graph shows the fold change in the cytokine production of TLR-DC and PGE_2-TLR-DC relative to cDC of five different donors. The table presents the mean ± SEM concentration (pg/ml) of each cytokine in absolute numbers for all conditions. **b** KLH-specific proliferation of PBL from patients vaccinated with KLH-loaded DC. Four patients were tested, one representative graph is shown. PBL were cocultured with autologous DC matured with the cytokine cocktail, TLR-ligands or TLR-ligands with PGE_2 with or without KLH. Proliferation was measured by incorporation of tritiated thymidine. *Filled symbols* represent DC loaded with KLH. *Open symbols* represent DC without KLH. *Error bars* show the standard deviation within the experiment

of IL-12p70 enabling skewing of the T cell response towards Th1.

To check whether the secondary production of IL-12p70 upon T cell contact indeed induces a Th1 type effector response, we analyzed the supernatants from co-cultures of cDC, TLR-DC and PGE_2-TLR-DC with PBL. It was demonstrated before, that cDC induce expansion of T cells and which subsequently secrete a broad array of cytokines, e.g. IL-2 and IFNγ [13]. Indeed, cDC induced the secretion of low levels of IFNγ by the PBL. Importantly, IFNγ production was approximately nine times higher when PBL were stimulated by TLR-ligand matured DC, both TLR-DC and PGE_2-TLR-DC (Fig. 5a). Secretion of Th2-directive IL-4 and IL-5 was comparable. Although TLR-ligand matured DC stimulated the secretion of IL-10, this secretion was decreased when PGE_2 was present during maturation. Thus, indeed TLR-DC and PGE_2-TLR-DC generated a more pronounced Th1 response. Both cDC, TLR-DC and PGE_2-TLR-DC are capable of stimulating antigen-specific T cells demonstrated by the KLH-specific proliferation of $CD4^+$ T cells isolated from patients that had been vaccinated previously with KLH-loaded DC (Fig. 5b).

Conclusion

Human DC consist of a heterogeneous population of cells that can be classified either according to their phenotype or to their function. It has been postulated that at a certain time point in their maturation, DC enter a crossroad where their fate, migratory-type or cytokine producing-type, is determined. The involved factors, as described above (pro-inflammatory cytokines, PGE_2, TLR ligands, CD40 L) at least demonstrate the possibility to modulate the properties of ex vivo generated DC. In a physiological situation these factors act on this process in the right time, at the right place, resulting in effective immune responses. Since vaccinations in cancer immunotherapy are an artificial situation, the DC used should potentially perform all the tasks needed. Our data show that the combination TLR3 and TLR7/8 ligands together with PGE_2, results in clinical grade mature and stable DC with a high migratory capacity and maintained IL-12p70 producing capacity. These DC fit the desired physiological role of migration to the lymph node and IL-12p70 production upon T cell contact to induce tumor specific IFNγ-producing Th1 cells and CTL. Future studies are needed to demonstrate whether this migratory potential indeed results in more efficient migration of DC into the lymph nodes and improved immune responses in cancer patients.

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