Induction of dendritic cell maturation in the skin microenvironment by soluble factors derived from colon carcinoma

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Abstract

Autologous tumor cell-based vaccines provide a wide range of tumor antigens and personalized neo-epitopes based on individual tumors unique antigenic mutanome signatures. However, tumor-derived factors may hamper *in situ* maturation of dendritic cells (DC) and thus interfere with the generation of effective anti-tumor immunity. As the skin is a preferred site for tumor vaccine delivery, we investigated the influence of primary colon carcinoma-derived soluble factors on the maturation state of migrating DC in a human skin explant model. Primary tumor-derived supernatants (TDSN) enhanced the phenotypic maturation state of skin-emigrated DC, resulting in an increased T cell stimulatory ability in an allogeneic mixed leukocyte response. In case of monocyte-derived DC a similar TDSN-induced maturation induction was found to entirely depend on cyclooxygenase (COX)-regulated prostaglandins. In contrast, the increase in skin-emigrated DC maturation was completely prostaglandin-independent, as evidenced by the inability of the COX inhibitor indomethacin to abrogate this TDSN-induced effect. Although TDSN conditioning effected a drop in IL-12p70 release by the skin-emigrated DC and induced a predominant Th17/Th22 transcriptional profile in subsequently stimulated T cells, Th cell subset differentiation, as assessed by intracellular cytokine expression upon polyclonal priming and re-stimulation, was not affected. Comparative analysis of phenotypic and transcriptional profiles suggests that the observed maturational effects in skin-derived DC may have been induced by tumor-derived GM-CSF. In conclusion, soluble factors derived from whole-cell colon tumor vaccines will not negatively impact DC migration and maturation in human skin, but rather induce DC maturation that will facilitate the priming of a poly-functional Th cell response.
Introduction

We have previously reported on the clinical efficacy of intradermally (i.d.) administered autologous whole-cell tumor vaccines for the treatment of colon cancer patients. A moderate, though significant, increase in disease-free survival was observed in a multi-center randomized Phase-III trial for Duke’s B patients. A recently published retrospective analysis of this trial revealed that patients with micro-satellite-stable tumors (MSS) in particular benefited from this vaccination, with long recurrence-free survival, whereas patients with micro-satellite instable tumors (MSI) did not. The latter group however showed prolonged survival regardless of vaccination, most likely due to the intrinsic immunogenicity of MSI tumors. Thus, intradermal vaccination with a live autologous whole-cell tumor vaccine may benefit patients with early-stage MSS tumors. Such autologous tumor-based vaccines may receive renewed interest in this era of next-generation sequencing which has demonstrated the immunogenic potential of neo-epitopes arising from unique mutations in individual tumors. Indeed, Segal and colleagues estimated that in each colon tumor anywhere between 40 and 60 neo-epitopes could be expected to arise with the potential to bind to MHC class-I molecules and to thus provide a target for cytotoxic T lymphocytes (CTL). It nevertheless remains costly and technically challenging to identify driver mutations with immunogenic potential to design targeted, personalized, and effective vaccines for each individual patient. Vaccination approaches based on autologous tumor cells would circumvent this need while encompassing all possibly relevant antigens and immunogenic neo-epitopes.

Whereas patients with Duke’s B MSS colon tumors experienced prolonged recurrence-free survival upon i.d. vaccination with an autologous tumor cell vaccine, no such increase in recurrence-free survival was observed in more advanced stages of the disease, indicating the need for enhanced vaccination efficacy. The pivotal role of dendritic cells (DC) in the initiation and the effector phase of the immune response is under intense investigation. In established tumors the immune response often proves to be ineffective. A number of publications has correlated poor clinical outcome with low numbers and activation state of tumor-associated DC. This has raised interest in DC and their complex interactions with stromal, immune and tumor cells, as well as their role of in the generation of antitumor immunity and the maintenance of immunosurveillance. Tumors utilize various mechanisms to subvert DC functions, e.g. by interfering with migration or proper DC maturation. To this end, tumor cells secrete a plethora of immunosuppressive soluble factors, but they may also induce their release by stromal cells or by infiltrating immune cells. We previously demonstrated the inhibitory effect of supernatants from primary colon tumors on the differentiation of monocyte-derived DC (MoDC) from blood monocytes. Here, we ascertained the effect of these supernatants on the maturation state of already fully differentiated DC, as interference of tumor-derived soluble factors with the activation of DC may seriously hamper tumor vaccination approaches. More specifically, soluble factors derived from intradermally applied autologous tumor-based vaccines may interfere with local DC activation in the skin vaccination site, either directly, or indirectly through conditioning of the dermal stroma. We therefore examined the effect of...
colon tumor-derived soluble factors on cutaneous DC in their natural tissue context, employing single-cell suspensions of primary colon tumors collected and stored for the purpose of vaccine preparation. We made use of a previously described ex vivo human skin explant model, which allowed the study of effects on the maturation and migration of skin-resident DC under near-physiological conditions.

Rather than inhibition, we report an enhanced phenotypic maturation of skin-emigrated DC under the influence of colon tumor-derived soluble factors. While for MoDC a similarly activating effect was found to be entirely prostaglandin-dependent, for skin-emigrated DC it was not. Based on similarities in effects we propose that the observed maturation-inducing effects in skin DC may be related to colon tumor-derived GM-CSF. Encouragingly, our data suggest that soluble factors derived from whole-cell colon tumor vaccines will neither interfere with migration and maturation of DC in human skin, nor with their T cell stimulatory ability.

Materials and methods

Cell lines

The CD40L-transfected J558 plasmacytoma cell line was a kind gift of Dr. M. Kapsenberg, Amsterdam Medical Center, Amsterdam, The Netherlands (J558-CD40L).

Monoclonal antibodies and recombinant cytokines

The following cytokines and reagents were used: Prostaglandin E2 (PGE2) (Sigma-Aldrich) and rhGM-CSF (Berlex Laboratories Inc). The following PE- or FITC-labeled mAbs were used: CD83 (Immunotech), CD80, CD86, CD40, CD54, CD14 and HLA-DR (all from BD Biosciences).

Tumor dissociation

Cell suspensions prepared from human primary tumors (colon carcinoma Duke's B or Duke's C) were handled within 24 hours of surgical removal as described. In short, viable tumor was minced with a scalpel and dissociated for 1 to 3 hours with 0.02 % DNase (Boeringer Mannheim GmbH) and 0.14 % Collagenase type IV (Boeringer Mannheim GmbH) in Hanks' Balanced Salt Solution (HBSS, Lonza). The cell suspension was filtered and washed to remove tissue debris and immediately cryo-preserved using a control rate freezing system and stored in liquid nitrogen. Before use, tumor cell suspensions were rapidly thawed and diluted in HBSS containing 0.02 % DNase. After three washes cell numbers and cell viability was determined by trypan blue exclusion and used to prepare tumor-derived supernatants (TDSN).

Preparation of tumor-derived supernatants

TDSN were prepared from freshly dissociated primary tumors as described. Cells were seeded at a density of 1x10^6 cells/ml in complete medium (IMDM containing 10 % FCS (Hyclone), 50 U/ml penicillin-streptomycin, 1.6 mM L-glutamine, 0.01 mM 2-ME). After 24 hours culture, tumor cells were centrifuged (1500 rpm, (530 g), 5 min), supernatants were run through a 0.22 μm Millex-GP filter (Merck Chemicals), and stored at -80 °C. Prostaglandin synthesis was
Dendritic cell maturation by colon tumor-derived soluble factors blocked through COX inhibition. TDSN were prepared, as described above, through culture of primary tumor cells in the presence or absence of a non-selective COX-1/COX-2 inhibitor, indomethacin (Merck Chemicals) at a concentration of 10 μM. Immune infiltrate was separated from the colon tumor suspensions through indirect panning on the basis of CD45 positivity as described previously. Remaining colon tumor cells were used to prepare supernatant at a concentration of 1x10^6 cells/ml, while isolated CD45^+ tumor-derived leukocytes were cultured at a concentration corresponding with 1x10^6 colon cells/ml to obtain supernatants.

**Generation and maturation of MoDC**

PBMC were obtained from healthy donors by density gradient centrifugation over Lymphoprep (Nycomed AS) and cryo-preserved as previously described. PBMC were thawed and resuspended in culture medium IMDM containing 10 % FCS and allowed to adhere to six-well tissue culture plates (3x10^6 cells/ml) for 2 hours at 37 ºC. Non-adherent cells were removed and adherent cells were cultured in complete medium supplemented with 100 ng/ml GM-CSF (specific activity 1.11x10^6 IU/mg, Schering-Plough) and 1000 U/ml IL-4 (specific activity 10^8 U/mg, Sanquin). Immature MoDC (iMoDC) were collected at day 6 and plated at a density of 0.3x10^6 cells/ml into a 12-well tissue culture plate. Maturation was induced by addition of TDSN 30 % (v/v) or PGE2 at 10 μM for 48 hours in the presence of 1000 U IL-4/ml and 100 ng/ml GM-CSF.

**Skin preparation and explant cultures**

Healthy human skin specimens were obtained after verbal informed consent at the time of hospital admission from patients undergoing corrective breast or abdominal plastic surgery at the VU University medical center (VUmc, Amsterdam, The Netherlands) or at the Tergooi hospital (Hilversum, The Netherlands) and used in an anonymous fashion. Patients that objected to this procedure signed a statement to this effect, in accordance with the "Code for Proper Use of Human Tissues" as formulated by the Dutch Federation of Medical Scientific Organizations (www.fmwv.nl) and following procedures approved by the IRB. Cytokines or TDSN (50 % v/v) were injected into the dermis with a Micro-Fine Insulin syringe (0.33 mm (29G) x 12.7 mm needle, BD Biosciences) at the indicated amounts and in a total volume of 20 μl serum-free medium (following a methodology that we previously described). A sham control was included by injecting skin with 20 μl serum-free medium (indicated as 'medium' in the text and figures). At the site of injection a 5 mm urtica appeared and an exact punch biopsy of 6 mm was taken. The biopsy was lifted from the specimen with a sterile forceps and with sterile scissors the dermis was cut at a depth of 2-3 mm to obtain skin explants. Biopsies were placed in 1 ml culture medium IMDM containing 5 % inactivated and (0.2 μm) filtered Human Pool Serum (HPS, Sanquin), either pure or supplemented with 3 % (v/v) TDSN. After a migration period of 48 hours cells were harvested and tested for phenotype, cytokine production, or T cell stimulatory capacity.
Flowcytometry
Immunophenotypic analysis was performed using FACS. In short, cells were washed in PBS supplemented with 1% BSA and 0.02% NaN₃ (PBA) and incubated for 30 min. at room temperature in the presence of appropriate dilutions of PE- or FITC-conjugated specific mAbs or with the corresponding isotype matched mAb. Excess mAb was removed by washing in PBA. The cells were subsequently analyzed, using FACS Calibur and CellQuest-Pro software (BD Biosciences). Results were expressed either as mean fluorescence or the percentage of positive cells.

Mixed Leukocyte Reaction
5x10⁴ Responder PBMC/well, isolated from buffy coats as described above, were incubated with titrated amounts of allogeneic stimulator DC. Cells were cultured in 96-well round-bottom plates (Costar) in complete medium supplemented with 10% HPS for 5 days at 37 °C and 5% CO₂ in a humidified atmosphere. Cells were pulsed with [3H] Thymidine (0.5 μCi/well (18.5X10³ Bq/well) Amersham) during the last 18 hours of culture. [3H] Thymidine incorporation was measured using a liquid scintillation counter (Wallac). Responses are shown as mean counts per minute (cpm) from triplicate wells.

IL-12p70 release
Mature MoDC or skin-emigrated DC were analyzed for functional IL-12p70 release as described previously. Briefly, 4x10⁴ DC were incubated with 4x10⁴ J558-CD40L cells in the presence of 1000 U rhIFNγ/ml (Sanquin) in 200 μl complete medium. We previously found IL-12p70 release for skin-emigrated DC to be optimal when J558-CD40L cells were added to the skin-emigrated DC at day 7 after the start of explant culture. All skin DC IL-12p70 release data were from this time point. After 24 hours co-culture of day 7 DC and irradiated J558-CD40L cells, the supernatants were collected and stored at -20 °C. IL-12p70 concentrations were determined by capture ELISA as previously described.

Th and Treg differentiation assays
40,000 skin-emigrated DC were incubated with 0.5 μg/ml anti-CD3 (OKT-3, eBioscience) in 200 μl complete medium (i.e. IMDM supplemented with 10% FCS, 100 IU/ml sodium penicillin, 100 μg/ml streptomycin sulfate, 2 mM L-glutamine, and 0.01 mM 2-ME for 15 minutes at 4 °C. After incubation, the DC were co-cultured with 20,000 CD4⁺CD25⁺ T cells (isolated by magnetic bead separation using the untouched CD4 isolation kit and anti-CD25 beads from Miltenyi, according to the manufacturer’s instructions) for 14 days. At day 7, 10 U/ml IL-2 (Strathmann Biotec) was added to the cultures. On day 14, T cells were harvested and analyzed by flowcytometry for CD3, CD4, CD25 and FoxP3 expression as previously described or 50,000-100,000 T cells were stimulated for 4 hours with 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of 0.5 μl/ml brefeldin A (GolgiPlug, BD Biosciences), stained for CD3 and CD4, and for IFNγ, IL-4, IL-17A, or IL-22 (all from BD Biosciences) after permeabilisation using the BD Perm-Fix kit and subsequently analyzed, using a FACS Calibur and Cellquest-Pro FACS analysis software (BD Biosciences).
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Alternatively, Th cells were stimulated with 0.5 μg/ml anti-CD3 (OKT-3) and 0.5 μg/ml anti-CD28 (clone 15E8) overnight. At that time stimulated Th cells were harvested and RNA isolated to also assess transcript levels of Th1-, Th2-, Th17-, Th22-, or Treg-associated transcription factors and cytokines.

**RNA Isolation and cDNA Synthesis**

Total RNA was isolated using the RNeasy Plus Micro kit (Qiagen). Contaminating genomic DNA was removed by using the gDNA Eliminator spin columns from the kit. The concentration and purity of the RNA was analyzed using the NanoDrop ND-1000 (Thermo Scientific). cDNA was synthesized using oligo(dT)20 primers and the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. Input of RNA was 240 ng. After cDNA synthesis nuclease-free water was added up to a final volume of 80 μl.

**Real-time qRT-PCR**

Transcripts were quantified by real-time quantitative polymerase chain reaction (qPCR) using an ABIPRISM 7900 Sequence Detector and pre-designed TaqMan Gene Expression Assays, reagents and probes according to manufacturer’s instructions (Applied Biosystems), as described previously 15,16. We validated all primers according to protocol. Mean relative mRNA expression was calculated using Pfaffl method 17.

**Statistical analysis**

DC subset frequencies and marker expression levels, cytokine levels, transcript levels, and T cell frequencies and responses were compared between conditions using the paired T test. Excel or Prism 4.0 statistical software (GraphPad Software Inc.) was used. Differences were considered significant when p<0.05 in two-sided analyses.

**Results**

**Primary colon tumor-derived supernatants induce DC maturation**

We and others previously studied the phenotype of DC spontaneously migrating from human skin explants and found DC migration to be optimal by day 2 after the start of explant culture 12,18. Migrated cells harvested at this time can be roughly subdivided into two major phenotypes: CD1a+CD83+CD14- mature DC and CD1a±CD83-CD14+ immature macrophage-like cells. The balance between these two populations can be shifted in favor of the mature skin-emigrated DC by the intracutaneous conditioning of DC by such maturation-inducing agents as GM-CSF or IL-4 14. I.d. injection of 50 % (v/v) TDSN prior to culture, and the inclusion of 3 % (v/v) TDSN in the medium during explant culture (both found to yield maximal effects in titration experiments), induced a significantly enhanced maturation state of skin-emigrated DC based on activation marker levels similar to those achieved by the known classic in vivo DC maturation inducer GM-CSF (Figure 1A,B). This maturation induction was accompanied by a shift from CD1a±CD83-CD14+ immature macrophage-like cells to CD1a+CD83+CD14+ mature DC among
Figure 1. Primary colon tumor derived supernatants (TDSN) promote maturation of DC migrating from human skin explants. Intradermal (i.d.) injection -prior to culture- of human skin explants with 100 ng GM-CSF or 50 % (v/v) TDSN (combined with 3 % (v/v) TDSN added to the culture supernatant) (A-B) enhanced the maturation state of migrated DC (harvested and analyzed two days after the start of skin explant culture; Mean Fluorescence Intensities (MFI) of the tested markers are listed in the histograms and the histogram markers indicate fluorescence levels obtained with the isotype controls) and (C-D) shifted migrated DC from an immature CD14+ phenotype to a mature CD1a+ phenotype. E) T cell stimulatory capacity in an allogeneic Mixed Leukocyte Reaction (MLR) of unmodulated or TDSN-matured DC, emigrated from human skin explants. Mature DC were cocultured at the indicated stimulator : responder ratio for 4 days and pulsed with [3H]-thymidine during the last 18 hours. Proliferative responses ([3H]-Thymidine incorporation) are shown as mean counts per minute (cpm) from triplicate wells. All data shown are representative or averages (± sd) of 3-8 independent experiments, *p<0.05.
Figure 2. Primary colon tumor derived supernatants (TDSN) promote maturation of monocyte-derived DC (MoDC) in a prostaglandin-dependent manner. A-C) 48 hours maturation induction of 7-day immature DC, generated with GM-CSF and IL-4, by either 10 μM PGE2 or 30 % (v/v) TDSN, revealed TDSN-induced maturation comparable with PGE2 induced maturation, as determined by flowcytometric analysis of CD83 and CD86 levels (Figure 2B, n=11), which was entirely abrogated by the use of Indomethacin (IM)-modulated TDSN (Figure 2C, n=4). Expression levels of the studied (maturation) markers are listed as Mean Fluorescence Intensities (MFI). D) Mixed Leukocyte Reactions were performed with MoDC, either unmodulated or matured by the indicated TDSN conditions. Data shown are representative or averages (± sd) of 3-11 separate experiments, *p<0.05.
Figure 3. Maturation-inducing effects of primary colon tumor derived supernatants (TDSN) on skin-emigrating DC are not dependent on prostaglandins. Colon TDSN were i.d. injected at 50 % (v/v) prior to explant culture, while 3 % (v/v) was added to the culture media of the explant cultures. Skin explants were removed at day 2 of culture, after which migrated DC were immediately harvested (or 5 days later for IL-12p70 release testing). A) Maturation induction of skin-emigrated DC by TDSN was not abrogated by indomethacin (IM) modulation of the TDSN during their generation. Expression levels of the studied (maturation) markers are listed as Mean Fluorescence Intensities (MFI). B) Mixed Leukocyte Reactions were performed with skin-emigrated DC, either unmodulated or matured by the indicated TDSN conditions. Data shown are representative of three to five separate experiments. C) DC harvested from the cultures at day 7 were stimulated by CD40L-transduced J558 cells in the presence of 1000 IU/ml IFNγ and supernatants were harvested 24 hours later. IL-12p70 concentrations were determined by ELISA; averages ± sd are indicated.*p<0.05 vs medium controls. Data shown are representative of 3-5 separate experiments.

Figure 4. Broad Th subset-inducing capacity of skin-emigrated DC i.d. modulated by primary colon tumor derived supernatants (TDSN). Colon TDSN were i.d. injected at 50 % (v/v) prior to explant culture, while 3 % (v/v) was added to the culture media of the explant cultures. Skin explants were removed at day 2 of culture, after which migrated DC were immediately harvested and loaded with anti-CD3 and co-cultured for 14 days with CD4+CD25− T cells. A) After 14 days the Th cells were restimulated with immobilized anti-CD3 and anti-CD28 for 24 hours after which mRNA was isolated and the indicated transcript levels were determined by qRT-PCR: values relative to medium controls, n=3. B-C) Alternatively 14 day-primed Th cells were restimulated with PMA/ionomycin for 4 hours after which intracellular cytokine production levels or FoxP3 expression in relation to high CD25 levels (i.e. Tregs) were assessed; representative results (B) and averaged data ±sem (C) are shown of three separate experiments; *p<0.05.
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the migrated cells (Figure 1C,D); overall, the effects of primary colon TDSN on the expression levels of CD83, CD40, CD54, and CD86 was significant (p<0.01, n=8). Beside the acquisition of phenotypic markers of maturation, treatment with TDSN also increased the T cell stimulatory ability of both MoDC and skin-emigrated DC in allogeneic Mixed Leukocyte Reactions (MLR, see figure 1E).

Addition of 30 % (v/v) TDSN to 7-day immature MoDC induced a level of phenotypic maturation comparable to the addition of the DC-maturation inducer Prostaglandin-E2 (10 μM), as judged on the basis of expression levels of the DC maturation markers CD83 and CD86 (measured after 48 hours of maturation induction, see figure 2A,B). Over a total of eleven experiments these maturation-enhancing effects reached significance for expression levels of both CD83 and CD86 (figure 2B), and also resulted in an increased allogeneic T cell priming capacity of the MoDC (Figure 2D). Interestingly, TDSN derived from colon carcinoma cell lines (A2233, Colon 320, HT 29, and WiDr, at 30 % (v/v)) did not have any effect on MoDC differentiation (data not shown). We previously identified prostaglandins as the factor in colon-derived TDSN responsible for the inhibition of MoDC differentiation 10. Here, we show that the TDSN-mediated maturation effects in MoDC are similarly prostaglandin-dependent, as shown by abrogation of the effects of TDSN generated in the presence of the COX-inhibitor indomethacin (IM, Figure 2A,C,D). In contrast, these TDSN-mediated effects in skin-emigrated DC appeared to be wholly prostaglandin-independent (Figure 3A,B). Of note, effectiveness of COX inhibition was
ascertained by measuring the PGE2 content in IM-modulated and unmodulated TDSN. PGE2 concentrations were absent or strongly reduced in IM-modulated TDSN, but did not influence the levels of other cytokines (e.g. IL-10 and IL-6) present in the TDSN (assessed by ELISA, data not shown) 4.

Although TDSN did not interfere in any way with the migration rate of skin-derived DC (data not shown), TDSN did significantly inhibit the ability of migrated DC to produce IL-12p70 in response to CD40L-stimulation, which, again, was not influenced by IM-mediated COX inhibition during the generation of the employed TDSN (Figure 3C).

**Effects on Th cell subset differentiation of TDSN-conditioned skin-derived DC**

To ascertain how i.d. delivery of TDSN affected the Th cell-stimulatory ability of subsequently migrated DC, they were loaded with anti-CD3 and co-cultured with allogeneic CD4+CD25- T cells over a period of 2 weeks. After polyclonal re-stimulation the Th cells were then profiled for the presence of Th1-, Th2-, Th17/22-, and Treg-associated transcripts (Figure 4A). These analyses suggested a modest induction of Th17/Th22 related genes such as RoR-γt, IL-17 and IL-22 in the TDSN conditions compared to the medium control. However, testing the Th cells for their actual ability to produce Th1-, Th2-, Th17-, or Th22-associated cytokines by intracellular staining, and simultaneously for Treg frequencies, demonstrated no major shifts induced by TDSN-conditioned DC in Th subset distribution profiles (Figure 4B,C). A predominance of Th1 cells was apparent both in control cultures and in cultures with TDSN-conditioned skin DC.

**GM-CSF as likely perpetrator of maturation induction in skin-emigrated DC by TDSN**

To ascertain whether the observed prostanoid-independent maturational effects on skin-emigrated DC were induced by tumor-derived soluble factors or rather by factors derived from the tumor infiltrate, tumor single cell suspensions were depleted of leukocytes by CD45-specific panning. Supernatants were subsequently generated from both the tumor and the infiltrate fractions and tested separately for maturing effects on skin-emigrating DC. As demonstrated in Figure 5, tumor-derived soluble factors were clearly responsible for the observed maturation induction and not infiltrate-derived factors. The employed primary colon TDSN contained a wide variety of factors, known for their DC-modulatory abilities (n=17, see Figure 6A). Among the tested factors, IL-6, GM-CSF, and PGE2 were known to have DC-maturational effects. As PGE2 was ruled out and we previously had observed no effects of IL-6 in the employed explant model 15, GM-CSF remained as a possible candidate. Indeed, in terms of phenotype-modulating effects, those of GM-CSF more closely mimicked the effects of colon TDSN than of PGE2, including the loss of CD14+ cells after migration, which was not apparent upon i.d. PGE2 delivery (Figure 6B). Like the employed TDSN, i.d. delivery of GM-CSF led to the induction of a transcriptional Th17/Th22-like profile in primed Th cells by skin-emigrated DC (Figure 6C). Although these observations make GM-CSF a likely causative agent for the observed effects, the overall immune-modulatory effects of the TDSN will obviously be determined by the combined activity of the multitude of factors they comprise.
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Discussion

Tumors interact with their microenvironment to promote their own survival and to escape immune surveillance. As part of these escape strategies tumors secrete suppressive factors that inhibit the development and functionality of DC. Intradermal vaccination with autologous colon tumor vaccines may be hampered by the same mechanisms. We previously reported that primary colon TDSN interfered with MoDC development in vitro. This impaired differentiation was caused by tumor-related upregulation of the COX-2 enzyme with concomitant PGE2 production by the primary colon tumor suspensions. In the present study we show that primary colon TDSN enhance rather than frustrate the maturation of skin-emigrated DC, ascertained in an organotypic skin explant system. In contrast to the previous and current in vitro MoDC studies, COX-induced prostaglandins did not play a role in the TDSN-induced enhancement of skin-emigrated DC maturation.

PGE2, the most likely culprit in the observed maturation induction of MoDC by colon TDSN, is constitutively expressed in the intestinal Lamina Propria and may serve to condition local DC to a non-inflammatory default setting, maintaining a general state of tolerance in the healthy gut. Further upregulation of PGE2 under the influence of tumor-associated COX-2 expression in colon tumors will reinforce this state and lock the DC in a state that is not conducive to the initiation of an anti-tumor immune response. Although PGE2 serves as a (co-)factor for phenotypic DC maturation, the functional consequences of this maturation may be detrimental in terms of anti-tumor immunity: it will skew cytokine production to a high type-2 immunity promoting IL-10 release and may lead to the expression of the tryptophan-catabolizing enzyme Indoleamine-2,3-Dioxygenase (IDO), known for its T cell suppressive effects. Discrepancy in prostaglandin dependence of the TDSN-mediated maturation induction between in vitro generated MoDC and skin-resident DC may in part be explained by a difference in the intrinsic susceptibility of these DC types to the effects of prostaglandins, since PGE2-induced maturation was more robust in MoDC than in skin-emigrated DC. Of note, as transcriptional levels of PGE2 receptors between MoDC and CD1a+ dermal DC are comparable these may...
not account for the observed difference in PGE2 susceptibility.

In addition, a possible explanation lies in the indirect effects through modulation of the skin microenvironment by the TDSN. Factors in the colon TDSN may have induced production of DC-maturing agents by keratinocytes, which upon activation are known to release such DC-activating cytokines as GM-CSF, IL-1β, and TNFα. Particularly the IL-1β-TNFα axis has been reported to play a vital role in DC migration from the epidermis, with IL-1β inducing TNFα release which acts as a direct mediator of Langerhans cell maturation and migration. We studied the possible role of the IL-1β/TNFα axis in the observed skin-emigrated DC maturation induction by TDSN, but could not block this effect by the i.d. injection of TNFα neutralizing antibodies (data not shown).

What exact factors in the primary TDSN are responsible for the enhanced maturation of skin explant-emigrated DC remains to be elucidated. Future studies should focus on the identity and source of the responsible soluble factors. GM-CSF is however a likely candidate and has previously been described to be secreted by colon tumors with both direct and indirect, immune-dependent antitumor effects. GM-CSF is known as an immune adjuvant, and potent DC-activator, especially when administered intradermally.

The finding that colon TDSN actually induce DC maturation may seem unexpected and counterintuitive since various studies have pointed to the reduced frequency of mature CD83+ DC infiltrating colon tumor fields in vivo and have identified this as a bad prognostic factor. However, this depletion of local CD83+ DC may be due to the inhibition of local DC precursor differentiation into bona fide DC (e.g. by tumor-derived prostaglandins and/or IL-6), rather than the inhibition of maturation of tumor-infiltrating DC. Such a dichotomous effect of tumor-derived factors on DC differentiation and maturation (inhibiting the first, enhancing the latter) as we have now observed for colon carcinoma, has previously been described for melanoma.

Others have also described (partially) maturing effects of TDSN on DC, in keeping with our own findings. The concomitant block in IL-12p70 release might have been expected to prohibit the generation of an effective T cell-mediated anti-tumor response and to rather have led to active tolerance induction. Indeed, a number of recent publications have suggested that mature CD83+ DC rather than immature DC are responsible for maintaining tolerance.

However, we found no evidence of hampered Th cell priming or Th cell differentiation induction by the TDSN-conditioned skin-emigrated DC, nor of increased Treg expansion. Although the modest induction of a Th17/Th22 transcriptional profile would be in keeping with recent reports relating IL-17 and IL-22 with colon cancer development, the maintained predominance of a Th1 response in co-cultures of TDSN-conditioned skin DC and allogeneic T cells bodes well for their projected ability to induce effective antitumor immunity.

Results from our study contrast with those from Michielsen et al., who demonstrated inhibition of MoDC maturation induction by colon tumor-derived soluble factors. In that study TDSN were derived from tumor explants. It is conceivable that 1) the intact 3D colon tumor environment led to a different secretome profile – both studies focused on different secreted factors present within the supernatants, or 2) interaction of tumor derived factors with elements in the skin microenvironment led to the differential effects on DC maturation observed in our
studies. Also, differences between the study results could be due to differences in methodology. In their study, Michielsen et al. pre-exposed the immature MoDC for 4 hours to the tumor explant-derived supernatants prior to adding LPS to induce DC maturation, whereas we added TDSN directly to immature MoDC and assessed their maturational effects per se. Of note, when we added TDSN and LPS simultaneously to immature DC we observed enhancement of LPS-induced maturation by the TDSN (data not shown). These differences in methodologies derive from differences in the research questions for both studies: whereas Michielsen et al. aimed to study what would happen to tumor-infiltrating immature DC once they would arrive in the tumor microenvironment, our study was designed to study the effects of whole tumor cell-based vaccines on DC in the human skin microenvironment. As such the model employed in our study carries certain advantages and disadvantages. It allows for an accurate and physiologically highly relevant assessment of the effects of conditioning of the human skin microenvironment by colon tumor-derived soluble factors on the T cell activating properties of subsequently migrating DC. However, it is also a relatively ill-defined, “dirty” system that is not readily amenable to delineation of immune-modulatory factors, mechanisms, and cross-talk events in the dermis, that underlie the observed stimulatory effects on migratory DC after i.d. delivery of primary colon tumor-derived supernatants.

We conclude that colon cancer-derived soluble factors in the skin microenvironment do not recapitulate the immune suppressive tumor microenvironment but in effect induce phenotypic DC maturation, which is accompanied by increased priming of a poly-functional Th cell response. As such i.d. delivery of autologous colon cancer-based vaccines should be fully compatible with the envisioned induction of anti-tumor immunity, certainly when further combined with powerful adjuvants that will ensure a strong type-1 T cell response and efficient cross-priming of tumor-specific CTL.
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