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New Target Genes for Tumor-derived Soluble Factors in Primary Monocytes

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Abstract. *Background: Tumor cells have developed several strategies to escape the immune system. One of these strategies consists of the secretion of immunosuppressive factors like interleukin-10 or prostaglandin E2 (PGE_2), which impair the immune system. We have demonstrated recently that tumor-derived PGE_2 down-regulates the expression of the integrin Mac-1 and the chemokine receptor CCR5 on primary monocytes, resulting in reduced adhesion and migration. Materials and Methods: In order to identify new target genes for tumor-derived factors in monocytes, we set up an in vitro system consisting of cDNA micro arrays and 2D gel electrophoresis. Results: We identified 25 genes that were differentially expressed upon incubation of cells in conditioned tumor cell supernatants as compared to cells incubated in cell culture medium. We describe in more detail that IL-1β secretion is induced by tumor supernatants and that IL-1β overexpression is also evident in monocytes from tumor patients in vivo, where expression correlates with the tumor stage. In addition, up-regulation of the plasminogen activator inhibitor-2, PAI-2, and down-regulation of the urokinase-type plasminogen activator receptor, uPAR, resulted in a reduced capability of monocytes to degrade and invade extracellular matrices. Conclusion: In summary, we describe interesting novel targets of soluble tumor-derived factors that are probably involved in the tumor-mediated immunosuppression commonly found in cancer patients.*

Tumors use several strategies to escape the host's immune system, including the loss and/or mutation of antigenic peptides (1), down-regulation of TAP-1 and MHC class I molecules (2), or defective death signaling using decoy receptors (3). Also, tumor cells secrete immunosuppressive factors like IL-10 and TGF-β or the eicosanoid PGE_2 (1). These tumor-derived substances have been shown to affect the immune system in multiple ways: they impede maturation of dendritic cells (4), reduce the proliferation and cytolytic capacity of lymphocytes (5) and may even interfere with essential monocyte/macrophage functions (6).

Monocytes/macrophages display a very important yet ambivalent relationship with tumors (7). On the one hand, tumor-associated macrophages (TAMs) have the capacity to directly kill tumor cells and to destroy tumor vessels, to inhibit angiogenesis and to professionally present tumor-derived antigens. On the other hand, TAMs may support disease progression by producing factors that promote tumor growth and neo-angiogenesis (8). This ambivalence of macrophages is only poorly understood so far but it is believed that the direct influence of tumor cells biases this dualism towards a more tumor-promoting one (9). In this respect, the influence of tumor-derived PGE_2 on cell surface receptors on monocytes was examined previously in our laboratory. We demonstrated that the chemokine receptor CCR5 as well as the adhesion molecule MAC-1 were down-regulated upon treatment with tumor-derived PGE_2, resulting in impaired monocyte functions (6). Also, Sica *et al.* recently demonstrated a defective expression of the chemokine receptor CCR2 in macrophages associated with ovarian carcinoma (10). Until now, only a few target genes of tumor-derived factors in monocytes/macrophages have been examined *in vivo* and *in vitro*.

Therefore, the aim of our present study was to identify additional tumor-regulated target genes in monocytes. For this analysis, we treated primary human monocytes with conditioned supernatants of tumor cell lines (TuSN) and compared these cells by means of nuclear run-on experiments and two-dimensional gel electrophoresis (2DE) to cells that were left untreated. Whereas expression profiling using cDNA array filters allowed for the identification of low abundant mRNAs, posttranslational modifications were detected using differential 2DE. In total, we identified 25 genes in primary monocytes as new

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molecular targets for tumor-derived immunosuppressive factors. The expression and functional consequences of the deregulation of two of these targets, interleukin-1β (IL-1β) and the urokinase-type plasminogen activator receptor (uPAR), were analyzed in more detail. Thus, we present new interesting targets of tumor-derived soluble factors, which may be involved in immune escape mechanisms.

Materials and Methods

Human cell lines. FaDu, Hlac-78, Tu179K, PCI-1 and PCI-13 are cell lines derived from squamous cell carcinomas of the head and neck. MCF-7 and SKBR3 are breast cancer cell lines, HCT-8 derived from a colon carcinoma and HeLa from a cervix carcinoma. WI 38 are fetal lung fibroblasts (ECACC, Cambridge, UK), AMFib and CaZe are primary skin fibroblasts (gift from A. Moosmann, Munich, Germany). Mono Mac-6 (MM-6) is a monocytic cell line (11) and NIH3T3 cells are murine fibroblasts (ATCC; Manassas, VA, USA). All cells were maintained at 37˚C as continuously growing monolayers in Dulbecco's MEM (DMEM) with 10% fetal calf serum (FCS, Seromed, Berlin, Germany).

Buffy coats and peripheral whole blood. Buffy coats, released for research purposes, were obtained from the Bavarian Red Cross (Munich, Germany). Whole blood was taken from tumor patients in full consent and after approval of the IRB. Separation of PBMCs was performed by Ficoll gradient centrifugation. For the enrichment of monocytes, PBMCs were incubated in DMEM for 20 min on cell culture dishes and non-adherent cells were removed by washing with PBS.

Generation of cell-free tumor cell supernatants (TuSN). Tumor cells or fibroblasts were seeded at 10^5 cells/ml and grown for 2 days. Conditioned supernatants (SN) were collected, centrifuged (340g, 5 min, RT), and passed through a 0.2μm Acrodisc low protein binding filter (Gelman Sciences, Ann Arbor, Mich, USA). Supernatants were used immediately.

Multiplex- ELISA. IL-1β and PAI-2 secretion was determined using commercial ELISA assays (R&D Systems; Wiesbaden, Germany and American Diagnostica; Greenwich, USA, respectively). TNF-α, IL-1β, IFN-γ, GM-CSF, IL-6 and IL-10 secretion was measured in a multiplex-assay using the Bio-Plex device (Bio-Rad, Richmond, USA) according to the manufacturer's instructions.

Expression profiling. Gene expression profiling was performed on primary human monocytes, incubated either in supernatants of PCI-1 cells or in cell culture medium only. Nuclear run-on assays were carried out as described (12,13). Briefly, 4 x 10^7 isolated nuclei in storage buffer (50mM Tris -HCl [pH 8.3], 40% [v/v] glycerol, 5mM $MgCl_2$, 0.1mM EDTA), were thawed on ice and subsequently incubated with the same volume of reaction buffer (10mM Tris-HCl [pH 8.0]; 5mM $MgCl_2$; 300mM KCl; 0.5mM ATP, GTP and UTP each; 100μCi of [α-^{32}P]CTP [800 Ci/mmol], Amersham Biosciences; Freiburg, Germany) for 15 min at 28˚C. Nuclear transcripts were isolated and labeled RNA was hybridized to Clontech's Atlas Array, Human 1.2 I (Clontech; Heidelberg, Germany) at 58˚C for 48 h in 5ml hybridization buffer (Clontech). After washing of the membranes, signals were visualized on a Kodak Biomax film and analyzed using the ImageJ software (http://rsb.info.nih.gov/nih-image/).

Table I. *List of differentially expressed mRNAs found in primary monocytes upon treatment with PCI-SN. Using the nuclear run-on technique with subsequent hybridization of the in vitro synthesized mRNAs to ATLAS cDNA arrays, we identified 16 differently expressed mRNAs, 14 of them were up-regulated and two down-regulated by TuSN (Reg.: regulation; n-fold up- (+) or down-regulation (-), * = not detectable in controls, de novo expressed upon treatment with PCI-SN).*

cDNA	Reg.	n-fold	Protein function
CDK4 InhibitorD	⇩	- 2.8	Inhibitor of CDK4 and CDK6
Janus Kinase 3 (JAK3)	⇩	- 1.4	Tyrosine-kinase
JunD	⇧	+ 1.6	Proto-oncogene
Neurogranin	⇧	+ 1.9	Substrate of PKC-mediated signal cascades, binds calmodulin
MCL-1	⇧	+ 2.6	Leukemia cell-differentiation protein
NF-Î B p100	⇧	+ 2.3	p52 precursor, involved in acute phase reactions and immune response
Tristetraproline	⇧	+ 1.4	Nuclear zinc finger protein
GADD45β	⇧	+ 2.2	Growth arrest and DNA-damage-inducible protein
LIF precursor	⇧	+ 6.0	Leukemia inhibitory factor
ENA78	⇧	+ 2.8	CXC chemokine attracting neutrophils
IL-8 precursor	⇧	+ 2.1	CXC chemokine
IL-1α precursor	⇧	*	Pro-inflammatory cytokine
IL-1β precursor	⇧	+ 41.5	Pro-inflammatory cytokine
IL-6 precursor	⇧	*	Cytokine
MMP-14	⇧	*	Matrix-metalloprotease
PAI-2	⇧	*	Plasminogen activator inhibitor-2

Two-dimensional polyacrylamide gel electrophoresis (2DE). Primary monocytes (1 x 10^7 cells per pH 4-7 linear strip and 1.5 x 10^7 per pH 4.5-5.5, pH 5.0-6.0 or pH 5.5-6.7 linear strip, respectively) were washed twice in PBS and once in PBS/H_2O (1:1 v/v) and lyzed in 350μl lysis buffer (9M Urea; 4% CHAPS; 1% DTE; 1mM EDTA). DNA was shreddered using QIAShredder-columns (QIAGEN;

Table II. *List of differentially-expressed proteins in untreated and TuSN-treated monocytes, identified by 2DE. Differentially-expressed proteins were excised from the gel, digested with trypsin and identified by MALDI-ToF mass spectrometry. Reg.: regulation; n-fold up- (+) or down-regulation (-) in Tu-SN.*

Protein	Accession Number	Mw. [kDa]	pI	Reg.	n-fold	Function
IL-1β precursor	P01584	30.8	4.80	⇧	+ 6.2	Pro-inflammatory cytokine
PAI-2 inhibitor-2	P05120	46.6	5.46	⇧	+ 4.3	Plasminogen activator
Ferritin heavy chain	P02794	21.1	5.30	⇧	+ 4.4	Storage of iron in cytoplasma
Vimentin	P08670	53.6	5.06	⇧	+ 3.6	Class-III intermediary filament
Adenosine deaminase	P00813	40.8	5.63	⇧	+ 3.6	Adenosine metabolism
Protein disulfide isomerase ER-60	P30101	56.7	5.98	⇩	- 2.3	Rearrangment of disulfide bonds
Pyruvate kinase M1/M2 isozyme	P14618 P14786	57.8	7.95	⇩	- 1.5	Glycolysis
PKC inhibitor-1	P29312	27.7	4.73	⇩	- 16.9	Regulator of PKC- and PKII-mediated signaling pathways
Cathepsin D	1LYA_B (P07339)	26.5	5.31	⇩	- 5.4	Acidic protease
Calgizzarin	P31949	11.8	6.56	⇩	- 1.9	Ca^{2+} binding protein
Alpha-enolase	P06733	47.0	6.99	⇩	- 6.9	Glycolysis

Hilden, Germany) and pelleted by centrifugation (42,000g, 1 h, RT). Bromphenol blue and ampholytes (2µl IPG-buffer, Amersham) were added. Isoelectric focusing was performed for 90,000 Vh using immobilized pH-gradient IPG-strips on an IPG-Phor unit (Amersham). Strips were incubated for 20 min in equilibration buffer (6M Urea; 3.3M Glycerol; 70mM SDS; 65mM DTE; 3.3% resolving-buffer [1.5M Tris; 0.4% SDS; 1.5mM NaN_3]). Second dimensions were run by vertical electrophoresis on 13% polyacrylamide gels (30 min: 3 watt/gel, 3-5 h: 18 watt/gel, 20˚C) in an Ettan Dalt II chamber (Amersham). Protein spots were detected using mass spectrometry compatible coomassie (14) or silver staining protocols (15). Differentially expressed proteins, *i.e.* proteins that were at least 1.5-fold up- or down-regulated in differently treated monocytes, as analyzed by the Image Master™ 2D software (Amersham), were excised and extensively washed in Millipore water, 50% acetonitrile and 50mM ammonium bicarbonate. Digestion was performed o/n with 50ng trypsin (Promega; Mannheim, Germany). Identification of proteins was performed by MALDI-ToF mass spectrometry (Reflex III Bruker; Heidelberg, Germany) and subsequent database search (www.matrixscience.com).

Immunoblotting. 3 x10^6 cells were resuspended in 30µl lysis buffer (1% Triton, 1mM PMSF, 0.5mM orthovanadate). For immunoblotting, equal protein amounts were separated by 13% SDS-PAGE and transferred to a PVDF membrane. Human IL-1β, PAI-2 and β-actin were detected using specific antibodies (α-IL-1β and α-actin: Santa Cruz Biotechnology, Heidelberg, Germany; α-PAI-2: American Diagnostica) in combination with an HRP-conjugated secondary antibody (Dako; Glostrup, Denmark). The signals obtained by conversion of the ECL reagent (Amersham) were visualized on X-ray films.

Flow cytometry. UPAR flow cytometry was performed using an uPAR-specific antibody and a FITC-labeled secondary antibody (Santa Cruz Biotechnology). The mean fluorescence intensity ratio (MFIR) was calculated as 'mean fluorescence (16) / mean fluorescence [isotype control]'.

Matrigel assays. MM-6 cells pre-treated with conditioned tumor supernatants or cell culture medium were cultivated in DMEM containing 0.1% FCS for 22 hours on ECM-covered matrigel migration inserts with 8 µm pores (Becton Dickinson; Heidelberg, Germany). Attraction of cells was achieved using supernatant of NIH-3T3 mouse fibroblasts. Non-invaded cells were removed using cotton swabs. Cells on the insert were fixed in 100% methanol and stained with toluidine blue (0.25% toluidine blue in 58% ethanol). The matrigel was washed three times in distilled water followed by an alcohol range (70%, 80%, 95%, 100% ethanol, xylol), and was finally fixed on object slides with Eukitt® (Kindler, Freiburg, Germany). The number of invaded cells was counted by light microscopy.

Immunohistochemical staining of cytospins. Cytospins were performed using 2x10^5 cells/slide of freshly isolated PBMCs from

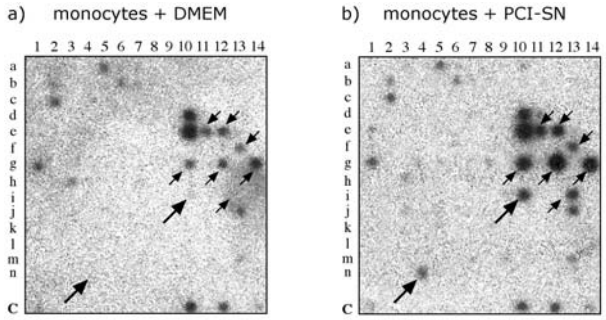

Figure 1. *Detail of Clontech Atlas Human 1.2 I cDNA arrays, showing expression patterns of untreated and TuSN-treated primary monocytes. Filters were hybridized with in vitro transcribed RNA obtained from run-on experiments using a) untreated and b) PCI-SN-treated primary monocytes. Large arrows indicate the two differentially-expressed genes IL-1β (coordinate i-10) and PAI-2 (n-4). Control housekeeping genes (row C) confirmed equal amounts of hybridized RNA. Shown is one experiment out of three.*

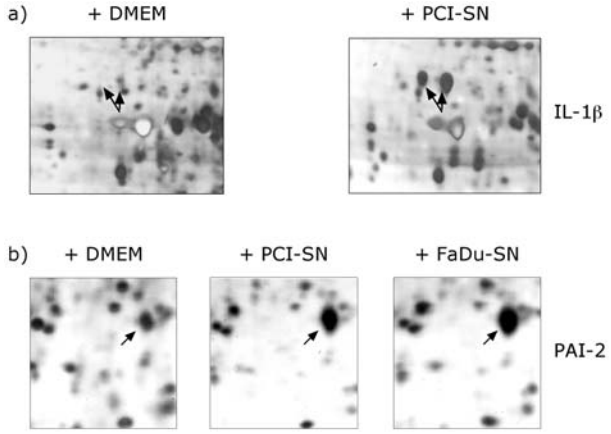

Figure 2. *Details of 2DE gels (pH 4-7) demonstrating different protein expression patterns in untreated and TuSN-treated primary monocytes. Shown is the increased expression of a) IL-1β precursor in monocytes following incubation in PCI-SN and b) PAI-2 following incubation in PCI- and FaDu-SN. Shown is one experiment out of ten.*

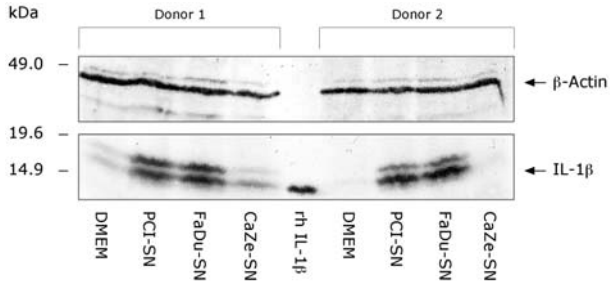

Figure 3. *Immunoblot analysis showing IL-1β expression in monocytes after incubation in different conditioned supernatants. IL-1β expression was strongly induced after incubation in TuSN (PCI-1, FaDu) but not in fibroblast-SN (CaZe-SN) compared to DMEM control in primary monocytes from two healthy blood donors (1 and 2). Recombinant human (rh) IL-1β was used as a control. Shown is one experiment out of four.*

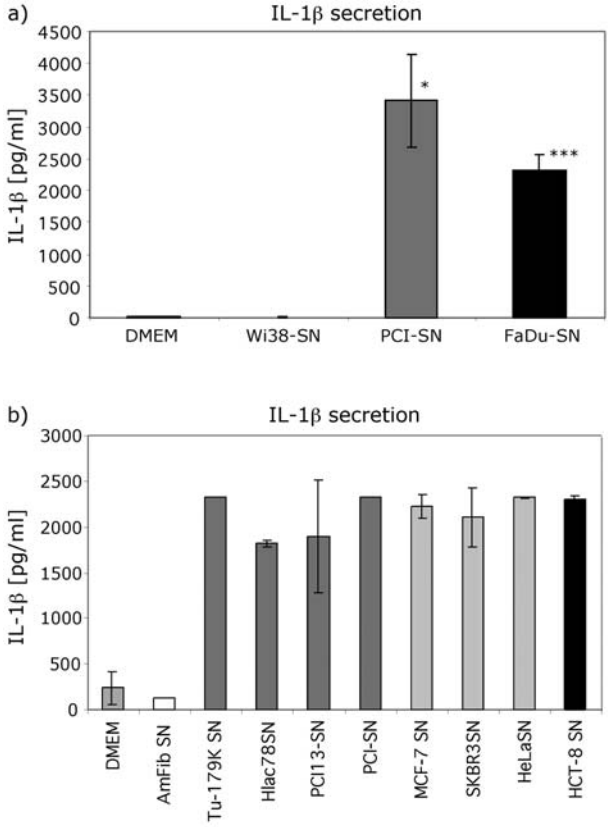

Figure 4. *TuSN derived from various carcinoma cell lines induce IL-1β expression in primary monocytes. a) IL-1β secretion of monocytes following incubation in DMEM, fibroblast-SN (WI 38) and TuSN (PCI-1, FaDu) as measured by ELISA. Mean values and standard deviations derived from three independent experiments. b) IL-1β secretion of monocytes following incubation in DMEM, fibroblast-SN (Am Fib) and different TuSN. Shown are mean values and standard deviations obtained from at least two independent experiments (*p<0.05; ***p<0.005).*

tumor patients or control donors. For the preparation of chamber slides, isolated PBMCs were allowed to adhere on plastic chamber slides and cultivated for 24 hours in either TuSN or standard cell culture medium. IL-1β antibody was detected using a standard avidin-biotin-peroxidase complex kit (ABC, Vectastain; Burlinghame, California, USA) and amino-ethylcarbazole (AEC, Sigma; St. Louis, USA) as chromogen. CD68 staining was carried out using the alkaline phosphatase anti-alkaline phosphatase method (APAAP, (17)) and Fast Blue salt (Sigma) as chromogen.

Results

TuSN alter gene expression in primary monocytes. In order to identify differentially-expressed genes and proteins in monocytes, we established a cellular system that relies on the incubation of primary monocytes in conditioned

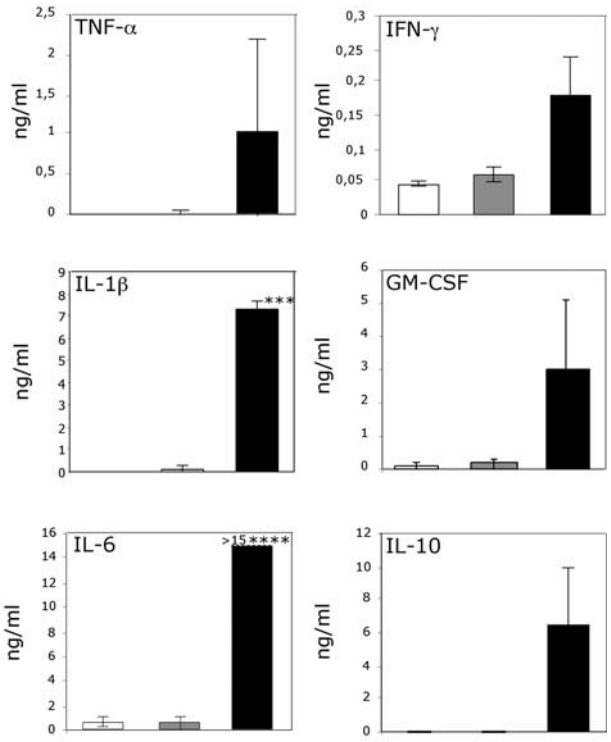

Figure 5. *TNF-α, IFN-γ, IL-1β, GM-CSF, IL-6 and IL-10 are induced in monocytes following incubation in TuSN. Histograms represent the amount of TNF-α, IFN-γ, IL-1β, GM-CSF, IL-6 and IL-10 secreted by tumor cells (white bars), primary monocytes (grey bars) and primary monocytes after incubation in TuSN (black bars) as determined by a multiplex (Bio-Plex) assay. Shown are mean values and standard deviations obtained from three independent experiments with PCI-SN. Similar results were obtained using the FaDu supernatants (***p<0.005, ****p<0.001).*

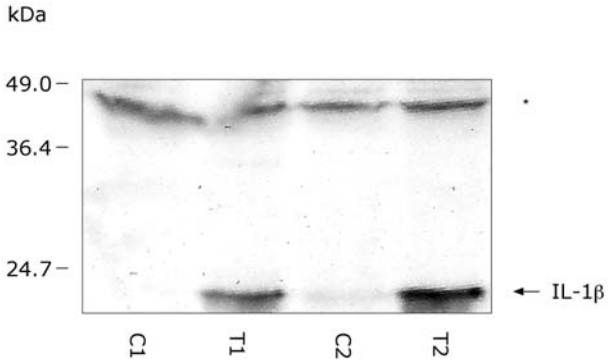

Figure 6. *Immunoblot showing elevated IL-1β expression in PBMCs from tumor patients compared to control donors. Immunoblotting was performed using freshly isolated PBMCs from 25 tumor patients at different stages of disease as well as from 13 healthy control donors. Shown is the IL-1β expression in PBMCs of two tumor patients (T1 and T2) as well as two controls (C1 and C2; *unspecific protein band). Results are also summarized in Table III.*

Table III. *Amount of IL-1β expression in PBMCs from 25 tumor patients and 13 healthy donors as revealed by immunoblot analysis. Tumor patients were grouped according to their tumor stage (T1-4, relapse). 0-+: undetectable to very weak expression, ++: moderate expression, +++: strong expression.*

Group	0 - +	++	+++
Control donors	13 (100%)	0	0
T1/2	5 (71%)	2 (29%)	0
T3	1 (25%)	2 (50%)	1 (25%)
T4	6 (60%)	0	4 (40%)
Relapse	2 (50%)	1 (25%)	1 (25%)
Total	27 (71%)	5 (13%)	6 (16%)

supernatants of tumor cell lines (TuSN) generated from either of two hypopharyngeal carcinoma cell lines, PCI-1 and FaDu. Monocytes were incubated in TuSN or in cell culture medium and were then harvested for nuclear run-on experiments after 24 hours or, for generation of whole protein lysates for 2DE, after 48 hours of incubation. Hybridization of *de novo* synthesized RNA derived from run-on assays to Clontech ATLAS Array Human 1.2 microarrays revealed about 200 genes expressed in monocytes, including 13 housekeeping genes. Of these genes, 16 were differentially expressed upon incubation in PCI-SN as determined by the ImageJ software (Table I). Using 2DE, we identified 11 differentially-expressed proteins, five being up-regulated and six down-regulated in TuSN-treated monocytes (Table II). Two of the TuSN-regulated target genes, IL-1β and PAI-2, were identified with both screening methods (Figures 1, 2) and were investigated in more detail.

TuSN enhances IL-1β production and secretion in monocytes. Since cDNA arrays and 2DE revealed an up-regulation of IL-1β in TuSN-treated monocytes, we performed immunoblot assays with differently pretreated monocytes. An IL-1β-specific monoclonal antibody stained two bands of 17 and 19kDa and detected a strong IL-1β induction in monocytes incubated in TuSN as compared to cells incubated in cell culture medium only (Figure 3). In contrast, no induction was observed upon incubation in conditioned supernatants from primary skin fibroblast (CaZe). Subsequent ELISA assays revealed that monocytes incubated in different TuSN secreted up to ten times more IL-1β than monocytes incubated in DMEM and in supernatant from human primary fibroblasts (Figure 4). Thus, both, IL-1β production and secretion were strongly induced in monocytes following incubation in TuSN, irrespective of the origin of the carcinoma cell line.

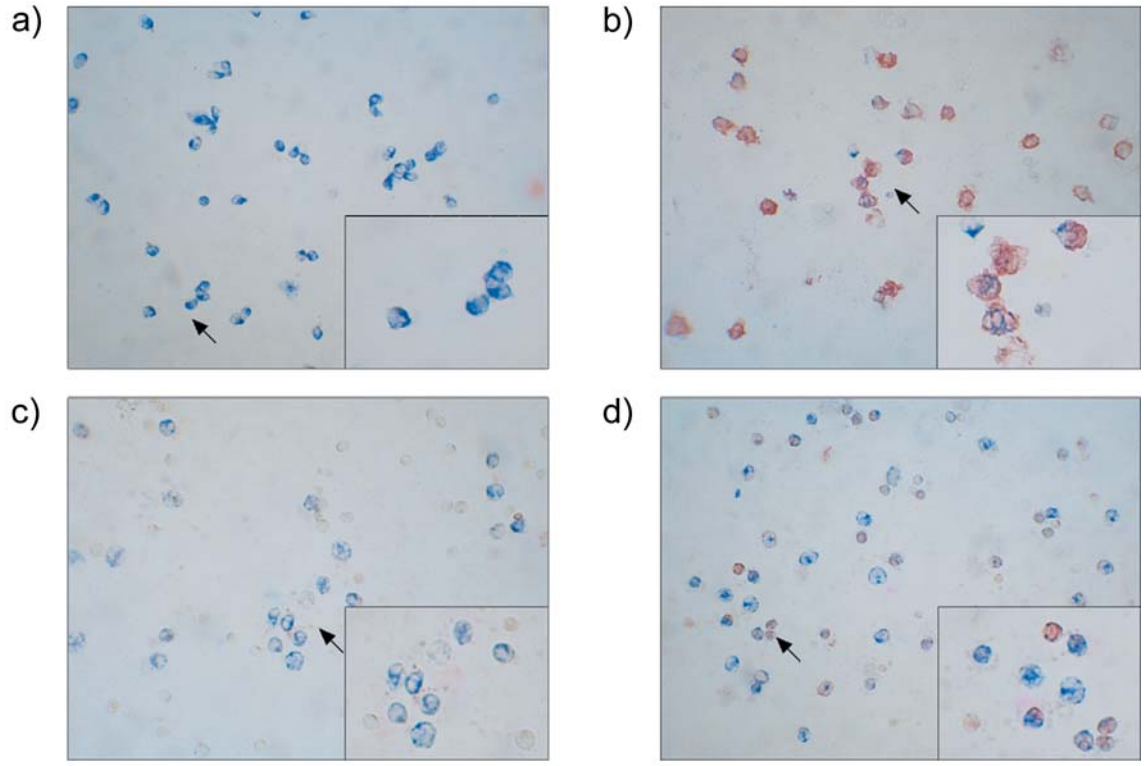

Figure 7. *PBMCs from cancer patients exhibit high amounts of intracellular IL-1β. a and b: freshly isolated PBMCs were allowed to adhere to chamber slides and were then incubated for 30 hours in (a) DMEM or (b) TuSN (FaDu). The pictures show the enhanced IL-1β expression (red) in CD68-positive monocytes (blue) upon treatment with TuSN. c and d: cytospins performed with freshly isolated PBMCs from a control donor (c) and a tumor patient with advanced stage disease (d). Inserts at higher magnification (1600x) were taken from a typical area (indicated by arrows) of each picture (400x).*

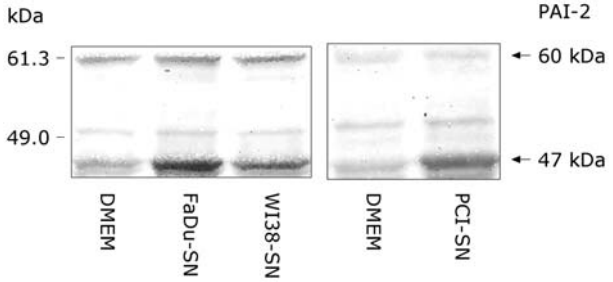

Figure 8. *Expression of intracellular PAI-2 is induced in monocytes following incubation in TuSN. Primary monocytes were incubated in standard medium (DMEM), TuSN (FaDu, PCI-1) or fibroblast-SN (WI 38) for 24 hours. The intracellular 47kDa form of PAI-2 was strongly induced in monocytes upon treatment with TuSN (FaDu: 2-fold, PCI-1: 2.3-fold), whereas the 60kDa form was not affected. Shown is of one representative experiment out of three.*

Besides IL-1β expression, profiling revealed additional cytokines being induced in monocytes following incubation in TuSN (Table I). In order to verify these results, we performed a multiplex Bio-Plex analysis, which demonstrated that levels of TNF-α, IFN-γ, IL-1β, GM-CSF, IL-6 and IL-10 were also significantly increased in supernatants of monocytes pre-incubated in TuSN as compared to cells cultured in DMEM (Figure 5). Again, none of these cytokines was induced upon incubation of monocytes in conditioned fibroblast-SN (data not shown). Secretion of IL-4 and IL-8 remained almost unchanged in all supernatants tested.

PBMCs from tumor patients contain high amounts of IL-1β. Since IL-1β expression was induced in monocytes following their incubation in TuSN *in vitro*, we wondered whether increased IL-1β levels were also present in PBMCs of tumor patients *in vivo*. To this end, we isolated PBMCs from 25 patients suffering from squamous cell carcinoma of the head and neck and from 13 healthy donors and determined the amount of IL-1β in PBMCs semi-quantitatively using immunoblot analysis. Elevated IL-1β levels were evident in PBMCs of tumor patients compared to healthy donors (Figure 6). It also became clear that IL-1β levels correlated directly to the tumor stage: whereas patients with T1/2 diseases displayed only moderate IL-1β levels, patients with advanced stage disease (T3) displayed strongly elevated intracellular IL-1β levels. Ambiguous results were obtained

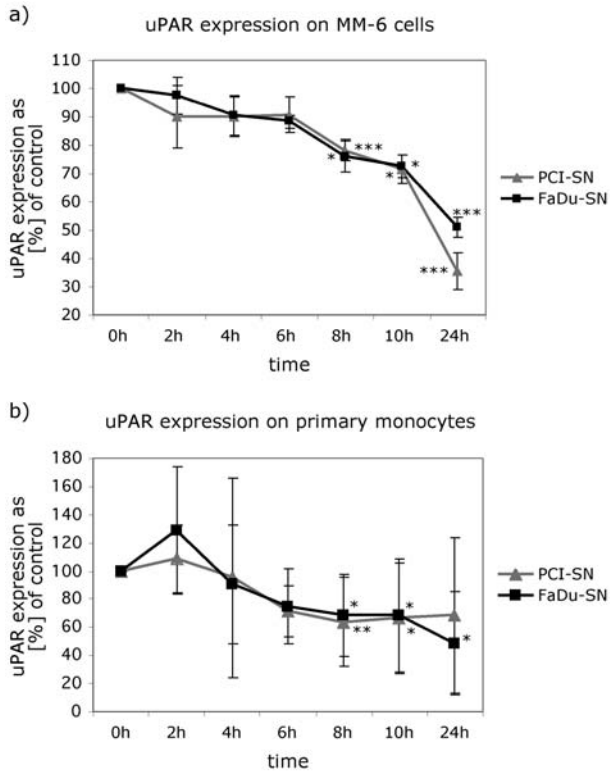

Figure 9. *UPAR surface expression is reduced on monocytes following incubation in TuSN as determined by flow cytometry. Monocytes were incubated in DMEM or TuSN (PCI-1, FaDu). The experiment was performed using either a) MM-6 cells or b) primary monocytes. Expression levels are given as the percentage of uPAR expression on TuSN-treated cells as compared to control (DMEM). UPAR expression in TuSN-treated cells started to decrease after about 6h of incubation. The resulting uPAR expression levels after 24 h of incubation were 36% (PCI-1) and 51% (FaDu) for MM-6 cells (9a) or 68% (PCI-1) and 49% (FaDu) for primary monocytes, respectively (9b). Mean values and standard deviations were calculated from three independent experiments (*p<0.05; **p<0.01; ***p<0.005).*

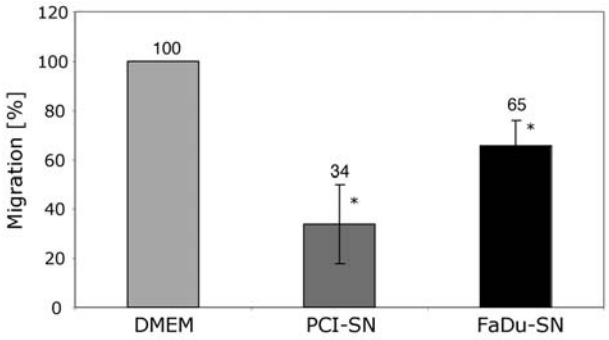

Figure 10. *ECM invasion capacity of MM-6 cells is reduced upon treatment with TuSN. MM-6 cells were incubated in TuSN (PCI-1, FaDu) or standard cell culture medium (DMEM-control) for 24 hours and then cultivated on migration inserts covered with a layer of ECM and allowed to transmigrate for 22 hours. Non-invaded cells were removed and the matrigel was fixed on object slides for microscopy. The amount of invaded untreated MM-6 cells was set to 100%. Shown are mean values and standard deviations calculated from three independent experiments (*p<0.05).*

from patients with stage T4 disease and patients with relapse: their PBMCs revealed either very high IL-1β levels or very low levels, a phenomenon that was probably due to a collapsing immune system (Table III).

In order to assess the nature of the IL-1β-producing cells in more detail, we performed double staining of cytospins of freshly-isolated PBMCs (Figure 7). IL-1β expression levels were increased in both, CD68-positive monocytes and CD68-negative lymphocytes from tumor patients compared to control donors (Figure 6, Table III).

TuSN increase PAI-2 expression in primary monocytes. Our analysis of monocytes also revealed a significant increase of PAI-2 mRNA and protein upon TuSN treatment. In order to validate PAI-2 overexpression in monocytes, we performed immunoblot analysis using monocytes treated with TuSN, supernatants from fibroblasts (WI38), or DMEM. Immunoblotting revealed a PAI-2 induction in monocytes incubated in TuSN, but not in cells incubated in WI38-SN. Interestingly, only the intracellular 47kDa form of PAI-2 was strongly induced whereas the secreted 60kDa form of PAI-2 was not regulated (Figure 8). This observation is in coincidence with results obtained by ELISA assays, which demonstrated no difference in PAI-2 secretion between TuSN-treated monocytes and control cells (data not shown).

TuSN decrease uPAR surface expression on monocytes. Since PAI-2 is an inhibitor of uPA (18) and thus involved in fibrinolysis and regulation of ECM degradation, we examined whether additional members of the plasminogen system were affected by TuSN. We therefore investigated by flow cytometry the surface expression of the urokinase receptor, uPAR (CD87), on MM-6 as well as on primary monocytes. Expression kinetics revealed that uPAR expression significantly decreased after six hours of incubation in TuSN, resulting in expression levels after 24 hours of 58% (FaDu) and 40% (PCI-1) for MM-6 cells or 49% (FaDu) and 68% (PCI-1) for primary monocytes, respectively, compared to control cells incubated in DMEM (Figure 9a).

TuSN impair the ECM invasion capacity of monocytes. UPAR plays an essential role in the directed proteolysis, whereupon receptor-bound pro-urokinase is activated to urokinase, which converts inactive plasminogen into its active form plasmin. Plasmin in turn degrades constituents of extracellular matrix

(ECM), enabling the cell to transmigrate tissues. In order to investigate the consequences of uPAR down-regulation onto the capacity of monocytes to invade ECM, we performed matrigel-invasion experiments. TuSN-treated and control MM-6 cells were cultured on matrigel inserts and tested for ECM invasion. After 22 hours, invaded cells were stained and counted by light microscopy. The number of invaded control monocytes was set to 100% (Fig. 10). We observed a decreased invasion capacity of MM-6 cells that directly correlated with the uPAR expression levels determined by flow cytometry (Fig. 9): in detail, only 34% and 65% of monocytes treated with PCI-SN or FaDu-SN, respectively, invaded the ECM (Fig. 10).

Discussion

Monocytes/macrophages play an important role in tumor development and maintenance, in that they can accomplish both anti-tumor and tumor-promoting effects, by providing a local milieu of cytokines, growth factors, angiogenic factors and proteases. On the one hand, a rich tumor infiltrate can correlate with a good clinical prognosis (19,20), on the other hand, immune cells can also be essential for tumor growth as in Hodgkin's disease (21). The interaction of monocytes with neoplastic cells is a complex and ambivalent relationship, which is still poorly understood. We have shown recently that tumor-derived PGE_2 down-regulates the surface receptors CCR5 and Mac-1 on monocytes, resulting in impaired monocyte function (6). Interestingly, a similar mechanism was also active *in vivo* in cancer patients and was reversible upon treatment of the patients with the cyclooxygenase-2 specific inhibitor Rofecoxib (22).

Here, we examined the effect of tumor-derived soluble factors on the gene and protein expression profile of primary monocytes. For this, we used an *in vitro* cellular system, relying on the incubation of primary monocytes in conditioned supernatants of different tumor cell lines. For the identification of TuSN-regulated genes and proteins we applied nuclear run-on assays with subsequent hybridization to Atlas array cDNA filters as well as 2D gel electrophoresis. In total, we identified 25 target genes as TuSN-regulated, 15 being up-regulated and 10 down-regulated. As has been described recently, some of these genes (IL-1β, IL-6, TNF-α and NF-Î B) are also up-regulated in LPS-treated monocytes (23), indicating similarities between TuSN and LPS. Interestingly, other genes that were up-regulated by TuSN were down-regulated by LPS (tristetraproline, MCL-1 and JunD), indicative of the complex relationship between tumor cells and the immune system.

The proinflammatory cytokines IL-1β, IL-6, GM-CSF and TNF-α, which play a central role in the initiation of immune responses, were expressed at significantly higher levels upon TuSN treatment. However, the same holds true for the immunosuppressive cytokine IL-10. Hence, this combination of cytokines could act together to affect monocyte (and possibly monocyte-derived DC) differentiation and function. The role of cytokines in cancer is complex. On the one hand they suppress tumor formation by controlling infection, inflammation and immunity and on the other hand they promote tumor growth, increase resistance to apoptosis and foster dissemination (24).

Other genes that were up-regulated by TuSN were either involved in apoptosis (MCL-1, GADD45β, PAI-2, JunD, neurogranin), cytokine- and growth factor-mediated signal transduction and response (Jak3, NF-Î B p100, tristetraproline), represent cytokines (IL-1β, IL-6, IL-10, TNF-α, IFN-γ, GM-CSF), or were components of protease complexes (MMP-14, PAI-2, uPAR). Two genes that were down-regulated upon TuSN treatment were involved in glycolysis (pyruvate kinase, α-enolase) and suggest a reduced metabolism of monocytes upon treatment with TuSN.

Increased IL-1β levels were not only seen *in vitro* but were also evident in peripheral monocytes of tumor patients *in vivo* (Figures 6, 7), indicating that tumors not only influence immune cells in their close vicinity but rather act systemically. IL-1β levels in cancer patients increased with disease progression (Table III) until collapse of the immune system in late stage disease.

The serpin PAI-2 exists in two different forms: whereas the glycosylated 60kd form is secreted and a well-known inhibitor of urokinase, the role of the intracellular unglycosylated form remains more enigmatic. Intracellular PAI-2 has been identified as an inhibitor of TNF-α induced apoptosis (25) and is involved in the differentiation of monocytes and keratinocytes (26). Only recently, it has been described that intracellular PAI-2 interacts with the retinoblastoma protein (Rb) and thereby impedes its degradation (27). Interestingly, TuSN exclusively induced the intracellular 47kD form of PAI-2 in monocytes, whereas the expression of the glycosylated secreted 60kD form was not increased (Figure 8). UPAR, a second member of the plasmin system, was also down-regulated by TuSN and ECM invasion of TuSN-treated MM-6 cells was significantly reduced. Moreover, uPAR has also been described to bind β1- and β2-integrins and is therefore involved in monocyte adhesion (16). Taken together, our data provide a first general analysis of the influence of soluble tumor-derived factors on gene and protein expression profiles in monocytes. Further analysis of two out of the 25 identified TuSN-regulated genes revealed that tumor-secreted factors have the capacity to decrease immune cell functions such as invasion capacity and influence immune cells not only in their close vicinity but also in the periphery.

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