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Functional characterization of a STAT3-dependent dendritic cell-derived CD14⁺ cell population arising upon IL-10-exposed maturation

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Abstract

IL-10 is a major cancer-related immune-suppressive factor with a unique ability to hamper Dendritic Cell (DC) maturation. We previously reported IL-10-induced conversion of activated, migratory CD1a⁺ DC from human skin to CD14⁺BDCA3/CD141⁺ macrophage-like cells. Here, as a model of tumor-conditioned DC maturation, we functionally assessed CD14⁻ and CD14⁺ DC that developed in vitro during IL-10-exposed maturation. IL-10-induced CD14⁺ DC were phenotypically characterized by a low maturation state and high levels of BDCA3 and DC-SIGN and as such closely resembled CD14⁺ cells infiltrating melanoma metastases. Compared to normally matured DC, CD14⁺ DC were found to express high surface levels of B7-H1, to secrete lower levels of IL-12p70, to more preferentially induce Th2 cells, to have a lower allogeneic Th cell and tumor antigen-specific CD8⁺ T cell priming capacity, and to induce proliferative T cell anergy. In contrast to their CD14⁺ counterparts, CD14⁻ monocyte-derived DC retained allogeneic Th priming capacity but induced a functionally anergic state, completely abolishing the release of effector cytokines. Transcriptional and cytokine release profiling further indicated a more profound angiogenic and pro-invasive signature of the CD14⁺ DC than normally matured DC or CD14⁻ DC matured in the presence of IL-10. Importantly, STAT3 mRNA interference prevented development of the IL-10-induced CD14⁺ phenotype, ensuring normal DC maturation, and providing a potential means of therapeutic intervention.
Introduction

Dendritic cells (DC) are professional antigen presenting cells (APC) that are unique in their ability to initiate and maintain primary immune responses. Immature DC (iDC) can efficiently capture and process antigens and upon activation migrate to the draining lymph node where they can present the processed antigens to naïve T cells, thereby inducing primary T and B cell responses. Since tumors frequently produce mutated proteins that can serve as targetable tumor-associated antigens (TAA) 1. DC should be capable of raising an immune response directed to these TAA, which could lead to eradication of tumor cells. Unfortunately, under tumor conditions hampered DC differentiation and activation has been reported, leading to an accumulation of iDC 2. Even in the presence of appropriate danger signals, DC often do not function efficiently in tumor patients due to this pronounced immune suppression that is mediated by high levels of tumor-derived soluble factors, resulting in the induction of T cell tolerance or anergy rather than T cell activation 2. In addition, tumor-derived factors can promote the generation of myeloid-derived suppressor cells (MDSC), a heterogeneous population of myeloid cells at early stages of differentiation. These MDSC can suppress T cell proliferation in vitro and can induce development of regulatory T cells in vivo 3.

IL-10 is secreted at high levels by tumors of many different origins 4,5 and is preferentially found in progressive and metastatic melanoma lesions 6. It is a potent regulator of DC maturation and DC effector function since IL-10 can down-regulate the expression of co-stimulatory molecules and the secretion of pro-inflammatory cytokines like IL-12 and TNFα 7-10. IL-10 has the unique ability to inhibit DC maturation as well as differentiation 7-9,12. In line with this, increased levels of IL-10 in serum of cancer patients correlate to higher frequencies of circulating immature DC (precursors) 13. Although IL-10 efficiently blocks phenotypic DC maturation and the ability of mature DC (mDC) to prime T cells, IL-10-conditioned mDC may still be able to migrate to lymph nodes, resulting in active T cell tolerance induction 14. Velten et al. described the development of a population of monocyte-like cells during the in vitro maturation of IL-10-exposed monocyte-derived DC (MoDC), which de novo re-expressed CD14 on the cell surface. Interestingly, these CD14+ cells also expressed BDCA3 (CD141/thrombomodulin), which was absent on monocytes and iDC and has since been linked to antigen cross-presentation ability 15-18. Previously, we demonstrated the migration of DC from human skin after intradermal delivery of IL-10 with the same phenotype, i.e. CD14+BDCA3+ 19. These macrophage-like cells displayed a poor T cell stimulatory ability, in agreement with the T cell-inhibitory characteristics of the BDCA3+ MoDC-derived subset described by Velten et al. 18. These data suggest that IL-10 is able to convert DC into macrophage-like cells with poor T cell-stimulatory abilities. This was recently confirmed in a mouse study, showing that fully differentiated DC can adopt a regulatory macrophage-like phenotype and that this commonly occurs under cancer conditions 20. Similarly, ovarian cancer cells were shown to induce a CD14+ subpopulation in maturing MoDC through the release of IL-10 21.

In this study, we show that CD14+BDCA3+ DC constitute a physiologically relevant subset that predominates myeloid infiltrating subsets in melanoma metastases, known to express
high levels of IL-10. Employing MoDC matured in the presence of a cytokine cocktail that included IL-10, we studied in more detail the distinct functional characteristics of this CD14 mDC subset in relation to T cell activation and potential for promotion of tumor growth and spread. In addition, we show that the IL-10-induced development of this immune regulatory subset is STAT3 dependent and can be blocked by RNA interference.

Materials and methods

Monocyte isolation and MoDC generation
Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation (Lymphoprep; Nycomed AS, Oslo, Norway) from buffy coats obtained from healthy blood donors (Sanquin, Amsterdam, The Netherlands). Monocytes were isolated from PBMCs using anti-CD14 magnetic microbeads according to the manufacturer’s instructions (Miltenyi Biotec). CD14+ cells were cultured at a density of 4.0x10⁵ cells/ml for 7 days in Iscove’s Modified Dulbecco’s Medium (IMDM) (Lonza BioWhittaker) supplemented with 10 % fetal bovine serum (FBS; HyClone), 100 IU/ml sodium penicillin (Yamanouchi Pharma), 100 µg/ml streptomycin sulfate (Radiumfarma-Fisiopharma), 2 mM L-glutamine (Invitrogen Life Technologies), and 0.01 mM 2-ME (Merck) supplemented with 100 ng/ml GM-CSF (Berlex) and 10 ng/ml IL-4 (Strathmann Biotec, Hamburg, Germany) and the cells were incubated at 37 °C, 95 % humidity and 5 % CO₂ for 7 days. Subsequently a cytokine maturation cocktail was added to final concentrations of 50 ng/ml TNFα (R&D Systems), 100 ng/ml IL-6 (R&D Systems), 25 ng/ml IL-1β (R&D Systems) and 1 µg/ml PGE2 (Sigma-Aldrich). To obtain IL-10 conditioned mature MoDC (IL-10-mDC) 40 ng/ml IL-10 (Strathmann Biotec) was also added to the maturation cocktail. After 2 days the cells were harvested.

FACS sorting
Two days after the addition of the maturation cocktail in the absence or presence of IL-10, MoDC were harvested and IL-10 mDC were labeled with an anti-CD14 PE-conjugated antibody (BD Biosciences, San Jose, CA), for 30 minutes on ice. Sorting was performed using a FACSARia (BD Biosciences CA, USA). The sorted cells were centrifuged, counted and used in functional experiments.

Tumor dissociation
Cell suspensions prepared from human melanoma metastases were handled within 24 hours after surgical removal from patients as described and after informed consent, in the context of an IRB-approved trial conducted at the VU University medical center in Amsterdam, The Netherlands. In short, viable tumor was minced with a scalpel and dissociated over the course of 1-3 1-hour cycles with 0.02 % DNase and 0.14 % Collagenase type IV in IMDM. The cell suspension was filtered through a 100 µm sterile cell strainer and washed to remove tissue debris prior to FACS analysis.
**Flow Cytometry**

Cells were incubated at 4 °C for 30 minutes in PBS with 0.1 % BSA and 0.01 % NaN₃, in the presence of appropriate dilutions of FITC- or PE-labelled mouse mAbs to CD83 (Coulter Immunotech, Marseilles, France), CD86, HLA-DR, CD1a, CD14, CD16, CD40, CD80 (all from BD Biosciences), B7-H1 (BD PharMingen), DC-specific ICAM-grabbing nonintegrin (DC-SIGN; BD PharMingen), BDCA3 (Miltenyi Biotec) and appropriate IgG isotype controls (BD Biosciences). After washing unbound antibody away, the cells were measured using a FACSCalibur and analyzed with CellQuest software (BD Biosciences). For intracellular staining of DC-SIGN, surface markers CD14, CD1a and CD11c were first stained as described above. Subsequently, cells were fixed and permeabilized using the BD Fix-Perm kit, following manufacturer’s guidelines. Intracellular was performed for 30 minutes at 4 °C in 1x permeabilization buffer. Cells were washed once with 1x permeabilization buffer and once with FACS buffer before analysis.

**Mixed leukocyte reaction (MLR) and Th cytokine release**

To determine the functional capacity of the different DC subsets to induce allogeneic T cell proliferation, the sorted stimulator DC were added at titered amounts to round-bottom 96 well tissue culture plates (Costar) pre-seeded with allogeneic responder peripheral blood lymphocytes (PBL), which were obtained from PBMC after removal of monocytes with CD14 microbeads (Miltenyi). Stimulations were performed in triplicate. Cells were cultured for 5 days in IMDM medium supplemented with 10 % HPS, sodium penicillin, streptomycin sulphate, L-glutamine and 2-ME. During the last 18 hours of culture, [3H]TdR was added (0.4 μCi/well) (Amersham, Aylesbury, U.K), after which the cells were harvested onto fiberglass filters, and [3H]TdR incorporation was determined using a flatbed liquid scintillation counter (Wallac). Alternatively, DC were added as stimulator cells to 10⁵ monocyte-depleted peripheral blood lymphocytes (PBL), labeled with 3 μM 5(6)-Carboxyfluorescein (CFSE, Sigma Aldrich, 21888). Stimulation of PBL was performed in triplicate. At day 6, samples were taken from each well and T cell proliferation was determined by FACS analysis. To determine the levels of T cell cytokines, supernatants were taken at day 6 and analyzed by flow cytometry using the Th1/Th2/Th17 CBA kit (BD, 560484).

**Th cell differentiation**

4x10⁴ cells MoDC were incubated with human anti-CD3 antibody (0.5 μg/ml) (OKT-3, eBioscience, San Diego, CA) in 200 μl complete medium (IMDM) for 15 minutes at 4 °C. After incubation, MoDC were co-cultured with 2x10⁴ CD4⁺CD25⁻ T cells (isolated by magnetic bead separation using the untouched CD4 isolation kit and anti-CD25 beads from Miltenyi, Bergisch Gladbach, Germany, according to the manufacturer’s instructions) for 14 days. At day 7, 10 U/ml IL-2 (Strathmann Biotec) was added to the cultures. To determine the cytokines that were released by the T cells co-cultured with the different MoDC subsets, 1x10⁵ cells were subsequently stimulated with 1 μg/ml anti-CD3 (16A9) and 1 μg/ml anti-CD28 (15A9) in 200 μl complete medium (both antibodies kind gifts from Dr René van Lier, Sanquin, Amsterdam, The
The supernatants were harvested the next day and tested for cytokine content with a Th1/Th2/Th17 cytometric bead array (CBA) according to the manufacturer’s instructions (BD Biosciences).

**DC Cytokine release**
The different DC subsets were analyzed for the release of IL-12p70, IL-10, IL-6, IL-1\(\beta\), TNF\(\alpha\), VEGF and IL-8. DC were cultured together with irradiated (5000 rad) mCD40L-transfected J558 cells (a kind gift of Dr. M. Kapsenberg, Amsterdam Medical Center) at a 1:1 ratio in complete medium supplemented with 1000 U/ml IFN\(\gamma\) (R&D Systems). After 24 hours supernatants were collected and tested with an inflammatory cytokine CBA (BD Biosciences).

**CD8\(^+\) T cell priming**
To determine the capacity of the different MoDC subsets to induce antigen specific CD8\(^+\) T cells, MoDC were loaded with 1 \(\mu\)g/ml Mart-1\(_{26-35}\) (ALGIGILTV) peptide in the presence of 3 \(\mu\)g/ml \(\beta\)2-microglobulin (Sigma-Aldrich) for 4-5 hours at room temperature. A total of 1x10\(^5\) peptide-loaded DC were cultured for 10 days together with 1x10\(^6\) CD8\(^+\) T cells and 1x10\(^6\) irradiated (5000 rad) CD8\(^+\)- autologous PBMCs in Yssel medium 23 supplemented with 1 % human AB serum (ICN Biochemicals) in a 24 well tissue-culture plate. After 10 days the T cells were analyzed by flow cytometry using PE-labeled tetramers presenting the relevant epitope, as previously described 24.

**Proliferative T cell anergy assay**
To assess proliferative T cell anergy induction, the different MoDC subsets were cultured with PBL (ratio 1:5) in IMDM supplemented with 10 % HPS for 8 days. After 8 days, the stimulated T cells were harvested, washed with PBS containing 5 % FBS and incubated with 5 \(\mu\)M CFSE (Molecular Probes, Eugene, OR) for 3.5 min at room temperature, and washed twice with PBS+5 % FBS. CFSE labeled T cells were incubated for 1 hour on ice with 2 \(\mu\)g anti-CD3 and 0.4 \(\mu\)g anti-CD28 per 1x10\(^6\) cells. After incubation and washing, the cells were placed in 24-well plates, coated with affinity-purified goat anti-mouse immunoglobulin (1:100; Dako, Glostrup, Denmark) in complete medium with 10 % HPS at a concentration of 1x10\(^5\) cells/mL/well for 1 hour at 4 \(^\circ\)C. The cells were subsequently cultured for 5 days in a humidified 5 % CO\(_2\) incubator at 37 \(^\circ\)C. After 3, 4 and 5 days of culture, T cell division was assessed by flow cytometry.

**RNA isolation and cDNA synthesis**
Total RNA was isolated from MoDC using the RNeasy kit (QIAgen, Venlo, the Netherlands) according to the supplier’s protocol. All isolations were subjected to on-column DNase treatment (QIAgen) to remove any genomic DNA contaminations. The concentration and purity of the RNA was analyzed using the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE). cDNA synthesis was performed with the iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands) on 1 mg of RNA according to the supplier’s protocol. After cDNA synthesis, nuclease-free water was added up to a final volume of 50 \(\mu\)l.
Primer design
Primer design were designed and validated as described previously. Primers were targeted against IDO, IL-4R-a, IL-6R, TGFβ, STAT3, VEGF-A, HIF1α, MMP3 and MMP9 (See Supplementary Table 1). The primers specifically target human sequences and were synthesized by Eurogentec.

Real-time PCR
Quantitative Real-time PCR (qRT-PCR) was performed essentially as described on the CFX96 (BioRad) using the iQ SYBR Green PCR master mix (BioRad). Each PCR reaction was performed in a 25 µL volume containing 30 ng cDNA, 12.5 µL 2x iQ SYBR Green PCR master mix and 1 µL of primer mix (10 µM forward primer, 10 µM reverse primer). The PCR profile was as follows: 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 30 seconds at 60 °C. Subsequently, a melting curve analysis was performed which consisted of 70 cycles of 10 seconds with a temperature increment of 0.5 °C/cycle starting at 60 °C. The obtained Ct value of each gene of interest was normalized to the Ct of the reference genes as follows: Ctnorm = Ctgoi - Ctreff with Ctreff = (CtbACT x CtbCycloA x Ctb2MG x CtbHPRT)^4(1/4) with norm = normalized, goi = gene of interest, and ref = reference gene. Data are shown as mean values ± SEM.

siRNA transfection of MoDC
Immature MoDC were resuspended in antibiotic free IMDM-10 % FCS supplemented with 1000 U/ml GM-CSF and 10 ng/ml IL-4) at 1x10^6/ml, then plated out at 1x10^5 cells per well in 96 well round bottom plates (Greiner: 650180) and transfected with 25 nM Dharmacon ON-TARGETplus SMARTpool RNAi for 48 hours at 37 °C, 5 % CO₂. The transfected siRNA conditions were CD83 (L-012680-00-0005), BDCA-3 (L-012551-00-0005), STAT-3 (L-003544-00-0005), non-targeting si RNA (D-001810-10-05) si Glo tranfection indicator (D-001630-01-05), and included a mock (non transfected) control. After 48 hours, transfection medium was replaced with complete medium supplemented with 1000 U/ml GM-CSF, 10 ng/ml IL-4 and a DC maturation-inducing cytokine cocktail (2400 U/ml TNF alpha, 100 ng/ml IL-6, 25 ng/ml IL-1 beta, and 1 ug/ml PGE2), with or without 40 ng/ml IL-10.

Western blot analysis
siRNA-transfected mDC were resuspended in lysis buffer containing 100 µl phosphatase inhibitor cocktail 1, 100 µl phosphatase inhibitor cocktail 2 (both Sigma-Aldrich, Steinheim, Germany) and 1 Complete Mini Protease inhibitor tablet (Roche Diagnostics, Indianapolis, IN, USA) in TBS-NP40 (1 %). The total protein content of the cell lysates was quantified in duplicate with the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Samples containing 15 µg protein were denatured before loading on the gel and were separated by SDS-PAGE, after which they were transferred to a poly vinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA) by electrophoresis. Membranes were blocked with milk powder and probed overnight with an anti-STAT3 antibody (Cell Signaling Technology Inc., Danvers, MA, USA). After washing, the membranes were incubated with horseradish peroxidase-conjugated
antibody (Dako, Glostrup, Denmark) for 1 hour at room temperature. Immunoreactive bands were visualized on film using the enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Buckinghamshire, UK). As loading control, membranes were also probed with a monoclonal anti-β-actin antibody (Sigma-Aldrich, Steinheim, Germany).

**Statistics**

DC subset frequencies, cytokine release levels, transcript levels, and specific T cell frequencies were compared between conditions using either the (paired) T-test or Wilcoxon Signed Rank test or the (repeated measures) one-way ANOVA with post hoc Tukey multiple comparison analysis or the Friedman test with post hoc multiple comparison Dunn’s test. Prism 4.0 statistical software (GraphPad Software Inc., La Jolla, CA) was used. Differences and correlations were considered significant when p<0.05 in two-sided analyses.

**Results**

**Immature CD14+ APC massively outnumber CD1a+ DC in melanoma metastases and co-express BDCA3 and DC-SIGN**

We previously showed that in situ conditioning of human skin by IL-10 led to a post-migrational trans-differentiation of mature CD1a+CD83+ DC with intermediate CD11c levels to immature CD14+ macrophage-like cells characterized by high levels of CD11c and BDCA3/CD141 as well as intracellular DC-SIGN (Lindenberg et al. submitted). As metastatic melanomas were previously shown to release high levels of IL-10 that could interfere with CD1 expression on developing DC 26, we assessed the balance between CD1a+ DC and CD14+BDCA3+ APC by 4-colour flowcytometric analysis in single-cell suspensions of melanoma metastases (n=6). As shown by representative results in Fig. 1A, CD1a+ DC were virtually absent whereas CD14+ cells were readily detectable. Moreover, these CD14+ cells expressed high surface levels of CD11c and BDCA3/CD141 and the majority also expressed intracellular DC-SIGN (Fig. 1A-B). Frequencies of metastasis-associated CD14+ myeloid cells averaged 8.78 % in a range of 1.54-29.5 %, which is consistent with recently reported frequencies by Gros et al. 27, and significantly outnumbered CD1a+ DC (0.05 %, range 0.005-0.1 %), see Fig. 1C. Beside the CD14+ APC, CD11c− myeloid cells that expressed neither CD1a nor CD14 were detectable at relatively low frequencies (in a range of 0.07-3.30 %, see Fig. 1C). Further phenotypic characterization showed both CD11c−CD14+ and CD14+ APC to be immature with high HLA-DR expression, but generally low levels of co-stimulatory markers; a notable difference between the subsets was the (low-level) expression of the co-inhibitory molecule B7-H1 by the CD14+ APC (Fig. 1D). These data are thus consistent with disturbed local DC maturation in metastatic melanoma leading to a rise in immature CD14+ APC with a distinct CD11c−BDCA3+DC-SIGN+ phenotype in the tumor microenvironment with as yet unclear functional ramifications.
Addition of IL-10 during MoDC maturation results in de novo development of a CD14⁺BDCA3⁺ subpopulation with a more tolerogenic macrophage-like phenotype

As comparative functional analyses of DC subsets from clinical samples are complicated by low cell numbers, we turned to an in vitro culture model to assess and compare the functionality of CD14⁺ and CD14⁻ cells arising during IL-10-conditioned DC maturation. Velten et al. previously reported the trans-differentiation of CD14⁺BDCA3⁺ cells from CD1a⁺ MoDC during IL-10 exposed DC maturation ¹⁸. As shown in Fig. 2A we made the same observation of de novo
CD14 expression on fully differentiated MoDC upon maturation induction (by an inflammatory cytokine cocktail of IL-6, TNFα, IL-1β and PGE2) in the presence of IL-10 (hereafter referred to as CD14+ IL-10-mDC). This CD14+ population also expressed high levels of BDCA3, lacked CD83 expression (Fig. 2A) and adopted a macrophage-like morphology (Fig. 2B), all of which contrasted with CD14+ IL-10-mDC, but was highly similar to the phenotype and morphology of skin-emigrated CD14+ APC that we previously showed to trans-differentiate from CD1a+ DC (see SFig.1). The percentage of post-maturation CD14+ IL-10-MoDC varied considerably between donors (as exemplified for two donors in Fig. 2A): in eight independent experiments CD14+ IL-10-mDC frequencies ranged from 3 to 95 % (with a mean of 35.9 %) which was significantly higher than the percentage CD14+ MoDC detected after normal maturation induction (1.5 %, range: 0-5 %, p=0.002 by Wilcoxon Signed Rank test, see Fig. 2C). Compared to control mDC and CD14+ IL-10-mDC, the CD14+ IL-10-mDC population expressed lower levels of the co-stimulatory molecules CD80, CD83, CD86, whereas expression levels of the inhibitory marker B7-H1 (PD-1L) were elevated (Fig. 2D). Similarly, surface levels of CD16 and DC-SIGN, both previously related to tumor-conditioned DC and macrophages, were specifically elevated on CD14+ IL-10-mDC. Again, absence of CD83 and CD80, combined with expression of B7-H1 and DC-SIGN, is consistent with the phenotype observed for CD14+ APC, emigrated from IL-10 conditioned skin explants, and confirms the validity of the employed in vitro trans-differentiation model of IL-10 exposed MoDC maturation. In addition, their immature state combined with BDCA3 and B7-H1 expression is consistent with the phenotype observed for the predominant CD14+ myeloid subset in melanoma metastases (Fig. 1).

**CD14+ IL-10-mDC have a reduced T cell stimulatory capacity and display tolerogenic traits**

In keeping with their immature phenotype, FACS-sorted CD14+ IL-10-mDC proved to be poor activators of naïve allogeneic Th cells (Fig. 3A). While CD14- IL-10-DC were more proficient in this respect, they were still inferior in comparison to normally matured MoDC (Fig. 3A). To assess the ability of IL-10-mDC to induce proliferative anergy, allogeneic T cells, stimulated by the differentially matured and sorted MoDC populations, were harvested at day 8 of co-culture, labeled with CFSE and re-stimulated with anti-CD3 and anti-CD28. Proliferation by CFSE dilution was determined after 3, 4, and 5 days. As shown in Fig. 3B, T cells primed by control mDC or CD14- IL-10-mDC displayed equivalent proliferation rates whereas CD14+ IL-10-mDC lagged behind with on average a 4-fold reduction in T cell proliferation levels, pointing to proliferative T cell anergy.

The poor ability of CD14+ IL-10-mDC to prime Th cells was also demonstrated upon anti-CD3/OKT-3 loading and co-culture with isolated CD4+CD25+ T cells, resulting in considerably lower expansion factors than those observed for control mDC or CD14+ IL-10-mDC (see Fig.3C). Next, release by the mDC populations of cytokines known to support and skew T cell responses were assessed upon CD40 ligation. Both CD14+ and CD14- IL-10-mDC released significantly reduced levels of IL-6 (Fig. 3D) and IL-12p70 (Fig. 3E) as compared to control mDC, the latter resulting in significantly lower IL12:IL-10 ratios (Fig. 3E). After a 14-day culture, the actual
Functional of IL-10/STAT3-dependent CD14+ DC cytokine release profile of Th cells induced by the anti-CD3 pulsed mDC populations was determined. As shown in Fig. 3F, control mDC preferentially induced Th1 cells which released IFNγ and TNFα and low levels of IL-17 and IL-6, whereas CD14+ IL-10-mDC also induced Th2 cells which released IL-4 and relatively high levels of the potentially immune suppressive...
cytokines IL-6 and IL-10. Remarkably, CD14+ IL-10-mDC failed to induce any cytokine release by the primed Th cells. Coupled to their ability to induce normal levels of Th cell expansion, this is a clear indication of selective and profound functional anergy induction.
To evaluate the antigen-specific CD8+ T cell priming ability of the different MoDC populations, they were loaded with peptides covering the immunodominant HLA-A2-binding epitope MART-126–35L derived from the melanoma antigen Melan-A/MART-1 and added to autologous CD8β+ T cell precursors together with irradiated CD8β- autologous peripheral blood mononuclear cells (PBMC). After a 10-day priming culture the frequency of MART-126–35L specific CD8+ T cells in the cultures was determined by tetramer (Tm) binding, examples of which are shown in Fig. 4A. Results from 5 independent priming experiments (average percentages of primed Tm+ CD8+ T cells shown in Fig. 4B) clearly demonstrated superior priming efficiency of normally matured MoDC and lowest frequencies of Tm+ T cells primed by CD14+ IL-10-mDC (p<0.05 vs mDC). Interestingly, Tm fluorescence intensity levels appeared lower on T cells primed by IL-10-mDC. This was confirmed by combined data analysis from the priming cultures, showing these differences in Tm binding levels to be significant (Fig. 4C) and indicative of priming of CD8+ T cells with lower target-binding avidity.

CD14+ IL-10-mDC display more profound angiogenic, pro–invasive and immune suppressive transcriptional profiles

As a further indication of the relative abilities of the CD14+ and CD14+ IL-10-mDC to influence tumor growth and spread in the tumor microenvironment we performed transcriptional profiling to assess expression of certain key genes in relation to angiogenesis, invasion and immune suppression. In Fig. 5A the fold changes of the mRNA expression levels in IL-10-mDC are shown in relation to control mDC. In both IL-10-mDC populations a relative increase in mRNA expression levels was observed for genes related to immune suppression (indoleamine 2,3-dioxygenase [IDO], IL-4R-a, IL-6R, STAT3, TGFβ and vascular endothelial growth factor [VEGF]-A, angiogenesis (STAT3, VEGFA, hypoxia-inducible factor [HIF]-1α, matrix metalloproteinase [MMP]-3, and MMP9), and tumor invasion (TGFβ, MMP3, and MMP9). Interestingly, CD14+ IL-10-mDC showed particular over-expression of TGFβ, HIF1α, MMP3, and MMP9, indicative of a specific ability of this subset to induce endothelial migration and tumor invasion. Elevated levels of TNFα and IL-8 release by CD14+ IL-10-mDC (Fig. 5B) further corroborate this, as pro-angiogenic and pro-invasive effects have been attributed to
both these cytokines.

![Graph showing CD8+ T cell percentage](image)

**Figure 4.** Induction of MART-1 specific CD8+ T cells by CD14+ and CD14+ IL-10-mDC. Autologous HLA-A2+ MoDC matured with or without IL-10 and sorted by CD14 expression were loaded with the 26-35L MART-1 peptide and co-cultured with MART-1 specific CD8β+ T cells in multiple (n=6) parallel cultures per condition per donor. A) Representative examples of MART-126-35L HLA-A2 tetramer binding by CD8+ T cells after a single 10-day *in vitro* stimulation for the different mDC cultures. B) Mean 10-day priming efficiencies (in % HLA-A2+ tetramer-bound T cells) from 5 healthy donors, *p<0.05. C) Tetramer binding levels (by mean fluorescence intensity) from tetramer positive priming cultures, *p<0.05.

**STAT3 mRNA interference blocks the development of CD14+ IL-10-mDC**

The JAK2/STAT3 pathway has previously been implicated in tumor-related DC suppression. To ascertain its possible involvement in the development of CD14+ IL-10-mDC, MoDC were transfected with STAT3 siRNA prior to maturation induction in the presence of IL-10. Non-targeted and CD83 siRNAs served as negative and positive controls, respectively. As shown in Fig. 6A, STAT3 mRNA interference resulted in an effective block of CD14+ DC development and facilitated up-regulation of CD80 and CD83 comparable to levels observed for normally
Figure 5. CD14+ IL-10-mDC display an angiogenic, pro-invasive and immune suppressive transcriptional profile. A) Relative mRNA expression of the indicated genes in CD14+ (open bars) and CD14- (closed bars) IL-10-mDC (related to mDC, values of which were set at 1) by qRT-PCR. Data are means from 6 experiments ± SEM, *p<0.05. B) Secretion of TNFα and IL-8 by mDC and IL-10-mDC sorted by CD14 expression, after CD40 ligation; means ± SEM from 5 experiments, *p<0.05.
Figure 6. mRNA interference reveals STAT3 dependent development of CD14⁺ IL-10-mDC. A) Effects of STAT3 siRNA transfection on CD14, CD80, and CD83 expression (by flow cytometry) of MoDC, matured with or without IL-10. A) representative results from 5 experiments, mean fluorescence intensities (MFI) and % positive cells are listed, B) mean percentages of positive cells ± SEM of 5 experiments, *p<0.05. Non-targeted and CD83 siRNA served as negative and positive controls respectively. C) Western Blot analysis of STAT3 expression after siRNA transfection in IL-10-mDC.
matured MoDC. In Fig. 6B combined data from five independent knock-down experiments are shown, clearly demonstrating that STAT3 mRNA interference completely abolished the suppressive effects of IL-10 on MoDC maturation. Although only partial STAT3 knock-down was achieved as evidenced by western blot analysis (Fig. 6C), this nevertheless proved sufficient for the prevention of CD14+ IL-10-mDC development. Importantly, STAT3 knock-down also prevented diminished functionality of IL-10-mDC as assessed by their allogeneic T cell priming capacity (Fig. 7A). Moreover, it resulted in maintained Th1 differentiation in the face of IL-10 suppression as evidenced by reduced IL-2 and increased TNFα and IFNγ release levels in the allogeneic T cell priming cultures (Fig. 7B). In addition, maintained IL-6 release upon STAT3 knock-down in these co-cultures (Fig. 7C) is consistent with interference in inhibition by IL-10 of the release of this T cell stimulatory cytokine at the DC level (see Fig. 3D).

**Figure 7** STAT3 mRNA interference maintains allogeneic T cell priming and Th1-inducing ability of IL-10-mDC. A) Mixed leukocyte reactivity after RNA interference for the indicated conditions, means ± SEM of 3 separate experiments are shown, *p<0.05. B) IL-2, TNFα, and IFNγ and C) IL-6 release levels at day 6 after RNA interference for the indicated conditions, n=2.
Discussion

A large number of studies attest to the remarkable plasticity of the myeloid lineage. Tumors abuse this phenotypic plasticity by secretion of locally and systemically acting factors that block normal myeloid differentiation and maturation pathways and re-direct them towards the development of immune suppressive subsets that effectively interfere with anti-tumor immunity through the induction of anergy and regulatory T cells (Tregs). Consequently, tumors are often characterized by an infiltrate of monocyte/macrophage-like cells and a coinciding lack of infiltrating DC, which is generally a poor prognostic indicator. A number of in vitro studies have shown that DC differentiation from monocytes can be blocked by tumor-derived supernatants (TDSN) resulting in the development of CD14+ M2 macrophage-like cells with poor T cell stimulatory abilities, or even MDSC-like cells with T cell suppressive activity. Beside monocytes, fully differentiated DC may also be recruited to the tumor microenvironment where they may lose their characteristic CD1 expression through the suppressive action of IL-10, as shown for melanoma metastases. We previously demonstrated an IL-10 dependent phenotypic shift of mature CD1a+ DC to CD14+ macrophage-like cells over the course of their migration from human skin explants. These CD14+ macrophage-like DC displayed poor T cell stimulatory properties and were characterized by expression of BDCA3/CD14. These observations have since been confirmed by Chu et al. who have additionally shown that these dermis-derived CD14+BDCA3+ DC induce inflammation-attenuating Tregs. Velten et al. reported conversion of CD1a+ DC to a CD14+BDCA3+ subset with immune regulatory properties during IL-10 conditioned MoDC maturation. This in vitro model enabled us to sort sufficient numbers of CD14+BDCA3+ IL-10-mDC to perform comparative transcriptional and functional studies in relation to CD14+ IL-10-mDC and normally matured mDC.

Previous studies had shown that addition of IL-10 during MoDC differentiation (prior to maturation, IL-10-iDC) resulted in high levels of CD14, DC-SIGN, CD68 and CD16. These macrophage-like cells induced Th2 cells by hampered IL-12p70 secretion. We found similar traits for CD14+ mDC arising during inflammatory cytokine-induced MoDC maturation in the presence of IL-10. In fact, the only apparent distinguishing features between CD14+ IL-10-mDC and IL-10-iDC were higher expression levels of the macrophage marker CD16 and the co-inhibitory receptor B7-H1 (PD-1L) on CD14+ IL-10-mDC; in all other respects, phenotypically, morphologically and functionally, IL-10-iDC proved very similar to CD14+ IL-10-mDC (see SFig. 2). In this regard, the finding of B7-H1 expression on CD14+ APC associated with melanoma metastases indicates that they may at least in part represent IL-10-mDC (Fig. 1D). In contrast, CD14+ IL-10-mDC differed considerably from CD14+ IL-10-mDC, with lower levels of CD83 and co-stimulatory markers, higher levels of BDCA3, CD16, DC-SIGN and B7-H1 and a typical macrophage-like morphology, whereas CD14+ IL-10-mDC were phenotypically and morphologically more reminiscent of control mDC. Compared to control mDC, both CD14+ and CD14+ IL-10-mDC secreted reduced levels of IL-6 and IL-12p70, a
feature shared with M2 macrophages. Altogether these features resulted in a more profound inability of CD14⁺ IL-10-mDC for both CD4⁺ and CD8⁺ T cell induction. In addition, CD14⁺ IL-10-mDC induced a measure of proliferative anergy in allogeneic T cells whereas CD14⁺ IL-10-mDC did not. On the other hand, CD14⁺ IL-10-mDC did not support any Th cell differentiation, inducing neither Th1 nor Th2 responses, whereas CD14⁺ IL-10-mDC induced a balanced Th1/Th2 response. This might be related to particularly low CD40 expression by the CD14⁺ IL-10-mDC (see Fig. 2). Both CD14⁺ and CD14⁻ subsets contrasted with control mDC in this respect, which primarily induced the release of Th1 cytokines, like IFN-γ and TNF-α. The relatively poor priming efficiency displayed by both CD14⁺ and CD14⁻ IL-10-mDC of MART-1\_26–35-specific CD8⁺ T cells with an apparently low avidity indicates that both subsets may be deficient in terms of supporting effective anti-tumor immunity. This is further supported by the increased expression by both subsets of transcripts like IDO, STAT3, TGFβ, VEGFA, IL-4Ra, and IL-6R, all of which have been related to immune-suppressive myeloid subsets. Of note, despite these suppressive features and their apparent anergy-inducing qualities, we were unable to unequivocally demonstrate either Treg induction or a direct suppression of T cell proliferation by either subset (data not shown). This is in keeping with a recent report by Gros et al. who showed that CD14⁺ cells infiltrating melanoma metastases did not suppress T cell proliferation nor showed a positive correlation to frequencies of infiltrating Tregs.

Beside a more profound inability to support T cell induction and expansion, CD14⁺ IL-10-mDC also displayed a transcriptional profile suggestive of the ability to induce blood vessel growth and tumor cell invasion; again features shared with tumor-conditioned M2 macrophages. Tumor-induced activation of a HIF1α transcriptional signature has been reported in macrophages, even under normoxic conditions. This is consistent with the transcriptional and cytokine profile observed for CD14⁺ IL-10-mDC, which revealed coordinated expression of HIF1α, TGFβ, VEGFA, MMP3, MMP9, IL-8 and TNFα, all of which can contribute to tumor-promoting processes such as endothelial cell migration and proliferation and tumor growth and invasion. These observations warrant further functional validation and investigation using both in vitro generated and primary tumor-derived CD14⁺BDCA3⁺ myeloid cells.

In our hands BDCA3 was also up-regulated on mDC that were matured by an inflammatory cytokine cocktail per se consisting of TNFα, IL-6, PGE2, and IL-1β, all of which are often present in tumor microenvironments and can activate tumor-conditioned DC, macrophages, and MDSC3. Although BDCA3 was expressed at higher levels in CD14⁺ IL-10-mDC, caution is clearly warranted in the sole use of BDCA3 to identify tumor-suppressed myeloid cells. Rather, combined expression of CD14, BDCA3, DC-SIGN, CD163, and CD16 appears to provide a phenotypic profile useful for the identification of macrophage-like subsets that arise during tumor-conditioned myeloid differentiation or maturation. We and others have found evidence of such subsets in breast, colon, head and neck, and melanoma tumors. A phenotypically distinct BDCA3⁺ human DC subset in blood and spleen has been found to efficiently cross-present soluble or cell-associated antigens to CD8⁺ T cells. As yet, the functional significance of BDCA3 in either cross-presenting or tumor-suppressed DC subsets remains unclear; however, some clues are emerging. BDCA3 or thrombomodulin is known as an anti-
coagulant factor that binds and neutralizes thrombin and is expressed on the membrane of endothelial cells from which it can also be shed; it consists of an extracellular Lectin-like domain and a short cytoplasmic tail which upon binding of thrombin activates a PI3K/NOS-3/Src kinase signalling axis. The Lectin-like domain can down-regulate NF-κB and mitogen-activated protein kinase (MAPK) pathways and thus interfere with endothelial cell activation. If BDCA3/thrombomodulin would mediate similar signalling effects in DC, this might interfere with DC maturation and drive IL-10 release and Th2 skewing. In support of this, BDCA3 levels on the mDC-2 subset in blood were shown to be upregulated upon allergen exposure and to promote Th2 skewing. In addition, in vitro generated or skin-derived CD14+BDCA3+ DC released elevated levels of IL-10. Of note, we did succeed in achieving siRNA-mediated knock-down of BDCA3 expression but did not observe any changes in the IL-10-induced CD14 expression during MoDC maturation (data not shown).

Importantly, STAT3 mRNA interference afforded complete protection from the suppressive effects of IL-10 exposure during DC maturation and ensured acquisition of a mature T cell-stimulatory DC phenotype with high levels of CD80, CD83, and CD86, while preventing the development of the CD14+ IL-10-mDC population. Accordingly, the allogeneic T cell priming and Th1-inducing ability of IL-10-exposed mDC was preserved. This is in accordance with previous reports that STAT3 inhibition or knock-down conferred resistance against the suppressive effects of tumor-associated factors like IL-6 and IL-10, prevented upregulation of B7-H1 (as observed on CD14+ IL-10-mDC), and preserved the Th1-skewing propensity of mDC.

In conclusion, IL-10 exposure of DC during their maturation may result in a STAT3-dependent acquisition of an immature CD14+BDCA3+ macrophage-like phenotype with immune suppressive and poor T cell-stimulatory traits as well as the potential to promote endothelial migration and tumor invasion. The development of such tumor-promoting cells within the tumor microenvironment may be therapeutically targeted by cytokine or STAT3 signalling interference.
SFigure 1. Phenotype and morphology of DC migrated from healthy donor skin explants, after intradermal delivery of IL-10, over the course of two days of culture. Cells were gated on characteristic high Forward and Side Scatter properties prior to marker analysis. Microphotograph shows typical stretched macrophage-like morphology (400x magnification). Healthy human skin specimens were obtained after informed consent and signing of a "non-objection statement" at the time of hospital admission from patients undergoing corrective breast or abdominal plastic surgery at the VU University medical center (Amsterdam, The Netherlands) in accordance with the “Code for Proper Use of Human Tissues” as formulated by the Dutch Federation of Medical Scientific Organizations (www.fmwv.nl). 50 ng IL-10 was injected intradermally in a total volume of 20 µl serum-free medium whereafter 6 mm punch biopsies were taken. The biopsies were transferred to a 48-well plate containing 1 ml IMDM supplemented with 5 % human pooled serum (HPS) (Sanquin), sodium penicillin, streptomycin sulfate, L-glutamine, and 2-ME. DC were allowed to migrate from the biopsies for 2 days and were subsequently harvested and phenotypically characterized by FACS analysis.

Supplementary Table 1 Employed qRT-PCR primers

| Target | Forward primer (5'-3') | Reverse primer (5'-3') |
|--------|------------------------|-----------------------|
| IDO    | TATTCAAGGCAATGCAAATG   | TGGGTTCACATGATCGTGG   |
| IL4Rα  | GTTCCTGGACCTGCTCGG     | AGGGCATGTCAGCACTCG    |
| IL6R   | TTCCCAGGAGTCCAGAAG     | GCCGAGATGCAGTCTACCAT  |
| STAT3  | CGGCGGCTCAGCTACTACT    | GCTGCGGTGTTGAGTTTCT   |
| TGFβ   | TCCTGGCGATACCTCAGC     | GAGGAGTGGCGCTAAAGG    |
| HIF1α  | CGTTCCCTTGAGCTGGTTGTC  | TCAGTGGTGCCAGTGGTATTG |
| MMP3   | TGGATGCAGCATAATGAG     | CAGAAATGGCTGCATCGA    |
| MMP9   | TACTGTCCTTGGAGTACG     | TTTCTCGGGGATAGGAAG    |
| VEGF A | AAGGAGGAGGGCCAGAATCAT  | CCAGGCCCTCGTCATTG     |
SFigure 2. CD14+ IL-10 mDC are phenotypically, morphologically and functionally similar to immature MoDC differentiated in the presence of IL-10 (IL-10-iDC). A) IL-10-iDC are CD14+BDCA3+ and have a macrophage-like morphology (400x magnification). B) Phenotypic (FACS) profile of IL-10-iDC vs CD14+ IL-10-mDC. Note differences in CD16, and most notably, B7-H1 levels. C) Mixed leukocyte reactivity instigated by IL-10-iDC vs CD14+ IL-10-mDC, means with SEM are shown from 3 experiments. D) Proliferative anergy induction by both IL-10-iDC and CD14+ IL-10-mDC. After 8 days of allo-stimulation of T cells with mDC, IL-10-iDC or CD14+ IL-10-mDC, they were pulsed with CFSE and re-stimulated by anti-CD3 and -CD28 mAbs; T cell proliferation was followed over time by CFSE dilution. The dotted line indicates CFSE levels of non-stimulated T cells. Data from one representative experiment out of three are shown.
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