Abstract
B7-H4 is a recently identified B7 family member. We previously showed that ovarian tumor and associated macrophages expressed B7-H4: tumor B7-H4+ macrophages and CD4+CD25+FOXP3+ regulatory T cells (Treg cells) suppressed tumor-associated antigen–specific T-cell immunity. To determine the pathologic relationship between B7-H4, macrophages, and Treg cells in the tumor environment, in addition to Treg cell numbers, we quantified B7-H4 expression in the tumor and tumor-associated macrophages in 103 patients with ovarian carcinoma. We observed that the intensity of B7-H4 expression in macrophages was significantly correlated with Treg cell numbers in the tumor. Further, both Treg cells and macrophage B7-H4, but not tumor B7-H4, were negatively associated with patient outcome. Tumor Treg cells enabled macrophages to spontaneously produce interleukin (IL)-10 and IL-6. Tumor macrophages stimulated B7-H4 expression in an autocrine manner through IL-10 and IL-6. Our previous work showed that tumor-associated macrophages spontaneously produced chemokine CCL22 to mediate Treg cell trafficking into tumor, and Treg cells induced B7-H4 on antigen-presenting cells (APC) including macrophages. Altogether, our data support the concept that there is a mechanistic interaction between Treg cells and macrophage, and that Treg cells may convey the suppressive activity to APCs through B7-H4 induction in human ovarian cancer.

Introduction
B7-H4 (B7x, B7S1) was identified in 2003 (1–3). B7-H4 mRNA expression was found to be widely distributed in the peripheral tissues, including kidney, liver, lung, spleen, thymus, and placenta. However, B7-H4 protein expression on the cells seems to be limited (1, 4). Interestingly, several groups have shown that human ovarian cancers express high levels of B7-H4 protein (4–9). Additionally, high levels of B7-H4 were found in non–small-cell lung cancer (10), ductal and lobular breast cancer (9, 11), and renal cell carcinoma (12).

Antigen-presenting cells (APC) are critical for initiating and maintaining tumor-associated antigen (TAA)–specific T-cell immunity. Tumor-associated macrophages markedly outnumber other APCs, such as dendritic cells, and represent an abundant population of APCs in solid tumors (13–16). We recently reported that human ovarian cancer–associated B7-H4+ macrophages inhibited TAA-specific T-cell immunity, in part through B7-H4 (5). We have now further quantified B7-H4 expression in tumor and tumor-associated macrophages, and analyzed the relationship between B7-H4 expression, Treg cells, and patient outcome.

Materials and Methods
Human subjects. We studied 103 previously untreated patients with epithelial ovarian carcinomas, International Federation of Gynecology and Obstetrics stage I to IV (Table 1). Patients gave written, informed consent. The study was approved by local institutional review boards. Clinical information is available for 70 patients.

Human cells and ovarian tumor tissues. Cells were obtained from ascites, fresh ovarian tumor tissues, and peripheral blood as previously described (17–19). CD3+ T cells and CD14+ cells were purified with paramagnetic beads (StemCell Technology) and sorted with FACSaria using DiVa software (Becton Dickinson Immunocytometry Systems). Lin−EpCam−CD45−CD14− epithelial ovarian tumor cells were sorted on a FACSaria using DiVa software (Becton Dickinson). Cell populations were >98% pure as confirmed by flow cytometry (LSR II, Becton Dickinson). Ovarian tumor tissues and associated cells were collected for immunofluorescence analysis and cytokine detection by reverse transcription-PCR (RT-PCR).

Immunofluorescent staining. Immunofluorescence analysis was done as previously described (19). Tissues were stained with mouse anti-human B7-H4 (hH4.1, IgG1, 4 μg/mL; ref. 1), mouse anti-human Ham56 (Ham56, IgM, 1/20; DAKO), followed by Alexa Fluor 488–conjugated goat anti-mouse IgG1 and Alexa Fluor 568–conjugated goat anti-mouse IgM (all 2 μg/mL, Molecular Probes). The same antibody concentrations were used for all the tissue staining throughout this work.

Quantification of B7-H4 expression. B7-H4 expression was detected with the Leica DMIRE2 confocal microscope. Laser intensity was calibrated for each observation session using a control test slide with CalibriTE beads (Alexa 488, Alexa 568). The intensity of the laser was adjusted to a normalized value of the mean fluorescent intensity (10 ± 0.1) using the same beam settings, Alexa 488 (10%), Alexa 568 (10%), PMT 1 and PMT 2 value (700), offset value PMT 1 and PMT 2 (0), Z thickness (10 μm), number of slices (10), and average of slices (2). Beam settings were adjusted using the slide with the most fluorescent intensity to Alexa 488 29%, PMT1 733, Offset-1; and Alexa 568 23%, PMT2 (701), Offset-1. The scanner was engaged and the peak fluorescence intensity of B7-H4 staining with Alexa 488 was located by adjusting the Z position to reveal the most intense pixel concentration. A series of eight slices containing fields with the most intense pixel concentration were selected with a total Z thickness of 3 μm. Two scans for each of the eight slices were captured and the average projection of all 16 slices was used to quantify mean fluorescent intensity. Five adjacent consecutive fields were captured for each of the specimens.

The Leica Confocal Simulator software contains functions to quantify specific values located in designated regions of interest (ROI). Background fluorescence was normalized adjusting the threshold of the average projection of each series. An ROI with an area of 2,400 ± 100 μm2 was

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designated in an observably negative stained area of each series average projection. The mean fluorescent intensity (MFI) of the background ROI was read by the Leica Simulator software quantify function by adjusting the threshold of the average projection such that the MFI of the ROI was equal to 0.04 ± 0.01. Using the threshold corresponding to the normalized background value, the entire average projection of the captured field area (149,260 μm²) MFI (B7-H4 expression) was read by the Leica quantify function in tumor cells or Ham56+ macrophages and recorded along with the SD of the mean and the intensity skew. The MFI reflected the quantity of mouse anti-human B7-H4 primary bound to antigen and anti-mouse IgG1–Alexa 488 conjugate bound to primary antibody. The SD reflects the variation between fields. The intensity skew reflects the differences in accumulation location of the antibody-bound antigen in each field. Five quantification recordings from each specimen were averaged using Microsoft Excel software.

Tumor environmental cytokines. Tumor tissues, tumor-associated macrophages (CD14+ cells), and blood CD14+ cells were used. Macrophages were sorted (5) and cultured for 72 h in different conditions. Neutralizing antibody (mAb) against human IL-6 (anti–IL-6, clone 6708, 10 μg/mL) in the presence of anti-human CD3 (2.5 μg/mL, clone, UCHT1, BD Biosciences) and anti-human CD28 (1.2 μg/mL, clone CD28.2, BD Biosciences) were added. Cytokines were detected by ELISA (R&D Systems) in the culture supernatants.

Statistical analysis. Differences in cell surface molecule expression were determined by χ² test, and in other variables by unpaired t test, with P < 0.05 considered as significant. Overall patient survival was the interval between diagnosis and death. The known tumor-unrelated death (e.g., intercurrent disease and accidental death) was excluded from death record for this study. Data were censored at the last follow-up for patients who were disease-free or alive at the time of analysis. Spearman correlation coefficients were computed to assess relationships between Treg cells, B7-H4, and overall survival. Median survival times were computed using Kaplan-Meier methods. 95% Confidence intervals were computed where possible. Differences in survival functions were assessed using the log-rank test. Confounding factors were assessed using Cox proportional hazards test. Stage was tested in each model; other variables, tumor histology, tumor grade, debulking (optimal versus not), and response to chemotherapy were included in a stepwise modeling procedure. All analyses were done using SAS 8.2 software.

Results

B7-H4 and patient outcome. We recently showed that ovarian cancer cells and tumor-associated macrophages highly expressed B7-H4 (5). We now extended this study to 107 patients with ovarian cancer. We observed that the intensity of B7-H4 expression was highly variable in ovarian cancer cells and tumor-associated macrophages among patients with ovarian cancer (Fig. 1A, i, ii, iii, three patients). We quantified the intensity of B7-H4 expression in all the patients studied and further analyzed the relationship between the intensity of B7-H4 expression in tumor and clinical pathologic data. We observed that B7-H4 expression in ovarian cancer cells was not associated with clinical and pathologic features, including patient survival, tumor size, stage, and grade (not shown).

Macrophages also expressed B7-H4 (Fig. 1A). We further quantified the intensity of B7-H4 expression in macrophages in the tumors. Based on the intensity of B7-H4 expression, we divided the samples into two groups, one group with low B7-H4 expression in macrophages and another group with high B7-H4 expression in macrophages. After adjustment for stage using Cox proportional hazards regression, low and high B7-H4 groups had significantly different survival times (P < 0.0004; Fig. 1B). The hazard ratio was
equal to 3.9 (95% confidence interval, 1.8-8.4). The different survival times remained significant in patients with advanced-stage diseases (Fig. 1C).

Stage and Treg cells are the well-defined variables affecting patient survival (18). We observed that the number of B7-H4+ macrophages was significantly increased in advanced disease stages (Fig. 1D). After adjustment for stage and Treg cells, there were still significant differences between the low and high B7-H4 expression groups \( (P = 0.0171) \). Hazard ratio was equal to 2.7 with 95% confidence interval of 1.2 to 6.1. The data indicate that B7-H4 in macrophages, rather than tumor cells, is related to patient outcome, suggesting the pathologic relevance of macrophage B7-H4.

**Relationship between tumor Treg cells and B7-H4+ macrophages.** We previously showed a mechanistic link between Treg cells and APCs (21). We showed that normal Treg cells conveyed suppressive activity to APCs through B7-H4 induction. Functional Treg cells were found in the tumor environment and predict patient survival (18). B7-H4+ macrophages were observed in human ovarian tumor and suppressed TAA-specific T-cell immunity (5). We thus tested the potential relationship between tumor Treg cells, identified as CD4+FOXP3+ T cells, and B7-H4+ macrophages. Spearman correlation of Treg cells and B7-H4+ macrophages was 0.39 \( (P = 0.0008; \text{Fig. 1E}) \). Thus, Treg cells and B7-H4+ macrophages are positively associated in ovarian tumors.
Tumor macrophages stimulate B7-H4 expression in an autocrine manner. We previously showed that macrophages in ovarian tumors expressed B7-H4, and that IL-10 and IL-6 in the tumor ascites stimulated B7-H4 expression (5). To determine the cellular source of IL-10 and IL-6 for B7-H4 induction in tumor environment, we incubated fresh blood monocytes from normal donors with primary ovarian tumor cells or tumor-associated macrophages. Unexpectedly, tumor macrophages, but not normal macrophages and primary tumor, significantly stimulated macrophage B7-H4 expression (Fig. 2A and B and not shown; n = 6, *P < 0.05). Consistent with previous findings (17), ovarian tumor macrophages, but not normal macrophages, spontaneously expressed IL-10 and IL-6 (Fig. 2C and D). LPS stimulation further enhanced the production of IL-10 and IL-6 by tumor-associated macrophages and normal macrophages (Fig. 2D). We next observed that B7-H4 induction mediated by tumor macrophages was partially and significantly reversed by anti–IL-10 neutralizing mAb and anti–IL-6 neutralizing mAb. Simultaneous blockade of IL-6 and IL-10 completely blocked B7-H4 induction by tumor-associated macrophages (Fig. 2A and B). GM-CSF and IL-4 reduces B7-H4 induction in APCs (5). We detected limited levels of GM-CSF and IL-4 in fresh ovarian tumor tissues (not shown; ref. 5). Thus, our published data (5) and current work indicate that tumor macrophages stimulate B7-H4 expression in an autocrine/paracrine manner through spontaneous production of IL-6 and IL-10.

Tumor Treg cells trigger the production of IL-10 and IL-6 by macrophages. We next examined why tumor macrophages spontaneously produced IL-10 and IL-6. We first studied whether primary tumor cells trigger spontaneous production of IL-6 and IL-10 by macrophages. To this end, blood monocytes from normal donors were cocultured with primary ovarian tumor cells. We observed that tumor cells had no effects on macrophage IL-6 and IL-10 production (not shown). As Treg cells were abundant in ovarian tumors, we further examined the potential role of tumor Treg cells on macrophages. Tumor Treg cells and fresh monocytes from normal donors were sorted and cocultured. As expected, we detected significantly higher levels of IL-10 and IL-6 in the coculture supernatants than in the macrophages or Treg cells alone (n = 7, *P < 0.05; Fig. 3A and B). FACS analysis showed that Tregs expressed little IL-10 and IL-6 (not shown). It suggests that IL-10 and IL-6 were predominantly from macrophages. To determine the pathologic relevance of the induced IL-10 (800 pg/mL), we cultured fresh monocytes from normal donors with different concentrations. We observed that IL-10 induced a dose-dependent B7-H4 induction on monocytes and 100 pg/mL IL-10 was able to induce significant amount of B7-H4 expression on monocytes (Fig. 3C). Thus, our data show that tumor Treg cells condition APCs to express functional IL-10 and IL-6 (Figs. 2A and B; 3A–C), and APC-derived IL-10 and IL-6 stimulate B7-H4 expression in APCs (Fig. 2A) and result in B7-H4–dependent suppressive APCs in the tumor microenvironment (Fig. 3D; ref. 5).

Discussion

B7 family consists of stimulatory and inhibitory molecules (22). Signals mediated by these B7 family molecules result in T-cell suppression or activation in different settings. B7-H1 and B7-H4 are the two identified inhibitory B7 family members (22). We previously showed that ovarian cancer and associated myeloid dendritic cells express B7-H1 (19, 23). Tumor-associated B7-H1 and

Figure 2. Tumor macrophages stimulated B7-H4 expression through IL-10 and IL-6. A and B, tumor-associated macrophages stimulated macrophage B7-H4 expression. Fresh normal blood monocytes were cultured for 72 h with ovarian tumor-associated macrophages in the presence or absence of anti-human IL-6 or anti-human IL-10 antibodies. B7-H4 expression was analyzed by FACS. Columns, mean of B7-H4+ cell percentage in total macrophages (*, P < 0.01); bars, SE. A, one representative histogram of six experiments. Results were expressed as the B7-H4+ cell percentage in total macrophages. Faded lines, isotypes. B, high levels of IL-6 and IL-10 mRNA expressed by tumor-associated macrophages. Paired tumor tissues, tumor-associated macrophages, and blood monocytes were subjected to RT-PCR for detecting IL-6 and IL-10. Results are shown for each paired patients. D, high levels of IL-6 and IL-10 protein expressed by tumor-associated macrophages. Tumor-associated macrophages or normal blood monocytes were cultured for 48 h with or without LPS stimulation (100 ng/mL). IL-6 and IL-10 were detected by ELISA in the supernatants. Columns, mean of cytokine concentration (8 normal donors; 11 patients; *, P < 0.01); bars, SE.
CD4+CD25bright T cells (Treg cells) were sorted by FACS from tumor ascites.

Figure 3. Tumor Treg cells trigger monocyte-dependent, high levels of IL-10 and IL-6. A and B, high levels of IL-10 and IL-6 triggered by Treg cells. CD4+CD25bright T cells (Treg cells) were sorted by FACS from tumor ascites. suppressive capacity was initially determined by suppressive assay (18, 26). Fresh monocytes were cultured with or without Treg cells for 72 h. The culture supernatants were collected. IL-6 (A) and IL-10 (B) were detected by ELISA kit. Columns, mean (n = 4); * P < 0.001; bars, SE. C, IL-10 stimulates macrophage B7-H4 expression. Fresh normal blood monocytes were cultured 72 h with recombinant IL-10. Cell surface B7-H4 was detected by FACS. Results were expressed as the B7-H4+ cell percentage in total macrophages (n = 8). D, proposed mechanistic interaction between APCs and Treg cells in the tumor environment. Treg cells enable APCs to produce IL-6 and IL-10, which stimulate B7-H4 expression on APCs in an autocrine or paracrine manner. B7-H4+ tumor APCs induce T-cell cycle arrest.

B7-H1+ myeloid dendritic cells reduce TAA-specific T-cell immunity through distinct mechanisms (19, 23). In this report, we focus on B7-H4, and the relationship between B7-H4, macrophages, and Treg cells in human ovarian carcinoma.

Consistent with previous reports, we show that primary ovarian cancer cells (4–9) and ovarian cancer–associated macrophages (5) express variable levels of B7-H4. Interestingly, the intensity of B7-H4 in macrophages, rather than tumor cells, is related to patient outcome. It has been reported that low levels of B7-H4 protein were found in all sera from ovarian cancer patients. The levels of serum B7-H4 were significantly higher in patients with ovarian carcinoma than healthy controls or women with benign gynecologic diseases (8). The median B7-H4 concentration in endometrioid and serous histotypes was higher than in mucinous histotypes (8). It remains unknown whether serum B7-H4 is derived from tumor or other cells, such as macrophages, and if so, how B7-H4 is released into sera. Nonetheless, the data suggest the pathologic relevance of B7-H4 in human ovarian cancer.

High levels of B7-H4 were found in non–small-cell lung cancer (10), ductal and lobular breast cancer (9, 11), and renal cell carcinoma (12). Vascular endothelial cells also express B7-H4 in renal cell carcinoma (12). Although we have observed that tumor B7-H4 expression is not associated to tumor pathology and clinical outcome in patients with ovarian cancer, it is unknown whether tumor–associated APCs express B7-H4 in renal cell carcinoma (12). Although the functional activity, the regulatory mechanism, and the signal pathways of tumor B7-H4 remain to be defined, the broad expression of B7-H4 in human tumors suggests a potential role for this protein in human tumor biology.

In addition to tumor cells, ovarian cancer–associated macrophages express B7-H4 and significantly inhibit TAA-specific T-cell proliferation, cytokine production, and cytotoxicity in vitro. These B7-H4+ macrophages also inhibit TAA-specific immunity in vivo and foster tumor growth in chimeric severe combined immunodeficient/nonobese diabetic mice bearing autologous human tumors, despite the presence of potential TAA-specific effector T cells (5, 21). We now show that the intensity of B7-H4 expression of macrophages is associated with tumor-infiltrating Treg cells and negatively predict ovarian cancer patient survival. These data indicate that macrophage B7-H4 signals contribute to tumor immunopathology.

Recombinant and tumor environmental IL-6 and IL-10 stimulate APC B7-H4 expression, whereas dendritic cell differentiation cytokines GM-CSF and IL-4 suppress APC B7-H4 induction (5, 21). We reason that tumor cells, tumor-associated macrophages, and regulatory T cells may be the source for IL-6 and IL-10 (17, 18).

In support of this, we observed that tumor macrophages spontaneously produce IL-6 and IL-10 and stimulate B7-H4 expression in an autocrine/paracrine manner. We further show that tumor–associated Treg cells trigger macrophage to produce IL-6 and IL-10, and IL-10 and IL-6 in turn stimulate B7-H4 expression on APCs. Thus, our data mechanistically link IL-10, B7-H4, Treg cells, and APCs in the context of tumor immunity, and suggest a complicated suppressive network in the tumor microenvironment (24, 25). Targeting this network may be therapeutically meaningful in treating patients with cancer.

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