Non-small Cell Lung Cancer Induces an Immunosuppressive Phenotype of Dendritic Cells in Tumor Microenvironment by Upregulating B7-H3

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Introduction: Tumors may shift the phenotype and function of dendritic cells (DC) toward the induction of tolerance. In the status of full maturity, DC express a multitude of T cell costimulatory molecules enabling them to induce immune reactions, whereas nonactivated resident DC lack these T cell stimulating capacities. Therefore, we investigated the changes in DC phenotype and expression of B7-H molecules induced by non-small cell lung cancer (NSCLC).

Methods: The expression of T cell coinhibitory B7 molecules (B7-DC, B7-1, B7-2, B7-H1, B7-H3) on DC isolated from malignant and nonmalignant lung and lymph node tissue from patients attending curative surgery for NSCLC ($n = 12$) was analyzed. T cell stimulatory functions of DC isolated from malignant and nonmalignant lung and lymph node tissue samples were measured by allogeneic mixed lymphocyte reactions. Furthermore, the secretion of IL-10 and IL-12p40 by DC was analyzed (enzyme-linked immunosorbent assay).

Results: B7-H3 was significantly upregulated in tumor-residing DC, whereas the expression of other B7 molecules, such as B7-DC, B7-1, B7-2, B7-H1, remained unchanged. Significantly reduced levels of T cell proliferation in mixed lymphocyte reactions with tumor-derived DC were recorded. Moreover, elevated concentrations of IL-10 were measured in tumor-derived DC, whereas IL-12 levels were reduced.

Conclusion: Our data indicate that (1) DC derived from NSCLC are immunosuppressive, and (2) under tumor conditions the coinhibitory molecule B7-H3 plays a crucial role in mediating the T cell suppressive effects of DC.

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Disclosure: The authors declare no conflicts of interest.

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ISSN: 1556-0864/11/0607-1162

Key Words: B7-H3, Dendritic cells, Lung cancer, Tumor microenvironment, Immunosuppression.

(*J Thorac Oncol.* 2011;6: 1162–1168)

Lung cancer is the leading cause of cancer death with growing incidence in both developed and developing countries. The prognosis remains poor, and the need for novel therapy approaches is apparent.[1,2] Particularly, in immunogenic malignancies, such as malignant melanoma and renal carcinoma, dendritic cell (DC)-based vaccinations have shown promising results but also in lung cancer clinical trials were successfully completed.[3–6] Indeed, DC are a highly heterogeneous cell population, and numerous functional subsets have been identified.[7] In the status of full maturity, DC express a multitude of T cell costimulatory molecules enabling them to induce immune reactions, whereas nonactivated resident DC lack these T cell stimulating capacities.[8] Despite the constant immune monitoring of the body by DC, apparently several tumors create a privileged microenvironment that protects cancer cells from immune destruction. The underlying means by which non-small cell lung cancer (NSCLC) escapes immune surveillance by DC are not completely understood; however, similar to other types of cancer such as melanoma, NSCLC has clearly immunosuppressive activity, as it raises the frequency as well as the suppressive capacity of regulatory T cells.[9,10] Moreover, NSCLC stimulates the release of immunosuppressive cytokines by PBMC and induces expression of suppressive surface molecules, such as the programmed cell death-1 receptor on $CD8^+$ T cells.[11,12] In addition to inducing immunosuppressive molecules that can act directly on effector cells of the immune system, NSCLC is also able to prevent the induction of immunity by influencing DC. Here, NSCLC inhibits the maturation of DC and keeps them in an immature and hence less immunogenic state.[13,14] Interestingly, the observed augmented frequency of Treg in NSCLC patients may be attributable to the effects of NSCLC exerted on DC, as NSCLC-derived DC produce high amounts of TGF-β and are strong inducers of Treg.[15] Thus, NSCLC is able to circumvent induction of immunity at least in part by affecting DC maturation and activation. Not only the abrogation of DC

maturation but also the induction of cell surface expression of T cell suppressive molecules on DC has been described in various tumors. Among those molecules that may inhibit immune reactions, members of the B7-H family are known to be upregulated in tumor conditions.[16] These molecules convey mainly suppressive signals inhibiting cytokine production and T cell proliferation in vitro and in vivo. In light of these data, we investigated the changes in DC phenotype and the expression of B7-H molecules induced by NSCLC. Therefore, we analyzed DC isolated from malignant and nonmalignant lung and lymph node tissue samples for surface molecule expression and T cell stimulatory functions and found that the inhibitory molecule B7-H3 and the secretion of the immunosuppressive cytokine IL-10 is upregulated in NSCLC-derived DC.

METHODS

Patients

A total of 12 patients who underwent curative resection of NSCLC (adenocarcinoma) were enrolled in this study; none were subjected to preoperative chemotherapy and/or radiotherapy. Lobectomy with radical mediastinal and hilar lymphadenectomy was performed in all patients. The postoperative pathologic stage for lung cancer was determined according to the seventh Tumor Node Metastasis classification system, revised in 2009.[17] The Ethics Committee of the University of Heidelberg approved this study (Study No. 270/2001). Each patient was informed comprehensively; written consent was given before participation.

Processing of Tissue Specimens

Immediately after surgical resection, tissue samples were extracted. Tumor tissue was taken from an area of solid tumor tissue lacking the gross aspect of massive necrosis. For the representative tumor-free lung tissue samples, tissue was taken from the surgically removed lung in farthermost position opposed to the tumor. In each patient, lymph node tissue was taken from a typically tumor draining position. Immediate frozen sectioning proved tumor infiltration in a total of six lymph node specimens.

Medium and Cell Reagents

All media and cell culture supply, if not stated otherwise, were obtained from PAA, Cölbe, Germany. Falcon tissue culture plasticware was obtained from Becton Dickinson, Heidelberg, Germany.

Single Cell Suspensions

Single cell suspensions of lymph nodes, lung tissue, and lung tumor samples were obtained using Collagenase/Trypsin digestion. Briefly, tissues were cut in pieces by scalpels and placed in petri dishes containing 4000 U/ml Collagenase IV (Cell systems, Troisdorf, Germany), 0.1% (wt/vol) Trypsin (Sigma, Deisenhofen, Germany), 1 mg/ml DNAse I (Sigma) and incubated at 37°C for 25 minutes. Thereafter, ethylenediaminetetraacetic acid (Sigma) was added to a final concentration of 1 mM. After additional 5 minutes incubation, the samples were placed into a cell grinder (Becton Dickinson, Heidelberg, Germany) and processed for 5 minutes. Thereafter, the suspension was filtered through a 0.2-μm cell strainer (Becton Dickinson, Heidelberg, Germany), centrifuged (1.200 rpm, 4°C, 7 minutes), and washed three times with RPMI medium.

FACS Analysis

For flow cytometry (FACS) analysis, cells were adjusted to 1×10^6/200 μl in phosphate-buffered saline (PBS) 1% fetal calf serum (FCS) (vol/vol) and kept on ice. For staining, cells were incubated with FITC-labeled anti-B7DC, anti-B7H1, anti-B7H3, anti B7–1, or anti B7–2 antibodies, respectively, at a final dilution of 1 μg/ml. PE-labeled anti-CD11c antibodies (1 μg/ml, final dilution) were added simultaneously, and cells were incubated for 45 minutes at 4°C (all antibodies were obtained from e-Bioscience, Frankfurt, Germany). Thereafter, samples were washed three times (PBS 1% vol/vol FCS) and analyzed using a FACS Canto (Becton Dickinson). To check the purity of the suspension and the quality of the obtained cells, additional FACS analyses were performed using antibodies defining granulocytes (CD16) and macrophages (CD14; both from Becton Dickinson, Heidelberg).

Isolation of DC and T Cells

The isolation of DC was performed using CD11c-specific MACS MicroBeads (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Briefly, single cell suspensions were diluted in MACS buffer (PBS 0.5% vol/vol BSA, 2 mM ethylenediaminetetraacetic acid) and CD11c micro beads were added. To increase specificity, half the number of beads as indicated in the manufacturer's protocol was added, resulting in a higher purity of the isolated DC. Samples were kept for 45 minutes at 4°C in a rotary shaker. Thereafter, cells were washed three times with ice-cold MACS buffer and placed into MACS buffer equilibrated paramagnetic columns. The columns were washed with 10 ml MACS buffer and the flow through was discarded. Subsequently, columns were removed from the magnetic field and the $CD11c^+$ cells were flushed out, washed, and collected by centrifugation. $CD4^+$ responder T cells were obtained from 20 ml blood of healthy volunteers using the "untouched T cell kit" (Miltenyi) according the manufacturer's protocol. Briefly, PBMC were incubated with a cocktail of biotinylated antibodies directed against $CD8^+$ T cells and non-T cells. After incubation, streptavidin-coated beads were added and the cells were subjected to a paramagnetic column. The flow through was collected and washed. Routinely FACS analysis of the purified $CD11c^+$ cells and of the $CD4^+$ T cells were performed, indicating yields of >80% $CD11c^+$ cells for the DC fraction and >95% for the $CD4^+$ T cell fraction.

Proliferations Assays and Cytokines

For proliferation assays, 5×10^4 DC were cocultivated with 1×10^5 $CD4^+$ T cells in 200 μl complete RPMI medium, supplemented with 10% FCS (vol/vol) in 96-well round bottom plates. As positive control, T cells were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (2 μg/ml) antibodies (e-Bioscience, Frankfurt, Germany). As indicated in the text, to some samples anti-B7-H3 antibodies and

respective isotype controls (both from e-bioscience) were added (final dilution: 10 μg/ml). Cultures were incubated at 37°C, 5% CO_2 for 4 days. For the last 18 hours of culture, 0.5 μCi/ml [^{3}H]-Thymidine (Amersham, Frankfurt, Germany) was added. Cells were harvested and Thymidine incorporation was determined using a PerkinElmer (Rodgau, Germany) scintillation counter. For the determination of cytokine production of DC, 10^5 DC were cultivated for 24 hours in 96-well plates in 200 μl complete medium, supplemented with 10 ng/ml lipopolysaccharides (Sigma, Germany). Thereafter, 100 μl of tissue culture supernatant was removed and analyzed in triplicates for IL-10 or IL-12, respectively, using conventional ELISA kits (e-Bioscience, Frankfurt, Germany) and a ELISA reader (Titertek, Turku, Finland).

Statistical Analysis

As indicated in the figures, differences were tested for significance using an unpaired, one-tailed Student's *t* test.

RESULTS

DC in Tumors Upregulate B7-H3 Expression

We analyzed the expression of different member of the B7 family (B7-DC, B7-H1, B7-H3, B7-1, B7-2) on DC

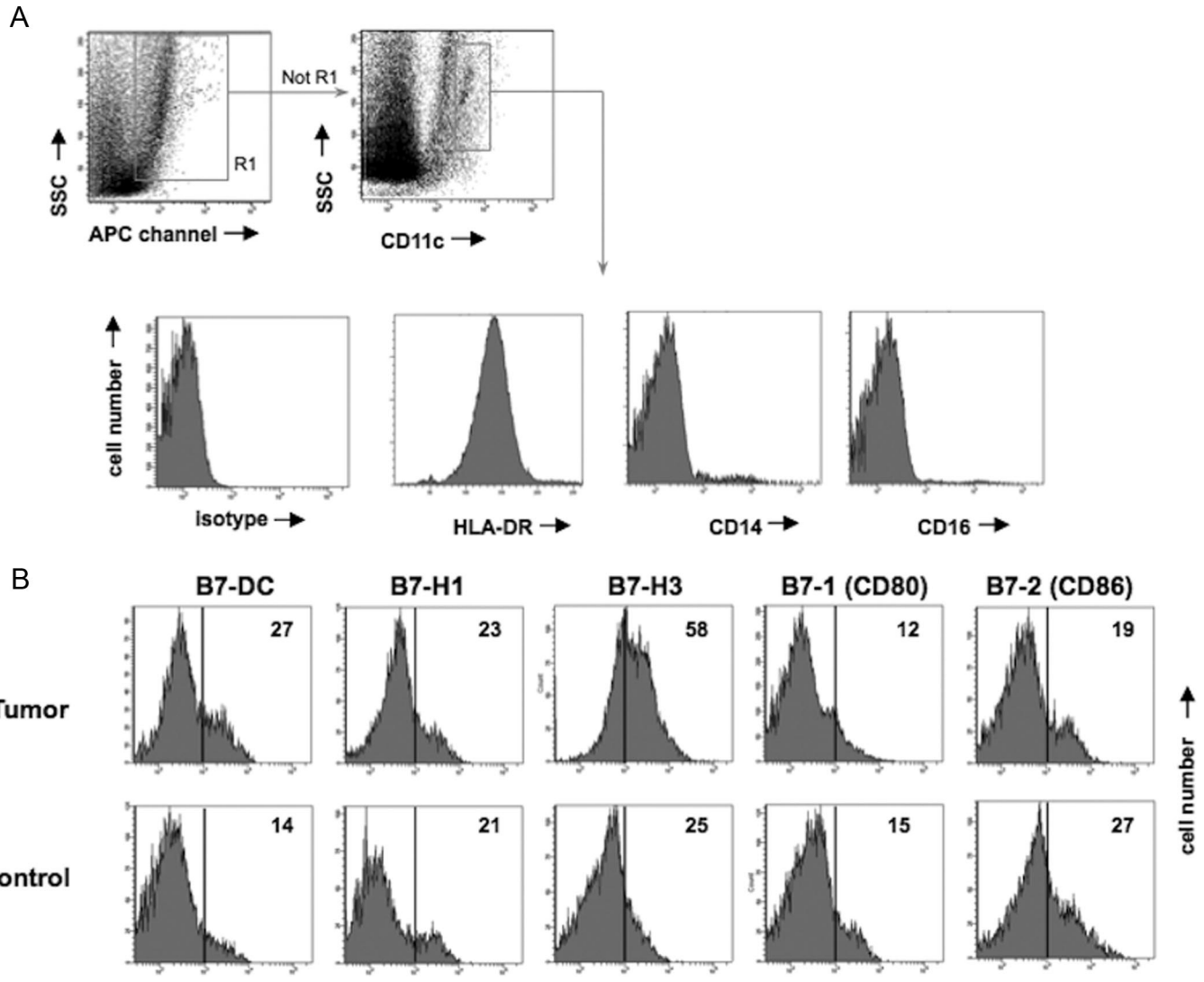

FIGURE 1. Expression of B7 molecules by dendritic cells (DC) in tumor and lung tissue. Single cell suspensions were prepared from lung cancer samples (tumor) and from unaffected lung tissue (control). Suspensions were stained with antibodies as indicated and analyzed by FACS. *A*, To gate for CD11c$^+$ DC, autofluorescent cells were determined first by measuring the fluorescence of the cells in the APC channel. These cells were excluded. Next, the SSC^{high}/CD11c$^+$ cells were gated and analyzed for typical DC (HLA-DR), granulocyte (CD16), and monocyte (CD14) markers. *B*, Cells gated as in (*A*) were further analyzed for expression of B7 markers. The vertical lines depict the border between negative and positive cells as determined by isotype controls and the number in the upper right corner displays the percentage of positive cells among the CD11c$^+$ population. A typical example for one representative patient out of four is shown.

isolated directly from tumor tissue in comparison to DC extracted from healthy lung tissue from one and the same donor. Among the molecules tested, B7-H3 was significantly upregulated in tumor-residing DC as compared with DC derived from healthy lungs. In contrast, expression of other B7 molecules was not significantly altered in tumor tissue compared with the healthy lung (Figures 1 and 2).

No substantial differences in the expression of B7 markers were apparent in tumor infiltrated lymph nodes versus tumor-free lymph nodes, when gating on $CD11c^+$ DC. The potentially immunosuppressive molecules, B7-DC, B7-H1, and B7-H3, were expressed by $CD11c^+$ DC in tumor infiltrated lymph nodes to a similar extend as in tumor-free lymph nodes. Moreover, also the expression of the T cell costimulatory molecules B7-1 (CD80) and B7-2 (CD86) did not differ between tumor infiltrated and tumor-free lymph nodes (Figure 3).

B7-H3 Expression by DC Impairs Their T Cell Stimulatory Capacity

In allogeneic mixed lymphocyte reactions (MLRs), a significantly reduced T cell proliferation was found in tumor-derived DC as compared with the control counterparts isolated from healthy lung tissue. As further controls, DC isolated from lymph node samples of the same patient were included; these DC were as potent in stimulating T cell proliferation as the lung DC. Finally, addition of anti-CD3/CD28 antibodies to the T cell cultures stimulated T cell proliferation maximally, indicating that the isolated $CD4^+$ T cells were viable and were able to respond to stimulation via their T cell receptors (Figure 4).

When B7-H3 antibodies were added to the cultures of tumor-derived DC and allogeneic T cells, increased T cell proliferation was observed, reaching similar levels of proliferation as obtained with DC purified from control lungs or lymph nodes, respectively. This effect was specific for blockade of B7-H3, as addition of isotype-matched, nonbinding antibodies did not affect the T cell proliferation. To rule out other unspecific T cell stimulatory effects of the B7-H3 antibodies, we added the antibodies and respective isotype controls to MLRs set up with lymph node-derived DC, which only express basic level of the B7-H3 surface molecules and thus should be unsusceptible to the blocking activity of B7-H3 antibodies. In these experiments, we measured similar T cell proliferation in antibody-treated and control samples, indicating that anti-B7-H3 antibodies do not stimulate T cell proliferation unspecifically (Figure 5).

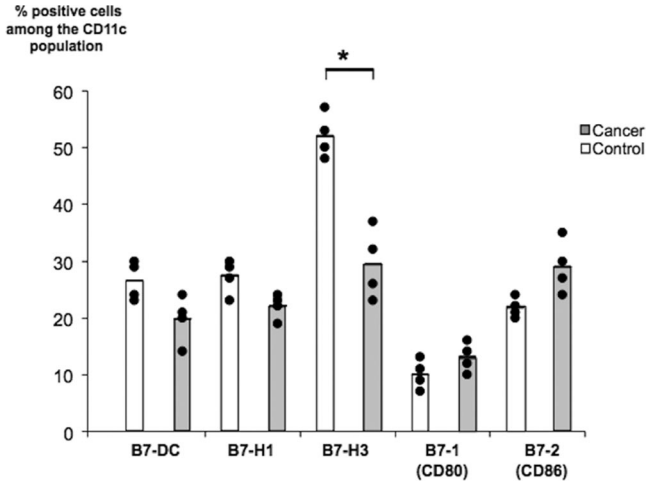

FIGURE 2. B7-H3 is upregulated in tumor-derived dendritic cells (DC). Upregulation of B7-H3 was found in all lung cancer samples compared with unaffected lung tissue. The figure shows the data of four experiments as individual dots. The mean values are indicated as bars. *Significant difference (*t* test; $*p < 0.05$).

Tumor-Derived DC Secrete More IL-10 and Less IL-12p70 as Compared with Controls

To further analyze the phenotype of the tumor DC, 1×10^5 magnetobead isolated DC were cultivated overnight together with 10 ng/ml LPS, and the supernatant was tested for IL-10 and IL-12p70 content by ELISA. Significantly elevated levels of IL-10 in cultures of tumor-derived DC were measured as compared with normal lung or lymph node DC. In contrast, IL-12 levels were significantly reduced in cultures of tumor DC as compared with the controls (Figure 6).

DISCUSSION

DC were originally defined as immune stimulatory antigen presenting cells that are able to induce robust T cell responses to various antigens. However, this function can be corrupted by tumors. Herein, we report that tumor-derived DC obtained from lung cancer patients express substantial amounts of the T cell coinhibitory molecule B7-H3 and are inferior in stimulating T cell proliferation as compared with controls. Thus, lung tumors may create an immunosuppressive environment by rendering DC immunosuppressive and preventing the induction on antitumor T cells.

The B7-H3 molecule is a member of the programmed death ligand (PD-L) family, which has structural similarities to the classical T cell costimulatory molecules B7-1 and B7-2.[18] B7-1 and B7-2 have clearly T cell costimulatory functions and are predominantly expressed by professional APC such as DC. In contrast, B7-H3 is broadly expressed on lymphoid organs, lung, bladder, and liver, and has dual functions as costimulator and also as a profound T cell coinhibitor.[16,19] Its activating properties are deducted from experiments showing that B7-H3 molecules increase proliferation of $CD4^+$ and $CD8^+$ T cells and stimulate IFNγ secretion.[20] Murine tumor models also revealed that natural or ectopic expression of B7-H3 on different tumor cells, such as melanoma, colon cancer, and mastocytoma, lead to induction of tumor-specific CTL and prolonged survival time significantly.[21–23] Moreover, in pancreatic cancer, expression of B7-H3 was associated with prolonged survival.[24] However, these results were in contrast to other reports that demonstrated immunosuppressive and therefore adverse effects of B7-H3 expression in pancreatic cancer patients.[24] Nevertheless, the T cell activating effects seem to be mediated by the triggering receptor expressed on myeloid cells (TREM)-like transcript 2 (TLT-2), which is constitutively

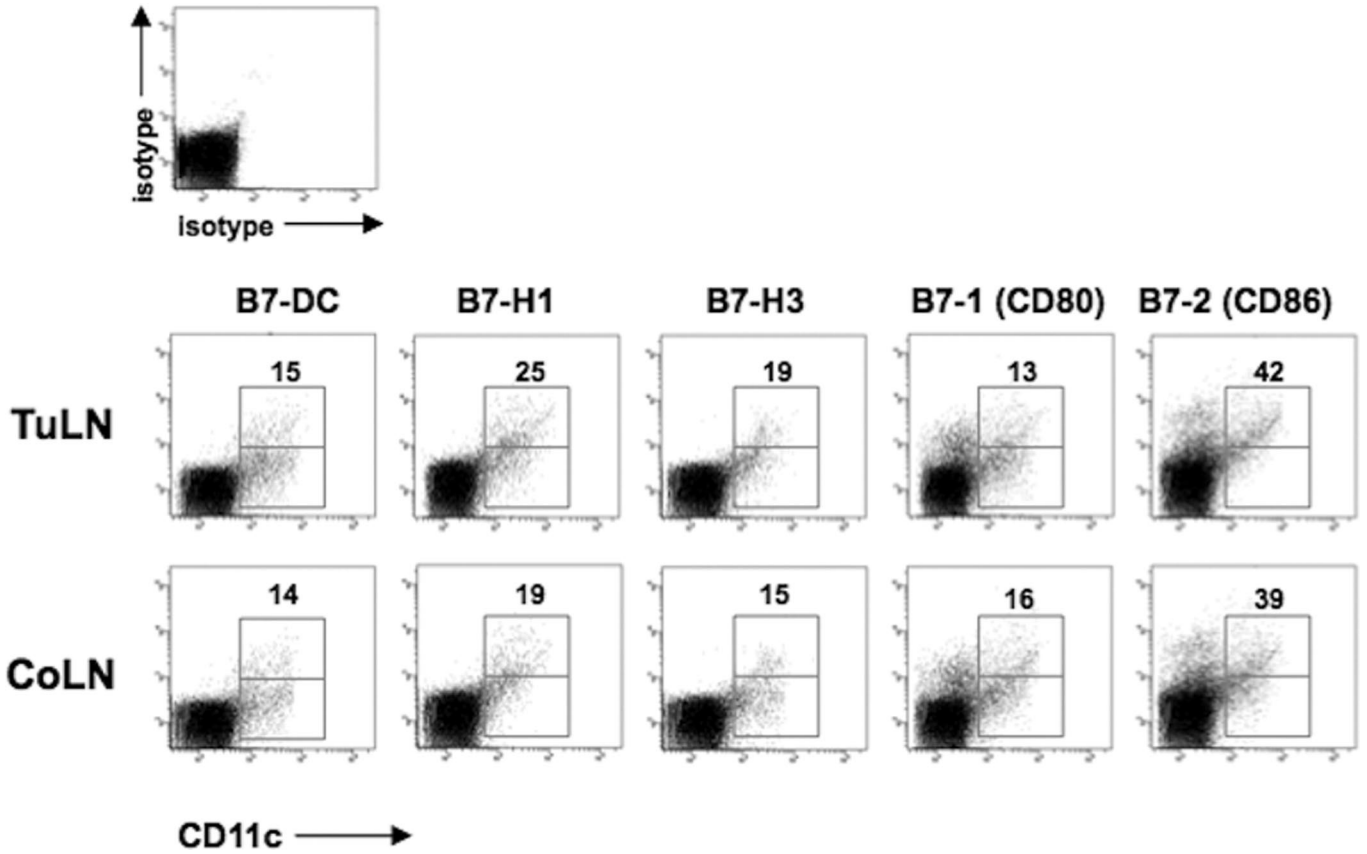

FIGURE 3. Expression of B7 molecules by dendritic cells (DC) in lymph nodes. Single cell suspensions from tumor-infiltrated lymph nodes from lung cancer patients (TuLN) and tumor-free lymph nodes (CoLN) were prepared and stained with the antibodies indicated. The isotype control is shown in the upper left panel and the big square gate indicates the $CD11c^+$ DC population. The upper gate depicts DC positive for the respective B7 markers. The numbers indicate the percentage of marker-positive cells among the $CD11c^+$ population. A typical example for one representative patient is shown.

expressed by DC, B cells, macrophages, NK cells, and neutrophils.[25] Thus, expression of B7-H3 by tumor cells may facilitate the cross talk between tumors and different types of leukocytes for the purpose of immune stimulation.

However, at the same time, accumulating evidence indicates that B7-H3 acts as a coinhibitory factor that is able to abrogate T cell response in various cancers and during autoimmunity.[26] In a murine autoimmune model for multiple sclerosis, the addition of recombinant B7-H3 molecules, which mimic the action of B7-H3 expressing cells, suppressed T cell proliferation in vitro.[27] Moreover, immunosuppressive effects of B7-H3 are also apparent in vivo in human cancer patients. Here, presence of B7-H3 in tumor vasculature of renal cell carcinoma (RCC) was investigated, and the data clearly indicate that B7-H3 expression was significantly associated with an increased risk of death from RCC.[28] Likewise, human pancreatic cancer patients displayed elevated expression of B7-H3 molecules on tumor tissues, which was even enhanced in cases with lymph node metastasis and advanced pathological stages, indicating a strong correlation between B7-H3 expression and a bad prognosis of survival for cancer patients.[24] The functional contribution of B7-H3 to the impaired antitumor immunity was further strengthened by observations in a murine pancreatic cancer model, showing that blockade of B7-H3 molecules on cancer cells by a monoclonal antibody resulted in a substantial antitumor effect that was mainly due to recruitment of $CD8^+$ T cells to the tumor site.[24] Our results are in line with this inhibitory function of B7-H3, as blockade of B7-H3 on DC did enhance T cell proliferation in MLRs. However, these differential effects of B7-H3 on immunity (inhibitory versus stimulatory) may be explained by different sets of activating and/or inhibitory counter-receptors expressed by target cells.[29] Indeed, the receptors involved in this T cell coinhibitory action of B7-H3, as well as the signal transduction, are largely unknown. Thus, the presence of either stimulatory or inhibitory counter receptors for B7-H3 on the target cells may determine the immunological outcome of B7-H3 engagement.[30] Consequently, in different tumors, the action of B7-H3 expressing DC may by different. In human NSCLC, B7-H3 was found in cell lines as well as tumor specimens; high expression of B7-H3 was associated with lymph node metastasis.[31] In addition, high levels of circulating B7-H3 were associated with tumor stage, nodal metastasis, and distant metastasis.[32] In addition to B7-H3 upregulation, we also observed decreased surface levels of the T cell costimu-

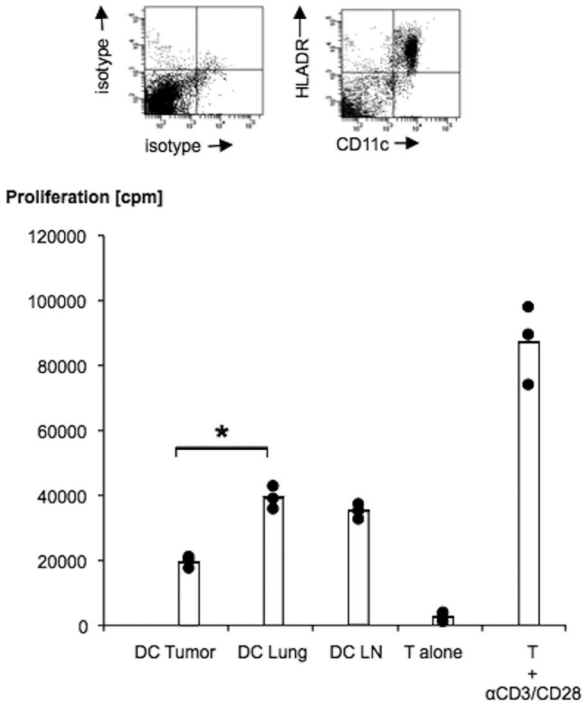

FIGURE 4. Tumor-derived dendritic cells (DC) are inferior in stimulating T cells. $CD11c^+$ cells were enriched by magnetobeads from organs as indicated. The upper panel shows a typical FACS plot for DC-specific markers. The lower panel shows cocultures of $CD11c^+$ cells with allogeneic $CD4^+$ T cells. After 96 hours, T cell proliferation was determined by pulsing with ^{3}H-Thymidine. As positive control, $CD4^+$ T cells were stimulated with a mixture of anti-CD3/anti-CD28 antibodies. The figure shows CPM values of three individual experiments as dots and the means are depicted in bars. *Significant difference (*t* test; $*p < 0.05$) between samples as indicated.

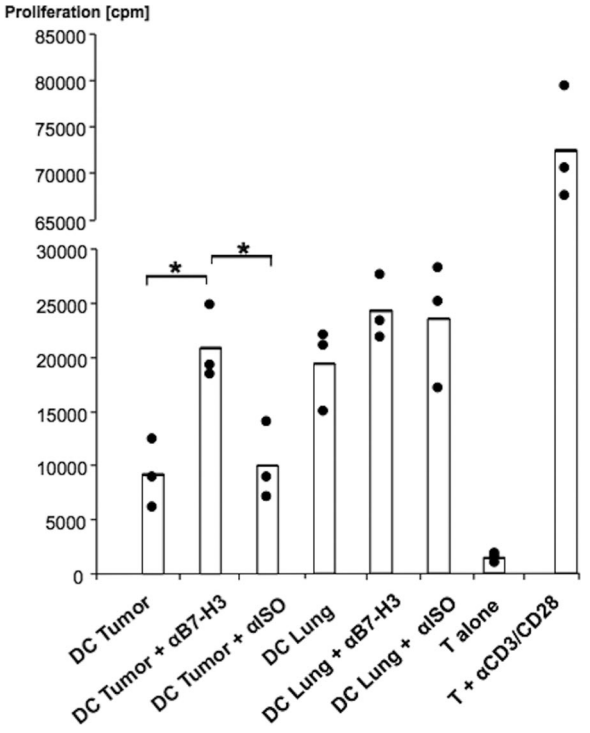

FIGURE 5. B7–H3 blockade results in enhanced stimulation of T cells. Dendritic cell (DC)-T cell cocultures were set up as in Figure 4 and anti-B7-H3 or respective isotype control antibodies (αISO) were added. After 96 hours, T cell proliferation was determined by pulsing the cultures with ^{3}H-Thymidine. The figure shows the individual data as dots and the means as bars. *Significant difference (*t* test, $*p < 0.05$) between tumor-derived DC and B7-H3-blocked tumor-derived DC.

latory molecules B7-1 (CD80) and B7-2 (CD86) on DC in all samples. Although theses differences were not statistically significant, these results are in agreement with findings published by Perrot et al.[14] Herein, the authors report an immature DC phenotype induced by tumors, which is indicated by reduced surface expression of B7-1 and B7-2. Moreover, these data further support our notion that an immunosuppressive phenotype of DC is induced, as immature DC are inferior in eliciting immune responses and have rather tolerogenic potential.

Interestingly in this series, NSCLC did not influence the expression of either inhibitory or costimulatory B7 molecules in the respective lymph nodes. However, in these experiments, we could only compare metastatic lymph nodes with nonaffected lymph nodes and we cannot exclude that even the nonmetastatic control lymph node was affected by tumors; for example, by soluble factors produced by the tumor, which may influence the DC phenotype because lymph drainage in lung cancer is variable. Due to this aspect and due to the limited number of lymph nodes examined in our series, further investigations are clearly needed to draw definite conclusions. Nevertheless, in the literature, no reports about B7 expression in lymph node-acquired DC in human cancer exist up to now.

Our data, showing that DC derived from NSCLC are immunosuppressive, as indicated by B7-H3 upregulation, are further supported by our results on IL-10 secretion. We have shown that tumor-derived DC secrete elevated levels of IL-10, which indicates a more tolerogenic and immature phenotype. In contrast, the secretion of the immune stimulatory cytokine IL-12 is decreased. Thus, these data further strengthen the notion that NSCLC is able to convert DC into a less immunogenic cell type. Under normal conditions, IL-10 secreting subsets of DC mediate tolerance and prevent exacerbated immune responses such as asthma. These effects are either mediated by IL-10 itself or by the induction of Tr1 cells, which are IL-10-producing T cells with regulatory properties. However, our data obtained in NSCLC show that antibodies to B7-H3 nearly completely restored the T cell stimulatory capacity of the tumor-derived DC. Therefore, we conclude that under tumor conditions, B7-H3 is crucial in mediating the T cell suppressive effects of the DC. Nevertheless, we observed elevated numbers of Treg in patients with NSCLC (data not shown), and reports by Dumitriu et al.[15] showed that NSCLC-derived DC produce high amounts of TGF-β and therefore are able to induce differentiation of Treg in peripheral tissues. Thus, beyond the observed direct

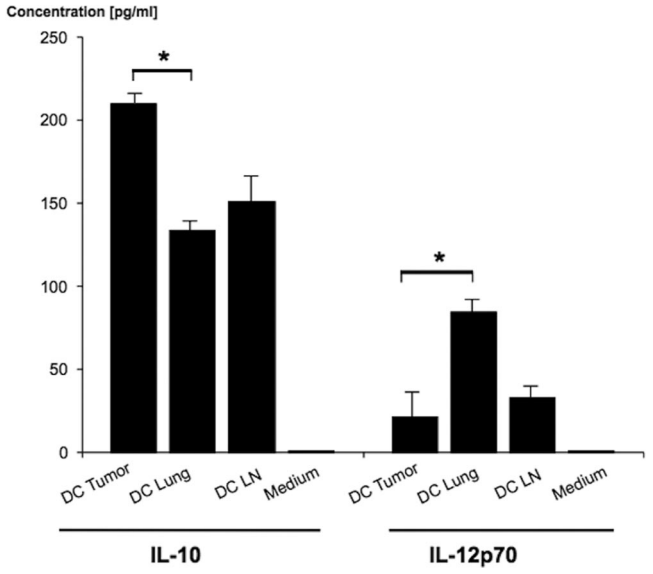

FIGURE 6. Tumor-derived dendritic cells (DC) produce less IL-12 but more IL-10. DC were purified with paramagnetic beads and cultivated overnight in medium containing 10 ng/ml LPS. Thereafter, tissue culture supernatant was harvested and analyzed for presence of IL-10 and IL-12p70 as indicated. Shown are the mean values ± SD of three patients. *Significant difference (*$p < 0.05$) between tumor and lung.

immunosuppressive effects of the B7-H3$^+$, IL-10-producing DC in NSCLC, further induction of Treg in vivo may also contribute to immune suppression in cancer patients.

CONCLUSION

Our data indicate that (1) DC derived from NSCLC are immunosuppressive, and (2) under tumor conditions the coinhibitory molecule B7-H3, among other so far unknown factors, is instrumental in mediating the T cell suppressive effects of dendritic cells.

ACKNOWLEDGMENTS

Supported by the Helmholtz Alliance against Cancer and the Tumor Center Heidelberg/Mannheim (to K.M. and S.R.).

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