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Human Dendritic Cells Produce TGF-β1 under the Influence of Lung Carcinoma Cells and Prime the Differentiation of CD4⁺CD25⁺Foxp3⁺ Regulatory T Cells¹

Ingrid E. Dumitriu,² Donald R. Dunbar, Sarah E. Howie, Tariq Sethi, and Christopher D. Gregory

Dendritic cells (DCs)³ belong to the innate immune system and are uniquely adapted to activate naïve T cells and initiate, coordinate and regulate adaptive immune responses (1, 2). DCs with an immature phenotype reside in peripheral tissues or circulate in blood, where they detect and capture Ags. The induction of immune responses is controlled by a process named DC maturation that is triggered by pathogens, tissue damage, and local inflammation (1, 2). The maturation of DCs increases the expression and production of molecules involved in Ag processing and presentation and further interaction with T cells (HLA-DR and costimulatory molecules like CD86). Furthermore, DCs dictate the fate of T cells that respond to Ag by sustaining and regulating their proliferation and differentiation into effector cells (3). Recent data suggest that DCs are pivotal not only for the generation of adaptive immune responses but also for the induction and maintenance of T cell tolerance (4–6). This phenomenon has important physiological roles in immune homeostasis and prevention of autoimmunity, but can also contribute to the subversion of the immune response in cancer (4–6).

Cancer is a chronic disease that becomes clinically visible only after a considerable interval of time. During this period, there is a mutual interaction between the tumor cells or their products and the host immune system, which often induces alterations that ensure the survival of the tumor (7). The generation of effective immune responses to tumors requires the coordinated action of DCs and effector T lymphocytes (8). Therefore, DCs might constitute an early target for subversion by developing tumors. One way in which tumors evade the immune system is by inhibiting the maturation and function of DCs (9). Soluble factors like vascular endothelial growth factor (VEGF) and PGE₂ present in the tumor microenvironment have been shown to inhibit the maturation of DCs (10–12), thereby promoting tolerance induction. Secretion of IL-6 by breast cancer cells biases the differentiation of monocytes into macrophages instead of DCs, thereby switching Ag presentation into Ag degradation at the expense of the immune response (13). Other tumors promote the differentiation of DCs producing IL-10 that induce the expansion of CD4⁺CD25⁺ regulatory T cells which contribute to tumor escape (14, 15). Another immune evasion mechanism used by tumors is the altered expression of costimulatory and inhibitory B7 molecules that prevents T cell-mediated destruction of tumors (16).

Lung cancer is one of the most common solid malignancies and is associated with very poor survival rates (17). The most frequent variant of lung cancer is the non-small cell lung
carcinoma (18). Patients with non-small cell lung carcinoma have been reported to have reduced frequency of circulating DCs (19). Also, tumor-infiltrating DCs have been shown to have characteristics of immature cells (20). However, the mechanisms by which lung cancer alters DC function are yet to be defined in detail.

In this study, we report the effects of lung carcinoma cells (LCCs) on human monocyte-derived DCs. We demonstrate that LCCs convert fully differentiated immature and mature DCs (mDCs) into cells producing TGF-β1. These TGF-β1-producing DCs are impaired in their ability to activate CD4+ T cells and are prone to generating CD4+CD25+Foxp3+ regulatory T cells.

Materials and Methods

Cell lines

The following human cell lines were purchased from American Type Culture Collection: SK-MES-1 (squamous cell carcinoma), A549 (adenocarcinoma), 16HBE (human bronchial epithelial cell line), and BJ (human primary fibroblasts). A549 cells were maintained in RPMI 1640 (Life Technologies/Invitrogen) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1.5 mM L-glutamine, and 10% (Life Technologies/Invitrogen) heat-inactivated FCS (PAA). The other cell lines were cultured in DMEM (Life Technologies/Invitrogen) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1.5 mM L-glutamine, 0.1 mM nonessential amino acids (Sigma-Aldrich), and 10% heat-inactivated FCS. All cell lines were routinely confirmed to be Mycoplasma negative.

Generation of DCs

PBMCs were isolated from healthy donor blood by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences). The method for the generation of DCs from PBMCs has been previously described (21). Briefly, monocytes isolated by adherence to plastic were incubated with GM-CSF (20 ng/ml; R&D Systems) and IL-4 (20 ng/ml; R&D Systems) for 6 days. Purity and maturation of DCs was routinely assessed by staining with Abs to CD1a, CD14, CD40, CD83, HELA-DR, and CD40 (BD Biosciences). Immature DCs (iDCs) were CD14+, CD1a+, CD11c+, CD83+, CD80+, CD86+, and HELA-DR+. DCs were used either immature or were stimulated with 1 μg/ml LPS (Escherichia coli 026:B6; Sigma-Aldrich). More than 90% of DCs expressed CD83 following maturation with LPS (data not shown). For microarray gene expression experiments, CD14− monocytes purified by immuno-magnetic selection using CD14 microbeads (Miltenyi Biotec) were used for DC generation. The purity of isolated monocytes was routinely above 95%.

Isolation of naive CD4+ T cells

Human CD4+ naive T cells were isolated from PBMCs by immunomagnetic selection using a naive CD4+ T cell isolation kit (Miltenyi Biotec) (21). Purity of naive CD4+ T cells was typically >90−95% as assessed by FACS analysis.

Coculture of DCs with LCCs or T cells

DCs (105) were incubated with LCCs (2×105) in RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1.5 mM L-glutamine, and 10% heat-inactivated FCS. Where indicated, Transwell inserts with 0.4-μm pore size membrane (Corning) were used to physically separate DCs and LCCs. For T cell activation experiments, DCs were extensively washed before incubation with naive allogeneic CD4+ T cells. T cells were mixed with DCs at a ratio of 10:1 and plated at 1.5×106 cells/ml in medium supplemented with 5% pooled human serum (BioWhittaker and Cambrex). On day 3, the cultures were supplemented with 25 U/ml IL-2 (Roche Diagnostics). In some experiments, naive CD4+ T cells were stimulated with 2 μg/ml Con A (Sigma-Aldrich). On day 8, cells were recovered and Th1 and Th2 polarization was assessed as previously described (21). To assess T cell proliferation, freshly isolated lymphocytes were labeled with CFSE (1 μM; Molecular Probes and Invitrogen) and cultured alone or in the presence of allogeneic DCs. Flow cytometry was performed on day 5 or 6 of coculture to assess proliferating cells.

Suppression assay

CD25+ or CD25− T cells were isolated from cocultures of naive CD4+ T cells and allogeneic DCs on day 7 using the CD25+ isolation kit (Miltenyi Biotec). Experiments were performed using immature or mature DCs cultured alone or in the presence of LCCs (as previously mentioned). The purity of CD25+ and CD25− fractions was checked by flow cytometry and was between 90 and 95%. For suppression assays, DCs (derived from the same donor as the CD25+ or CD25− T cells) were matured with LPS (1 μg/ml) and used as stimulators for CFSE-labeled allogeneic naive CD4+ T cells (responders) (22). To test the dependence of suppression on cytokines, 10 μg/ml neutralizing Abs against TGF-β1 or IL-10 (R&D Systems) was added as indicated elsewhere (23). The ratios between the stimulator DCs and the responder allogeneic T cells was 1:10. The suppression function of CD25+ (suppressor) or CD25− (control) cells was tested on the proliferation of CFSE-labeled responder CD4+ T cells (22). The ratio of responder:suppressor (CD25+ or CD25−) T cells varied between 1:1/2 and 1:1/32. The proliferation of CFSE-labeled T cells was assessed at day 5 of coculture by flow cytometry.

Microarrays

For coculture with DCs, LCCs were placed in the inner chamber of Transwell inserts with a 0.4-μm pore size membrane and cultured for 40 h. RNA was isolated from DCs using a RNeasy mini kit (Qiagen). The Scottish Centre for Genomic Technology and Informatics processed the RNA samples according to the Affymetrix protocol and performed the hybridization to Affymetrix Human Genome U133_2 GeneChip arrays. The data were extracted and analyzed by the Scottish Centre for Genomic Technology and Informatics team and the Bioinformatics team in the Centre for Inflammation Research, University of Edinburgh (accession no. E-MEXP-1958; at ArrayExpress http://www.ebi.ac.uk/microarray-as/ae).

Detection of cytokines

The IL-12p70, TNF-α, and TGF-β1 ELISA kits were purchased from R&D Systems and the quantification of the cytokines was performed according to the manufacturer’s instructions. The supernatants used for the cytokine ELISAs were collected immediately or stored frozen until analysis. Samples and standards were analyzed in duplicate. For the TGF-β1 ELISA, cells were cultured in X-Vivo 20 serum-free medium (BioWhittaker and Cambrex) and the samples were treated as per the manufacturer’s instructions to measure active TGF-β1.

Flow cytometry

Surface Ag staining was performed using mouse mAbs against CD4, CD11c, CD25, CD86, and HELA-DR (BD Biosciences). For the experiments in which the tumor cells and the DCs were cocultured directly and the expression of various surface markers by DCs was determined, the DCs were gated as CD11c+ cells. The expression of CD86 and HELA-DR by the CD11c+ cells was then analyzed. To investigate the expression of TGF-β1 by DCs, cells were stained with TGF-β1/latency-associated peptide (LAP; R&D Systems) or isotype-matched control Abs (24). Intracellular cytokine content was determined after stimulation of cells with 50 ng/ml PMA (Sigma-Aldrich) and 1 μg/ml ionomycin (Calbiochem and Merck Chemicals). GolgiStop (BD Biosciences) was added during the last 2 h of culture to prevent cytokine secretion. Cells were fixed and permeabilized using Cytofix/Cytoperm (BD Biosciences) and then stained with FITC-conjugated anti-IFN-γ and PE-conjugated anti-IL-4 mAbs (BD Biosciences). Foxp3 expression in T cells was assessed at various time points using a human naive T cell staining kit (eBioscience) as per the manufacturer’s instructions. Cells were simultaneously stained with CD25allophycocyanin and CD4-FITC (BD Biosciences). The samples were acquired on a BD Biosciences FACSCalibur. Data analysis was performed using FlowJo software (Tree Star).

Statistical analysis

Statistical analysis was performed using the two-tailed Student’s t test for unpaired samples with unequal variance. Probability values (p) of less than 0.05 were considered statistically significant.

Results

LCCs decrease the expression of CD86 and HELA-DR and the production of IL-12 and TNF-α by DCs

We evaluated the effect of SK-MES-1 and A549 cell lines (LCCs) on DC maturation by culturing iDCs alone or in the presence of LCCs.
and monitoring the expression of surface markers after 48 h. These two cell lines are characteristic of the most frequent histopathological types of non-small cell lung carcinoma, which accounts for 70–80% of all lung cancer cases (18). The expression of CD86 and HLA-DR was significantly reduced on DCs cultured with LCCs compared with iDCs alone (Fig. 1, A and C). This effect was specific to the LCCs...
since the control cells tested (BJ primary human fibroblasts) did not alter significantly the phenotype of iDCs (Fig. 1A). Additionally, in the presence of LCCs, the expression of CD11c was not significantly changed, ruling out a generalized inhibitory effect of LCCs on DCs (Fig. 1A). DCs stimulated with LPS are known to up-regulate the expression of surface markers (25). We therefore assessed whether LCCs had an effect on mature DCs as well. For this purpose, DCs were stimulated with LPS alone or in the presence of LCCs for 48 h. In keeping with the observations on iDCs, LPS-stimulated DCs expressed significantly decreased levels of CD86 when cultured with LCCs (Fig. 1, B and C). However, the expression of HLA-DR and CD11c remained unchanged (Fig. 1, B and C). LCCs had no effect on the survival of DCs as similar numbers of viable immature and mature DCs were obtained from DCs cultured alone or in the presence of tumor cells (6.15 ± 0.07, 6.6 ± 0.96, and 6.3 ± 0.72 million cells, mean ± SD for iDCs, iDCs plus SKM, and iDCs plus A549, respectively; 6.72 ± 0.38, 7.27 ± 0.85, and 7.64 ± 1.31 million cells, mean ± SD for mDCs, mDCs plus SKM, and mDCs plus A549, respectively).

In addition to changes in the expression of surface markers, mature DCs also secrete cytokines like TNF-α, a potent inflammatory stimulus, and IL-12 that regulates the differentiation of CD4+ T cells into Th1 effectors (1, 26, 27). Interestingly, LCCs decreased both the production of IL-12p70 and TNF-α by mDCs (Fig. 1D). This effect was specific to LCCs because primary human fibroblasts (BJ) and human bronchial epithelial cells (16HBE) failed to suppress the secretion of cytokines (Fig. 1D). No effect on the production of IL-12p70 and TNF-α by iDCs was observed in the presence of LCCs (Fig. 1D).

The inhibitory effects of LCCs on DCs are not dependent on contact

The effects observed with LCCs on the maturation of DCs could be attributed either to a contact-dependent inhibition or to soluble factors released by LCCs. We used Transwell inserts to prevent direct contact between DCs and LCCs in culture. As described in Fig. 1, LCCs cultured directly with iDCs inhibited CD86 and HLA-DR expression (Fig. 2A). Interestingly, this suppression was maintained even when direct contact between LCCs and DCs was prevented (Fig. 2A). Similarly, preventing direct contact between LCCs and DCs in the cocultures still resulted in a significant reduction in the production of IL-12p70 and TNF-α by mature DCs (Fig. 2B). This decreased cytokine production was comparable to that observed when DCs were cultured with LCCs directly (Fig. 2B). These results suggest that the effects of LCCs on DCs are not dependent on contact, but are mainly mediated by soluble factors.

LCCs induce DCs to produce the anti-inflammatory cytokine TGF-β1

To dissect potential mechanisms by which LCCs alter DC maturation, we performed DNA microarray experiments. We analyzed gene expression in iDCs cultured alone or in the presence of LCCs. DCs induced to mature by stimulation with LPS were also included in the analysis. D. SK-MES1 and A549 up-regulate the expression of TGF-β1 and TGF-βRII genes by iDCs. LPS induces the down-regulation of these genes. Results are expressed as the percentage increase of the expression values in comparison to iDCs cultured alone. Error bars indicate mean ± SD of three independent experiments performed with DCs from different donors. *, Significant difference from controls; *, p < 0.05 and **, p < 0.01.
In this work, we focused on genes for cytokine/cytokine-receptor pairs that exhibited significant changes in their expression levels. Examples of cytokine/cytokine-receptor pairs showing changes in gene expression are listed in Fig. 2C. Interestingly, the microarray gene analysis revealed a coordinated increase in the expression of the genes for TGF-β1 and its receptor TGF-βRII in iDCs cultured with the two types of LCCs in comparison to iDCs alone (Fig. 2D). In contrast, the expression of TGF-βII and TGF-βRII genes was down-regulated in DCs treated with LPS. This pattern (up-regulated gene expression in the presence of LCCs and down-regulated expression following LPS treatment) was not noted with other cytokines like VEGF and IL-10 and their receptors. Additionally, VEGF and IL-10 could not be detected at protein level in iDCs alone or following coculture with LCCs (data not shown).

The increase in TGF-β1 mRNA in DCs cultured with LCCs also translated into an increased production of the protein. This was assessed by measuring the secretion of TGF-β1 by DCs using ELISA. Both iDCs and LCCs were noted to spontaneously secrete TGF-β1 (Fig. 3A). However, when iDCs were cultured with LCCs, the TGF-β1 production was significantly increased over the baseline secretion by DCs alone (Fig. 3A). Furthermore, the TGF-β1 detected in the cocultures of DCs with LCCs was significantly higher than the sum of TGF-β1 derived from DCs and LCCs alone, ruling out a mere additive effect (Fig. 3A). Similarly, mDCs cocultured with LCCs also exhibited increased production of TGF-β1 (data not shown).

We next verified whether this increase in TGF-β1 was due to elevated production by DCs or by LCCs. For this purpose we used monensin, a reagent that inhibits the secretion of proteins by altering their trans-Golgi transport (28). We observed that monensin abrogated the production of TGF-β1 by LCCs, while leaving the TGF-β1 from DCs unaltered (Fig. 3B).

### FIGURE 3. LCCs increase TGF-β1 production by DCs. A. The effect of SKM and A549 on the production of TGF-β1 by DCs was monitored by ELISA. iDCs were cultured alone or in the presence of SKM/A549 cells (iDC + SKM co and iDC + A549 co, respectively) for 24 h. SKM and A549 designate the production of TGF-β1 by cell lines cultured alone. The numerical sum of TGF-β1 secreted by either DCs alone or SKM/A549 cells alone is indicated (iDC + S sum and iDC + A sum). B. iDCs were cultured alone or with SKM/A549 cells (iDC + SKM cocult. and iDC + A549 cocult., respectively) for 24 h. Where indicated, monensin was added to the cultures after 6 h. The numerical sum of TGF-β1 secreted by either DCs alone or SKM/A549 cells alone is indicated (iDC + SKM sum and iDC + A549 sum, respectively). C. DCs stimulated with LPS (mDCs) were cultured as above and TGF-β1 was measured in the supernatants. Data represent the average of duplicate samples ± SD and are representative of three independent experiments. *, Significant difference from controls; **, p < 0.05 and ***, p < 0.01.
DCs with LCCs was sustained in the presence of monensin (Fig. 3B), indicating that the DCs were indeed the source of increased TGF-β1 in the cocultures. These findings were in keeping with the results obtained in the gene expression microarray experiments (Fig. 2D). Additionally, we observed that mature DCs cultured with LCCs in the presence of monensin similarly produce significantly more TGF-β1 (Fig. 3C). The precise mode of action of monensin in blocking TGF-β1 secretion from LCCs but not from DCs is not known. Because DCs are specialized cells that secrete various cytokines in addition to processing and presenting Ags, it is possible that the mechanisms that regulate the secretion of TGF-β1 from DCs differ from those present in tumor cells.

Neutralization of TGF-β1 restores the production of IL-12 and TNF-α by DCs cultured with LCCs

We next investigated whether TGF-β1 was indeed the factor responsible for the inhibitory effects observed on DCs following treatment with LCCs. For this purpose, we used Abs against TGF-β1 (1 µg/ml) to neutralize TGF-β1 in the cultures. The neutralizing Abs per se did not have any effect on cytokine release by iDCs. Interestingly, the production of IL-12p70 and TNF-α by mature DCs cocultured with LCCs was restored in the presence of TGF-β1-neutralizing Abs to levels comparable to those produced by mDCs cultured alone (Fig. 4, A and B), suggesting that the triggered TGF-β1 alters the cytokine production by DCs. Isotype-matched control Abs did not influence the inhibitory effects of LCCs on the secretion of cytokines by mDCs (Fig. 4, A and B). To confirm the results obtained in the presence of monensin, which indicated that DCs were the potential source of the functionally active TGF-β1, we designed experiments using Transwell cocultures. In these experiments, the LCCs were added in Transwells (0.4-µm pore size) and cultured with DCs for 24 h. Then, the Transwells containing the LCCs were removed and the DC medium was replaced with fresh medium. These tumor-conditioned DCs were then stimulated with LPS alone or in the presence of neutralizing Abs against TGF-β1. IL-12p70 was quantified in the supernatants after 24 h. Tumor-conditioned DCs produced less IL-12p70 than DCs cultured alone (Fig. 4C). Treatment with anti-TGF-β1-neutralizing Abs significantly restored the production of IL-12p70 by tumor-conditioned DCs (Fig. 4C). These results indicate that the LCCs trigger the production of TGF-β1 by DCs and that this TGF-β1 is functionally active because it interferes with the ability of the DCs to secrete cytokines. We further confirmed that LCC-conditioned DCs were indeed the source of TGF-β1 by evaluating the expression of surface-bound TGF-β1. For this purpose, we used an Ab directed against cell-bound TGF-β1, which is known to associate noncovalently with LAP (24). Tumor-conditioned DCs expressed higher levels of TGF-β1/LAP on their surface than DCs cultured alone (Fig. 4D). The average expression of TGF-β1/LAP was 64 ± 3.08 (iDCs) and 80 ± 3.04 (mDCs plus SKM) (mean ± SD, p < 0.01; 37 ± 1.06 (mDCs), and 45 ± 5.3 (mDCs plus SKM). These data lend further support to our hypothesis that LCCs trigger TGF-β1 production by DCs.

LCCs impair the ability of DCs to drive the proliferation and differentiation of CD4+ T cells into Th1 effectors

DCs are the most potent APCs capable of activating naïve T cells (1–3). Upon activation, T cells proliferate and differentiate into effectors that mediate immune responses (29). Because we observed that LCCs induce changes in DC maturation, we examined whether there was an effect on the activation of CD4+ T cells. DCs were assessed for their capacity to trigger the proliferation of allogeneic naive T cells. We monitored the proliferation of T cells by measuring the dilution of CFSE. Both immature and mature DCs induced expansion of CD4+ T cells resulting in four to five rounds of cell division (Fig. 5A, upper and middle panels). As expected, mature DCs were more efficient at driving CD4+ T cell proliferation compared with iDCs (1). The capacity of both immature and mature DCs to sustain the proliferation of CD4+ T cells was significantly decreased when the DCs were cultured with LCCs (Fig. 5A, upper and middle panels). This observation is in keeping with the decreased expression of CD86 and HLA-DR by DCs cultured with LCCs. However, the proliferation of naïve CD4+ T cells induced with the mitogen Con A remained unchanged in the presence of LCCs (Fig. 5A, bottom panel). This suggested that the decreased proliferation of CD4+ T cells noted in the allogeneic cultures was mediated via an inhibitory effect of LCCs on the DCs and not by direct action on T cells.

Production of IL-12 by mDCs regulates the differentiation of CD4+ T cells into Th1 effector cells which produce IFN-γ (27, 29, 30). As one of the effects of LCCs on DCs was decreased production of IL-12p70, we monitored the differentiation of CD4+ T cells into Th1 effectors. mDCs efficiently induced the differentiation of CD4+ T cells into IFN-γ+ Th1 effectors (Fig. 5B). When DCs cultured with LCCs were used for priming of allogeneic CD4+ T cells, a significant reduction in the percentage of IFN-γ+ Th1 cells was observed (Fig. 5B). No significant changes were noted in the percentage of IL-4+ Th2 cells.

LCCs inhibit the ability of DCs to generate CD25+ activated T cells and increase the induction of CD4+CD25+Foxp3high T cells

Upon activation, T cells up-regulate the expression of CD25, the α-chain of the IL-2R that associates with the βγ-chains to form the high-affinity receptor and makes the T lymphocytes more sensitive to IL-2 (31). Very few of the freshly isolated naïve CD4+ T cells express low levels of CD25 (Fig. 6A). Priming of naïve CD4+ T cells with allogeneic DCs (immature or mature) resulted in the appearance of T cells expressing high levels of CD25 (Fig. 6A). When DCs cultured with LCCs were used for priming, the percentage of activated CD25+ T cells was significantly lowered (Fig. 6A). Similar results were obtained with both immature and mature DCs. These results demonstrate that LCCs decrease the ability of DCs to sustain the activation of CD4+ T cells.

As mentioned previously, LCCs increased TGF-β1 production by both immature and mature DCs (Fig. 3). TGF-β1 is involved in the differentiation of CD4+ T cells into Foxp3+ regulatory T cells (32). We therefore examined the ability of DCs cultured in the presence of LCCs to generate Foxp3+ T cells. Immature and mature DCs were found to induce the differentiation of CD4+ T cells into CD4+CD25+Foxp3+ T cells (Fig. 6B). Interestingly, LCCs significantly enhanced the capacity of DCs to generate CD4+CD25+Foxp3+ T cells (Fig. 6B). Indeed, a significant increase in the percentage of CD4+CD25+Foxp3+ T cells was detected when the DCs used for activation of T cells were cultured with LCCs (Fig. 6B). In addition, the expression of Foxp3, as measured by the mean fluorescence intensity (MFI) of the whole population of T cells, was significantly increased when DCs cultured with LCCs were used to prime the T cells (Fig. 6B). LCCs did not influence Foxp3 expression when Con A was used to induce the activation of T cells (Fig. 6B).
The CD4⁺CD25⁺Foxp³⁺ T cells induced by DCs under the influence of LCCs function as regulatory T cells and suppress the proliferation of T lymphocytes

To test whether the CD4⁺CD25⁺Foxp³⁺ T cells induced by DCs were regulatory T cells, we assessed their suppressive function. For this purpose, we used a suppressor assay described by Steinman and Dhodapkar (22), in which naïve CD4⁺ T cells (responder) are activated by allogeneic DCs and then the suppressive effect of CD4⁺CD25⁺Foxp³⁺ T cells on the proliferation of responder T cells is quantified. CD4⁺CD25⁺Foxp³⁺ T cells were generated as before from cocultures of DCs and allogeneic naïve CD4⁺ T cells. On day 7 of culture, CD25⁺ (suppressor) or CD25⁻ (control) T cells were isolated by immunomagnetic selection using a CD25⁺...
The purity of CD25+/H11001 or CD25+/H11002 T cells was between 90 and 95% as assessed by FACS analysis (Fig. 7A).

The sorted CD25+/H11001 or CD25+/H11002 T cells were then used as suppressors (responder:suppressor ratio, 1:1/2–1:1/32) in the suppressor assay mentioned above (22). The mDCs used to drive the proliferation of naive responder CD4+/H11001 T cells were from the same donor as the one used to generate the CD4+/H11001 CD25+/H11001 Foxp3+/H11001 T cells (22). The proliferation of responder CD4+ T cells but not the CD25− T cells inhibited the proliferation of responder T cells (Fig. 7, B–E). Similar results were obtained when either mature (Fig. 7, B–D) or iDCs (Fig. 7E) were used for the generation of CD4+/H11001 CD25+/H11001 Foxp3+/H11001 T cells. These results demonstrate that the CD4+/H11001 CD25+/H11001 Foxp3+/H11001 T cells have regulatory function since they suppress T cell proliferation. Using this suppressive assay, we then compared the regulatory function of CD4+/H11001 CD25+/H11001 Foxp3+/H11001 T cells generated by DCs cultured in the presence of LCCs with those generated by DCs cultured alone. The CD25+/H11001 T cells generated using DCs cultured with LCCs were more efficient as suppressors in comparison to the CD25+ T cells generated by DCs alone (Fig. 7, A).

FIGURE 5. LCCs impair the ability of DCs to induce the proliferation and differentiation of CD4+ T cells. A, Naive CFSE-labeled CD4+/H11001 CD45RA+ T cells were cultured with allogeneic iDCs and mDCs. DCs cultured in the presence of LCCs (DC + SKM, DC + A549) were also used as stimulators. The effect of LCCs on Con A-induced T cell proliferation was also monitored. Proliferation was measured by flow cytometry on day 5 or 6. This figure represents one of three similar experiments. The bar graphs represent the mean ± SD of triplicate samples. Results are representative of three independent experiments. B, Allogeneic mDCs cultured alone or in the presence of LCCs (DC + SKM, DC + A549) were used to activate naive CD4+/H11001 CD45RA+ T cells. Differentiation of activated T cells into Th1 (IFN-γ+) or Th2 (IL-4+) effectors was assessed by intracellular staining and flow cytometry on day 8. The numbers in the dot plots indicate the percentage of T cells. The bar graphs depict the percentage of IFN-γ+ T cells as mean ± SD of triplicate samples. Results are representative of three independent experiments. * Significant difference from controls; ** p < 0.01 and *** p < 0.001.
FIGURE 6. LCCs decrease the capacity of DCs to activate CD4+ T cells and increase the generation of CD4+CD25+Foxp3+ T cells. A. Naive CD4+CD45RA+ T cells were activated with allogeneic iDCs and mDCs cultured alone or in the presence of LCCs (DC + SKM, DC + A549). The activation of T cells was monitored by flow cytometry by analysis of CD25 expression on day 8. Control staining with isotype matched Abs (iso) and CD4/CD25 labeling of naive T cells (T naive) are also shown. Numbers adjacent to the rectangular gates indicate the percentage of CD4+CD25+ T cells. The bar graphs depict the percentage of CD4+CD25+ T cells as mean ± SD of triplicate samples. Results are representative of three independent experiments. B. Naive CD4+CD45RA+ T cells were activated as above and the presence of CD4+CD25+Foxp3+ T cells was monitored by flow cytometry on day 8. The effect of LCCs was also tested when Con A was used for activation of the naive T cells. Open histograms display the expression of Foxp3 on CD4+CD25+ gated T cells, while filled histograms represent staining with isotype-matched control Abs. The numbers adjacent to the upper linear gate indicate the percentage of CD4+CD25+Foxp3high T cells. The numbers adjacent to the lower linear gate indicate the MFI of Foxp3 on the entire T cell population. The bar graphs on the right display the percentage of CD4+CD25+Foxp3high T cells and the MFI of Foxp3 (mean ± SD of triplicate samples). Results are representative of three independent experiments. *, Significant difference from controls; **, p < 0.001.
FIGURE 7. LCCs increase the capacity of DCs to generate CD4+CD25+Foxp3+ regulatory T cells. A, Naive CD4+CD45RA+ T cells were activated with allogeneic iDCs and mDCs cultured alone or in the presence of LCCs (mDC + SKM, mDC + A549). On day 7, CD25+ and CD25+ T cells were isolated by immunomagnetic sorting. The plots display the post-sort purity of the fractions used as suppressor cells in the suppressor assay. B, CD25+ and CD25+ T cells isolated as in A were tested for their capacity to suppress the proliferation of CFSE-labeled responder T cells. mDCs (from the same donor as the one used to generate the CD25+ or CD25+ T cells) were cultured with CFSE-labeled allogeneic responder naive CD4+ T cells at a 1:10 ratio. CD25+ (suppressor) or CD25+ (control) T cells were added to the cultures at a responder:suppressor ratio of 1:1/2–1:1/32. Five days later, flow cytometry was performed to assess the proliferation of responder cells in the cultures. The numbers indicate the percentages of proliferating and nonproliferating T cells. C, Graphs display the percentage of proliferating responder T cells following addition of CD25+ (suppressor) T cells generated in the presence of mDCs cultured alone or with LCCs (m + SKM, m + A549). D, Graphs display the percentage of proliferating responder T cells following addition of CD25+ (suppressor) or CD25+ (control) T cells generated in the presence of mDCs cultured alone or with LCCs (m + S. m + A). E, Graphs display the percentage of proliferating responder T cells following addition of CD25+ (suppressor) T cells generated in the presence of iDCs cultured alone or with LCCs (i + SKM, i + A549). F, Anti-TGF-β or anti-IL-10 Abs were added to suppressor assays performed as in C. G, Contact between CD25+ (suppressor) and responder T cells was prevented using Transwells (0.4-μm pore size) and suppressor assays were performed as in C. Results (mean of triplicate cultures ± SD) are representative of five independent experiments performed with cells from different donors. *, Significant difference from controls; **, p < 0.01 and ***, p < 0.001.
These results are in agreement with the increased percentage and expression of Foxp3 in the T cells generated by DCs under the influence of tumor cells compared with DCs cultured alone (Fig. 6B). We further evaluated whether the suppressive effects of the generated CD25+ T cells were dependent on cytokines. For this purpose, neutralizing Abs against IL-10 or TGF-β were added to the cell cultures in the suppression assay. We found that the suppressive function of CD25+ T cells remained unchanged following neutralization of these effector cytokines (Fig. 7F). We then performed suppressor assays in which direct contact between regulatory CD25+ and responder T cells was prevented using Transwells. Of note, regulatory CD25+ T cells failed to suppress the proliferation of responder T cells when direct cell-cell contact was prevented (Fig. 7G). These results suggest that the suppressive function of regulatory CD25+ T cells generated by tumor-conditioned DCs is contact mediated and not influenced by IL-10 and TGF-β effector cytokines.

Discussion

During cancer development, a complex and reciprocal interaction occurs between tumor cells and cells of the immune system (7). Chronic activation of innate immune cells, like macrophages, at neoplastic sites has been shown to enhance the development of tumors (33, 34). However, the eradication of tumor cells requires full activation of adaptive immune cells like T lymphocytes, a crucial step performed by DCs (8). Inhibition of DC maturation and function is probably one of the means by which tumors escape immune surveillance (9). Production of immunosuppressive mediators like VEGF and PGE2 by tumor cells has been shown to inhibit the maturation of DCs (10–12). Additionally, tumor cells may also signal inflammatory cells in their vicinity to release suppressive cytokines such as IL-10, enabling the tumor to escape immune surveillance (14, 15). The precise alterations induced in human DCs in the lung cancer microenvironment are relatively unexplored. In this study, we demonstrate that LCCs modify DCs and increase their capacity to secrete the anti-inflammatory cytokine TGF-β1. Our microarray gene expression analysis revealed an increase in the TGF-β1 transcripts in DCs exposed to LCCs, which was also validated at protein level by ELISA and by flow cytometry. This is an interesting and novel finding because it clearly indicates that human lung cancer cells reprogram human DCs and convert them into TGF-β1-secreting cells. TGF-β1, a cytokine with complex functions, modulates cell growth and survival and has important roles in the immune system (35). It regulates lymphocyte proliferation, differentiation, and survival. Furthermore, TGF-β1 has been shown to inhibit the differentiation of CD4+ T cells into Th1 effectors (36). Indeed, we found that LCCs reduced the ability of DCs to activate naive CD4+ T cells and sustain their differentiation into Th1 (IFN-γ) effectors.

Secretion of IL-12 by DCs dictates the differentiation of activated T cells into effectors producing IFN-γ. TGF-β1-secreting DCs were found to have an impaired production of IL-12, which would explain their poor capacity to drive Th1 differentiation. Tumor cells secrete a variety of factors such as VEGF, MCP-1, and IL-10 capable of impairing the DC function. It remains to be assessed whether such soluble factors either individually or in various combinations could also enable human DCs to produce TGF-β1.

Increasing evidence supports the presence of higher numbers of CD4+ CD25+ Foxp3+ regulatory T cells in solid tumors and hematologic malignancies (37). Accumulation of Foxp3+ regulatory T cells is one of the mechanisms preventing the function of antitumor effector T lymphocytes (38–40). However, little is known about the molecular and cellular mechanisms responsible for the increase and maintenance of elevated levels of regulatory T cells in cancer. DCs have pivotal roles in the induction of tolerogenic/regulatory T cells (41). We observed...
that LCCs increased the capacity of iDCs to generate CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T cells from CD4\(^+\)CD25\(^-\) naive T lymphocytes. This observation might be explained by the low levels of Ag-presenting and costimulatory molecules on iDCs following interaction with LCCs. In addition, the increased production of TGF-\(\beta\) could render the LCC-modulated iDCs more prone to induction of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T cells.

In contrast to iDCs, mDCs are best equipped for triggering Ag-specific T cell responses (1, 2, 25). However, it has recently been shown that mature DCs can also activate and expand naturally occurring CD4\(^+\)CD25\(^+\) regulatory T cells (22). Indeed, we observed that mDCs were capable of generating CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T cells. Interestingly, upon exposure to LCCs, mDCs were even more potent in generating CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T cells. Because LCCs increased TGF-\(\beta\)1 production by mDCs, this might explain the augmented generation of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T cells.

Our data indicating that TGF-\(\beta\)1 production by human DCs drives the generation of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T cells is supported by some previous reports using recombinant TGF-\(\beta\)1 in murine models. It has been reported that recombinant TGF-\(\beta\)1 can convert murine CD4\(^+\)CD25\(^-\) naive T cells into CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T cells following TCR stimulation with anti-CD3 Abs (32). An increased generation of Foxp3\(^+\) T cells was also observed when murine DCs pulsed with specific peptides were supplemented with rTGF-\(\beta\)1 (42). It has also been shown in murine models that tumors can induce DCs to express TGF-\(\beta\)1 and promote the expansion of regulatory T cells (43).

We also demonstrated that the CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T cells generated in the presence of LCCs by human DCs are endowed with suppressive function and that the suppressive effects were dependent on contact and independent of the production of IL-10 and TGF-\(\beta\) by CD4\(^+\)CD25\(^+\) T cells. Even more, the CD25\(^+\) suppressor T cells induced by DCs cultured in the presence of LCCs were more efficient suppressors compared with the CD25\(^+\) T cells induced by DCs alone. This might be explained by the increased percentage and expression of Foxp3 by T cells cocultured with LCC-modulated allogeneic DCs.

To the best of our knowledge, this is the first report that human DCs can be licensed by tumor cells to produce TGF-\(\beta\)1. This has important implications for the use of DCs in vaccine therapy, as tumor cells seem capable to render fully differentiated DCs, whether immature or mature, into TGF-\(\beta\)1 producers that hamper the generation of Th1 effector cells and promote the induction of CD4\(^+\)CD25\(^+\)Foxp3\(^+\) regulatory T cells.

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Disclosures
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