Increased Frequency of CD4\(^+\) Cells Expressing CD161 in Cancer Patients

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Abstract

Purpose: Although the function of natural killer receptors on T cells infiltrating tumors and their potential effect on antitumor immunity has been investigated, little is known about T cells expressing NKR-P1A (CD161) in cancer patients. In the present study, we examined T cells expressing CD161 in the peripheral blood, the tumor tissue and in malignant effusions of patients with several types of malignancies.

Experimental Design: Expression of CD161 in CD4\(^+\) or CD8\(^+\) (lacking CD56) T cells isolated from peripheral blood (\(n = 61\)), tumor specimens (\(n = 8\)), and malignant effusions (\(n = 37\)) of cancer patients was examined using four-color flow cytometry. Proliferative capacity and cytokine production of purified CD4\(^+\)CD161\(^+\)CD56\(^-\)/C0 cells were studied after weak or strong stimulation, with or without costimulation, in the presence or absence of interleukin 2. The possible regulatory function of activated CD4\(^+\)CD161\(^+\)CD56\(^-\) cells on T-cell alloresponses was also investigated.

Results: CD4\(^+\) cells expressing CD161 were increased in cancer patients, compared with healthy individuals. This increase in the peripheral blood of cancer patients positively correlated with disease stage and was augmented at the tumor site. Phenotypic analysis revealed that CD4\(^+\)CD161\(^+\) cells are memory T cells, with low expression of activation markers. CD4\(^+\)CD161\(^+\) cells play an immunoregulatory role through cytokine production, because upon receiving costimulatory signals via CD28, they exert suppressive activity on autologous peripheral blood mononuclear cell alloresponses.

Conclusions: CD4\(^+\)CD161\(^+\)CD56\(^-\) cells represent a distinct memory T-cell population significantly increased in cancer patients. Depending on the type of signals provided by the tumor microenvironment, CD4\(^+\)CD161\(^+\) cells may regulate the immune response.

During the last decade, attention has focused on the expression of activating and inhibitory receptors of natural killer (NK) cells on T-cell subsets. Both types of NK receptors, namely killer cell immunoglobulin-like receptors and C-type lectin receptors, have been detected on peripheral T lymphocytes (1–6), as well as on tumor infiltrating lymphocytes in melanoma (7–9), renal cell carcinoma (10, 11), and cervical carcinoma patients (12). The potential role of inhibitory NK receptor on T-cell function has been extensively examined, whereas the concept that NK receptors are involved in the dysfunction of T lymphocytes against cancer cells has been established as a novel escape mechanism of tumors from immunosurveillance.

Human NKR-P1A (CD161) is a type II transmembrane glycoprotein with characteristics of the C-type lectin superfamily, lacking a clear ITIM/ITAM (immunoreceptor tyrosine-based inhibitory/activating) motif. Although CD161 is considered a receptor of NK cells, it is also expressed on 25% of adult peripheral T cells, mostly of effector/memory phenotype (6). It has also been detected on >90% of peripheral blood monocytes and in vitro derived dendritic cells (13). Engagement of CD161 on the latter cell population results in strong intracellular calcium release, as well as in IL-1\(\beta\) and IL-12 production (13).

A small subset of T cells coexpressing CD161 and an invariant Va24JaQ T-cell receptor (TCR) \(\alpha\) chain, paired predominantly with V\(\beta\)11, is defined as analogous to murine NK1.1\(^+\) T cells and possesses immunoregulatory features through production of both Th1 and Th2 cytokines. CD161 on this subset has been reported to function as a costimulatory molecule (14). Nevertheless, Va24\(^+\)CD161\(^+\) cells represent only a minority (0.01–0.1%) among peripheral blood T cells (15). In contrast, CD161\(^+\) T cells (mostly CD8\(^+\)), not skewed to Va24 TCR, were found to accumulate in human liver and were able to exhibit strong cytotoxicity against various tumor cell lines (16). Over one

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half of T cells (either CD4 or CD8), isolated from epithelial and lamina propria layers of duodenum and colon, express CD161 and are capable of producing IFN-γ and tumor necrosis factor-α, but not IL-4, upon activation with phorbol 12-myristate 13-acetate and ionomycin (17). Accumulation of CD161+ T cells (57.9% CD8+ and 31.2% CD4+) has also been observed in human intestinal epithelium. Only 14% of intestinal epithelium CD161+ T cells expressed CD56, in contrast to CD161+ hepatic T lymphocytes that coexpressed CD56 (18). Increased numbers of CD4+CD161+CD56− cells have been detected in peripheral blood mononuclear cells (PBMC) of rheumatoid arthritis patients compared with healthy donor PBMC (15.8% versus 12.5%, P = 0.01) (19). Others have shown that the CD161 molecule on CD4+ T cells is involved in transendothelial migration, because CD4+CD161+ cells also express high levels of β1 and β2 integrins (20). Recently, it was reported that CD161 expression defines subsets of CD4+ and CD8+ T cells with different functional activities (21). Thus, CD4+CD161+ and CD8+CD161+ T cells secreted IFN-γ and tumor necrosis factor-α (TNF-α), whereas the CD161− subset secreted more interleukin 2 (IL-2) upon stimulation via the TCR. In addition, CD4+/CD8+CD161− subsets contained mainly naïve T cells in contrast to CD161+ cells, which contained predominantly effector and central memory T cells.

Thus far, no study has described the potential role of CD161+ subsets in cancer patients. In the present study, we investigated T cells expressing CD161 in PBMC, tumor infiltrating lymphocytes (TIL) and malignant effusions (ME) from patients with several types of cancer. Further phenotypic characterization of CD4+CD161+CD56− cells was conducted. Proliferative capacity and cytokine production of purified CD4+CD161+CD56− cells from normal donor PBMC and ME mononuclear cells (ME-MNC), following TCR triggering, was also examined. Finally, the possible regulatory function of activated CD4+CD161+ cells on T-cell alloresponses was investigated.

**Materials and Methods**

**Patients and normal donors.** Heparinized blood samples were collected from 12 healthy donors and 61 cancer patients (47 breast cancer, 4 ovarian, 4 lung, 2 colon, 2 pancreas, and 2 stomach). Tumor samples from eight breast cancer patients were obtained by surgical excision. MEs from 37 cancer patients (25 ascites and 12 pleural effusions) were also collected during routine aspirations. Information regarding patients’ type of cancer and tumor stage (according to the American Joint Committee on Cancer) was also recorded. All samples have been acquired under the informed consent of patients and normal donors, according to the Declaration of Helsinki.

**Cell isolation.** PBMCs were isolated by Ficoll-Hypaque centrifugation using standard procedures. Tissue obtained from tumor specimens of breast cancer patients was mechanically fragmented with scissors and scalpel. The resulting small pieces were digested for 2 to 4 hours in MEM-α (Life Technologies, Ltd., Paisley, Scotland) with 5 mg/mL collagenase IV (Life Technologies). A part of the cells acquired, after three washes in HBSS (Life Technologies), was used for flow cytometry and the remaining cells were kept frozen in liquid nitrogen. MEs (250-1,000 mL) were spun at 1,300 rpm (rounds per minute) for 10 minutes. Part of the cells acquired (MNC and cancer cells) were used for flow cytometry or further cell isolations and the remaining cells were kept frozen in liquid nitrogen.

**Flow cytometry.** The phenotype of lymphocytes isolated from peripheral blood, tumor specimens, and MEs was determined using four-color flow cytometry. Monoclonal antibodies (mAb) specific for CD3, CD8, CD45RA, CD45RO, CD25, CD38, CD95, HLA-DR conjugated with fluorescein isothiocyanate (FITC), CD4 conjugated with allophycocyanin (APC), and isotype-matched controls were purchased from Becton Dickinson (Mountain View, CA). mAbs against CD161 (clone 191B8) conjugated with phycoerythrin (PE), and CD56 conjugated with PE-cy5 were obtained from Immunotech (Marseille, France). FITC-conjugated mAbs against TCR Vβ1, Vβ7, Vβ11, Vβ13.6, Vβ14, Vβ16, Vβ18, Vβ20, Vβ21, Vβ22, and Vα24 were purchased from Serotec (Oxford, United Kingdom), whereas Vβ3.1, Vβ5(a), Vβ6.7, Vβ8(a), Vβ12, and Vβ13 were obtained from Endogen (Woburn, MA).

Cells to be immunostained were washed twice with ice-cold PBS (Life Technologies) 1% bovine serum albumin (Sigma, St. Louis, MO)/0.05% NaN3 followed by incubation with saturating concentrations of the appropriate mAbs for 15 minutes at room temperature. Thereafter, cells were washed twice in ice-cold PBS/1% bovine serum albumin/0.05%NaN3 and fixed with 1% paraformaldehyde (Sigma) in PBS.

Samples were analyzed using FACS Calibur (Becton Dickinson, Heidelberg, Germany) and CellQuest analysis software.

**Isolation of CD4+CD161+ cells.** For negative selection of CD4+ T cells, PBMC from normal donors or MNC derived from patients’ MEs were stained with saturating concentrations of mouse anti-human mAb specific for CD8, CD14, CD19, CD16, and CD56 surface molecules. Elimination of CD8+, CD56+, CD14+, CD19+, and CD16− cells was accomplished after incubation with rabbit anti-mouse IgG microbeads and exclusion using LS and LD separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of this CD4+ cell population was >95%. Further isolation of CD4+CD161+ and CD4+CD16− cells was achieved after staining with saturating concentrations of the phycoerythrin-labeled mAb, specific for CD161 molecule, followed by incubation with rabbit anti-mouse IgG microbeads and positive selection using LS separation columns. Purity of CD4+CD161+ cell preparations gained following the above isolations ranged from 90% to 98%. To remove debris (platelets or erythrocytes left in the negatively selected cell population), positive selection using CD4 microbeads was conducted. Purity of isolated CD4+CD161− cells was 95% to 98%.

**Proliferation assay.** Isolated CD4+CD161+ cells (5 × 10^4) were seeded into 96-well round-bottomed plates. T-cell expander beads (CD3/CD28 Dynabeads, Dynal Biotech, Oslo, Norway) or CD3 Dynabeads (Dynal Biotech) were added at a bead/cell ratio of 4:1 (strong signal) or 1:10 (weak signal) in the presence or absence of 100 IU/mL IL-2 (Proleukin, Chiron B.V., Amsterdam, the Netherlands) and cultured for 72 hours. Proliferation of stimulated cells was estimated by adding 1 μCi [3H]thymidine (30-40 Ci/mmol, Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) per well for the last 16 hours of culture. Subsequently, cells were harvested and [3H]thymidine uptake was measured in a microbeta counter (Wallac, Perkin-Elmer, Inc., Boston, MA). Results are expressed in cpm. All proliferation assays were performed in triplicates.

**Cytokine detection.** Cytokine production was measured in supernatants of CD4+CD161+ cultures, seeded as described above. Supernatants were kept frozen at −75°C until analysis. Levels of IFN-γ, IL-10, IL-4, TNF-α, and granulocyte macrophage colony-stimulating factor (GM-CSF) were determined by multiplexed particle-based flow cytometric assay, according to the recommendations of the manufacturer (R&D Systems, Abingdon, United Kingdom). Transforming growth factor-β (TGF-β) secretion was measured by ELISA (R&D Systems), according to the instructions of the manufacturer.

**Mixed lymphocyte reaction.** PBMC (5 × 10^4 per well) and irradiated (3 × 10^6 per well; 3,000 rad) allogeneic lymphoblastoid cell line (LCL) were cocultured in 96-well round-bottomed plates. Autologous to PBMC-purified CD4+CD161+ cells, strongly (four beads per cell) stimulated for 3 days with T-cell expander beads or CD3 beads in the absence or presence of 100 IU/mL IL-2, were treated with mitomycin C (Kowa, Tokyo, Japan), as previously described (22). Mitomycin C-treated CD4+CD161− cells (5 × 10^4 or 10^5) were added in PBMC/LCL cocultures.
Alternatively, $5 \times 10^8$ per well normal donors' or cancer patients' PBMCs were cocultured with $3 \times 10^6$ irradiated (3,000 rad) allogeneic LCL in the lower section of transwell systems (Nunc A/S, Roskilde, Denmark). Autologous to PBMC $S \times 10^6$ per well, CD4*CD161* or CD4*CD161* (used as a control) cells isolated from normal donors' peripheral blood or patients' ME were stimulated for 3 days with high concentration (four beads: cell) of T-cell expander beads in the presence of 100 IU/ml IL-2 and seeded in the transwell inserts, at equal to PBMC numbers. For neutralization of IL-10, 10 μg/ml anti-IL-10 mAb (R&D Systems) were added. Neutralization of IL-4 and TGF-β was accomplished by adding 1 μg/ml anti-IL-4 (Becton Dickinson) or anti-TGF-β (R&D Systems) mAb.

Cultures were incubated for 4 days and labeled with 1 μCi/well [³H]thymidine for the last 16 hours. [³H]Thymidine incorporation was measured in a microbeta counter (Wallac). Results are expressed in cpm. All mixed lymphocytes reaction (MLR) assays were done in triplicates.

Statistical analysis. Statistical analysis was done with Graphpad Prism version 4.00 (Graphpad Software, Inc., San Diego, CA). Data were analyzed by Student's $t$ test and Pearson coefficient analysis. $P < 0.05$ was considered significant.

Results

CD4* cells expressing the CD161 receptor in cancer patients. Lymphocytes isolated from the peripheral blood of 12 healthy donors and 61 cancer patients (17 at stage I-II and 44 at stage III-IV), tumor samples from eight breast cancer patients (7 at stage II and 1 at stage III), as well as ascites and pleural effusions from 37 cancer patients (stage IV), were analyzed by immunofluorescent staining and multicolor flow cytometric analysis to determine CD56*, CD4*, and CD8* cells expressing CD161. It has previously been reported (6) that >90% of peripheral blood NK cells (CD56*CD3–) express CD161. In this study, we detected an average of 75% of total CD56* cells (both CD3– and CD3+) expressing CD161 in normal donors’ PBMC as well as in patients’ PBMC, tumor infiltrating lymphocytes, and ME-MNC (data not shown).

Regarding expression of CD161 on T cells, most CD8*CD161* cells coexpressed CD56 (70% of CD8* in PBMC, 63% in ascites, 51% in pleural effusions). The percentage of CD8*CD161*CD56* cells among lymphocytes was very low in all samples tested (average 1.57 ± 0.82, range 0.27–9.3). Recently, a population of CD8*CD56*CD161*high cells has been identified in normal donors’ PBMC that is anergic, does not proliferate, secrete cytokines, or mediate cytolytic activity (21). In our samples, this CD8*CD161*high cell population was either not detected or represented a minor fraction (<1%) of lymphocytes (data not shown).

Interesting findings emerged when analysis of CD4*CD56* cells expressing CD161 was done. As shown in Fig. 1, a significant increase in the percentage of CD4* cells expressing CD161 was observed in cancer patient–derived MNC compared with healthy individuals’ PBMC. In particular, whereas 18 ± 6.1% of normal donor CD4* cells express CD161, this percentage in cancer patients reaches an average value of 23.5 ± 9.6% and 27 ± 9.8% in PBMC of early- and late-stage cancer patients, respectively. In malignant samples, this dissimilarity further increases, revealing 35.6 ± 5.2% in breast tumor infiltrating lymphocytes, 36.3 ± 12.1 in ascites MNC, and 28.4 ± 10.9% in pleural effusion MNC. A statistically significant difference ($P = 0.02$) was observed in the amount of CD4* cells expressing CD161 when comparing patients’ PBMC at early (I-II) and late (III-IV) cancer stage. This observation indicates a positive relationship between circulating CD4*CD161* cells within the CD4* T-cell population and cancer progression (Fig. 1A).

Furthermore, PBMCs and MEs were obtained at the same time from 17 cancer patients and a positive correlation was observed in the percentage of CD4* cells expressing CD161 between circulating and ME-MNC ($r = 0.83$, $P < 0.001$, Pearson coefficient; Fig. 1B).

Phenotypic characteristics of CD4*CD161* and CD4*CD161* cells. Phenotypic characterization of peripheral blood CD4*CD161* and CD4*CD161* cells from normal donors and cancer patients, as well as from MEs, was achieved using four-color flow cytometry. Expression of CD25, CD38,
HLA-DR, CD95, CD45RA, CD45RO, and CD28 was examined on gated lymphocytes after exclusion of CD56+ cells and selection of CD4+CD161+ or CD4+CD161- cells. Figure 2A shows the percentage expression of these markers on CD4+CD161+ and CD4+CD161- cells. Both cell populations expressed CD28 (data not shown).

No significant difference was observed in the percentage of CD4+CD161+ and CD4+CD161- expressing CD25 in the peripheral blood of normal donors and cancer patients, whereas expression of this surface molecule was decreased in ME CD4+CD161+ cells ($P < 0.05$). Mean fluorescence intensity (MFI) of CD25 was significantly lower in patients' peripheral CD4+CD161+ cells (mean MFI 59 ± 27 in normal donors and 24.4 ± 5.5 in patients' PBMC, $P < 0.0001$) as well as in ME (mean MFI 31.1 ± 8.2, $P < 0.0001$, compared with normal donors' PBMC). CD38 expression deviated between CD4+CD161+ and CD4+CD161- cells in healthy individuals' PBMC ($P < 0.05$), whereas this divergence was not detected.

Fig. 2. Phenotype of CD4+CD161+ cells. A, PBMC from healthy donors, cancer patients, and ME-MNC were analyzed by four-color flow cytometry using mAbs specific for CD4, CD161, CD56 and CD25 or CD38, HLA-DR, CD95, CD45RA, CD45RO. Analysis of gated CD4+CD161+ and CD4+CD161- cells was done after exclusion of CD56+ lymphocytes. Percentage of gated cells expressing the indicated markers as well as mean values (horizontal lines) are presented. B, gated CD4+CD161+CD56- cells were analyzed with regard to TCR$\delta$ repertoire and compared with CD4+CD161-CD56- gated cells, using four-color flow cytometry. Representative results from two donors out of five tested. C, TCR$\delta$ repertoire was also examined in the peripheral blood (left) and ME (right) of three cancer patients. Representative results from one patient.
in cancer patients’ ME-MNC. Interestingly, although CD38 expression on CD4+CD161+ cells did not vary among samples from different sources, CD4+CD161+ cells exhibited great heterogeneity among different samples. ME CD4+CD161+ cells expressing CD38 were significantly lower compared with normal donors’ PBMC (P < 0.01). CD38 MFI on CD4+CD161+ cells was also decreased in cancer patients’ PBMC and ME-MNC (P < 0.01). CD4+CD161+ and CD4+CD161+ cells expressed the same levels of HLA-DR surface molecule. However, the percentage of CD4+ cells (both CD161+ and CD161+) expressing HLA-DR was increased in ME (P < 0.01) compared with healthy donors’ PBMC. The increase in the percentage of HLA-DR positive CD4+ cells in ME was not characterized also by increase in the MFI.

CD4+CD161+ and CD4+CD161+ cells differed in their expression of CD95, CD45RA, and CD45RO. CD4+CD161+ cells expressed higher amounts of CD95 and CD45RO and lower amounts of CD45RA compared with CD4+CD161+ cells, which seem to represent a heterogeneous cell population. In particular, CD95 expression on CD4+CD161+ cells did not alter among types of samples, whereas it increased in CD4+CD161+ cancer patients’ PBMC and ME-MNC. No difference in the MFI of CD95 was detected among the two cell subsets. However, a decrease in the MFI of CD95 molecule was observed in CD4+CD161+ cells of patients’ PBMC and ME-MNC (58 ± 27 in normal donors’ PBMC, 37.7 ± 8.8 in patients’ PBMC, and 40.8 ± 8.4 in ME-MNC, P < 0.05) as well as in CD4+CD161+ cells (57 ± 25 in normal donors’ PBMC, 38 ± 8.6 in patients’ PBMC, and 38.5 ± 9.2 in ME-MNC, P < 0.05).

As also shown in Fig. 2A, CD4+CD161+ cells comprise a mixture of naive (CD45RA+) and memory (CD45RO+) cells, characterized by variation in the expression of these markers among different types of samples. On the contrary, CD4+CD161+ cells possess a defined memory phenotype with low CD45RA and high CD45RO expression, in agreement with previous reports (14, 21). Intriguingly, significant decrease in CD4+CD161+ cells expressing CD45RA was observed in ME-MNC (P < 0.01). The intensity of expression of CD45RA was also reduced in cancer patients’ CD4+CD161+ cells (mean MFI 92 ± 27 in normal donors’ PBMC, 58.6 ± 36.9 in patients’ PBMC, and 58.5 ± 36.7 in ME-MNC, P < 0.05). The percentage of CD4+CD161+ cells expressing CD45RA was similar in both normal donors and cancer patients.

To examine whether CD4+CD161+ cells comprise an oligoclonal cell population, the TCR repertoire of gated CD4+CD161+ cells was examined in five healthy donors as well as three cancer patients and compared with CD4+CD161+ cells. As shown in Fig. 2B and C, CD4+CD161+ cells were not biased toward a restricted TCR phenotype and therefore cannot be considered as oligoclonally expanded cells.

Proliferative capacity and activation signal requirements of isolated CD4+CD161+ cells. Proliferative capacity and activation requirements of isolated CD4+CD161+ cells were investigated in 3-day cultures in the presence of strong or weak signal via CD3/CD28 or CD3 alone, with or without IL-2. As shown in Fig. 3, costimulation via CD28 considerably increased proliferation of CD4+CD161+ cells compared with stimulation via CD3 alone (P < 0.01). In the absence of costimulation, strong TCR triggering or IL-2 presence were required for
proliferation of CD4⁺CD161⁺ cells. Proliferation pattern of isolated cells from MEs did not differentiate from the respective pattern of normal donors’ isolated cells. [³H]Thymidine incorporation of CD4⁺CD161⁺ cells, under most stimulating conditions, was similar to CD4⁺CD161⁺ cells (data not shown). Because CD4⁺CD161⁺ cells used in the proliferation assay were isolated using a specific mAb directed against CD161, triggering of this receptor might have occurred under all culture conditions tested. However, due to contradictory data concerning the effect of CD161 ligation with the used mAb, the actual role of this interaction on the isolated cell population cannot be estimated.

Cytokine production of activated CD4⁺CD161⁺ cells. To investigate the effect of different stimulatory signals on the functional activity of CD4⁺CD161⁺ populations, isolated cells from normal donors’ peripheral blood and patients’ MEs were
cultured for 48 hours with high and low concentrations of anti-CD3/anti-CD28 or anti-CD3 beads, in the presence or absence of IL-2. Supernatants were collected and examined for the presence of IFN-γ, TNF-α, GM-CSF, IL-4, IL-10, or TGF-β. Figure 4 shows the levels of cytokines produced by CD4+CD161+ cells from normal donors (A) and cancer patients (B). In agreement with previously reported data (21), CD4+CD161+ cells were found to produce overall higher amounts of cytokines, compared with CD4+CD161− cells (data not shown). The levels of cytokines produced highly varied from donor to donor, in both healthy individuals and cancer patients, although a similar pattern is observed, as far as their signal requirements for cytokine production is concerned. Isolated cells were capable of producing all cytokines tested, when costimulation via CD28 or IL-2 were provided. Production of Th1 and Th2 cytokines is expected because CD4+CD161+ cells comprise a memory T-cell population. Different stimulation signals alter the amount of cytokines produced, but no effect on cytokine profile is observed. The amount of cytokines produced correlates with signal strength and reaches a maximum level in the presence of IL-2 and strong costimulation. Similar pattern in cytokine production is observed in both healthy individuals and cancer patients. As stated in the previous paragraph, the possible contribution of CD161 ligation with the mAb used for the isolation of this subpopulation cannot be evaluated under these conditions.

Suppressive activity of CD4+CD161+ cells stimulated via CD3/CD28 on alloresponses mediated by autologous PBMC. Because CD4+ cells expressing CD161 were increased in cancer patients, we sought to investigate whether CD4+CD161+ cells possess immunosuppressive characteristics, either due to cell-to-cell contact or soluble factors, and therefore contribute to a putative dysfunction of the immune system against cancer cells. Having established that CD4+CD161+ cells isolated from peripheral blood and MLEs were similar, in terms of phenotype, activation signal requirements, and cytokine profile, we first did MLR assays using CD4+CD161+ cells purified from normal donors' PBMC. Normal donors' total PBMC were stimulated with allogeneic LCL in the presence of autologous CD4+CD161+ cells, at 5:1 and 1:1 PBMC/CD4+CD161+ cell ratios (Fig. 5). CD4+CD161+ cells, in these series of experiments, were pretreated with mitomycin C to prevent their proliferation. Freshly isolated CD4+CD161+ cells had no suppressive activity on autologous PBMC proliferation (data not shown). However, when CD4+CD161+ cells were previously stimulated for 3 days with a high concentration of CD3/CD28 beads, in the presence or absence of IL-2, suppression of autologous PBMC proliferation was achieved. This suppressive effect was not observed when CD4+CD161+ cells were stimulated with CD3 beads alone. CD4+CD161+ cells, stimulated via CD28, induced higher levels of suppression (44% ± 4.2) in the absence than in the presence (26.6% ± 3.2) of IL-2. These data suggest that CD4+CD161+ cells may exert suppressive function on PBMC proliferation upon CD28 costimulation.

Suppressive activity of CD4+CD161+ cells is partly mediated by soluble factors. Because CD4+CD161+ cells, receiving strong costimulatory signals, secrete immunosuppressive cytokines (i.e., IL-10, IL-4 and TGF-β), we were interested to determine whether the suppressive function of cultured cells was mediated by these soluble factors. For this reason, MLR assays were done in which PBMC and allogeneic LCL were cultured in the lower section of transwell systems, whereas CD4+CD161+ cells, previously stimulated for 3 days with high concentration of CD3/CD28 beads in the presence of IL-2, were added to the upper section at equal amounts with those of PBMC.

Fig. 5. CD4+CD161+ cells stimulated via CD3/CD28 suppress autologous PBMC proliferation. Normal donors' total PBMC were stimulated with irradiated allogeneic LCL in the presence of autologous CD4+CD161+ cells, at 5:1 and 1:1 PBMC/CD4+CD161+ cell ratios. CD4+CD161+ cells were previously stimulated for 3 days with a strong (four beads per cell) CD3/CD28 signal in the presence (A) or absence (C) of IL-2 or a strong CD3 signal in the presence (B) or absence (D) of IL-2 and pretreated with mitomycin C before addition to the MLR. Proliferation of PBMC was estimated after 4 days by [3H]thymidine incorporation. Columns, mean of triplicate cultures from a representative healthy donor (out of three); bars, SD.

www.aacrjournals.org 6907 Clin Cancer Res 2006;12(23) December 1, 2006
**Discussion**

In the present study, we report for the first time increased frequencies of CD4+CD56- cells expressing CD161 (NKR-P1A) among cancer patients' CD4+ T cells from peripheral blood and tumor-involved sites (primary tumor and MEs). These cells were distinct from CD4+CD161-CD56- cells, which constitute a heterogeneous cell population, and exhibited similar phenotypic and functional characteristics in both cancer patients and healthy individuals. CD4+CD161-CD56- cells were found to represent a memory T-cell population, in agreement with data reported by Takahashi et al. (21). Upon TCR triggering and costimulation, CD4+CD161+ cells secrete both Th1 and Th2 cytokines. Moreover, we have shown the potential suppressive property of CD4+CD161+ cells, which is mediated through soluble factors, mainly through high IL-10, IL-4, and TGF-β, suggesting that CD4+CD161+ cells may play an immunoregulatory role. In parallel, we examined CD8+ cells expressing the CD161 receptor and found that CD8+ cells, expressing CD161 but not CD56, were detectable at very low numbers among lymphocytes from all sources tested. Furthermore, the anergic CD8+CD161high cell population previously described by Takahashi et al. (21) was either not detected or slightly represented.

Thus far, CD8+ T cells have been extensively studied for NKR expression in cancer (3, 4, 7, 9, 23). CD8+ T cells expressing either immunoglobulin-like or C-type lectin-like receptors (except CD161) have been mainly found to lack CD28 expression and have been considered to reflect the currently ongoing immune response (1). On the contrary, in accordance with others (1, 21), we have found that both CD4+ and CD8+ T cells expressing CD161 still express CD28. Although it has previously been reported that CD4+CD161+ T cells contain oligoclonal populations preferentially expressing TCR Vα24 and Vβ11, recent data (15, 21), as well as our own study, revealed that the vast majority of CD4+CD161+ cells were not skewed to Vα24Vβ11 TCR. Moreover, we found that CD4+CD161+ cells, derived either from the peripheral blood of healthy donors and cancer patients or ME, are polyclonal cells, not restricted to a specific TCR repertoire. Polyclonality of CD4+CD161+ cells in cancer patients possibly indicates that they are not directed toward a specific tumor antigen. Tumor cells may express a variety of tumor-associated antigens. In addition, patients with ME are at late stages of the disease, and many tumor variants, expressing a variety of tumor antigens, might have been developed. Thus, the large number of existing epitopes may be responsible for TCR polyclonality of the increased percentages of CD4+CD161+ cells in cancer patients. However, the TCR repertoire of CD4+CD161+ cells is not sufficient to validate tumor specificity of these cells.

Phenotypic and functional characterization of CD4+CD161+ cells revealed that they represent memory cells in healthy donors (21). Accordingly, we found that CD4+CD161+ cells are mostly CD45RO+CD45RA-, followed by high expression of CD95, which is consistent with a memory phenotype, both in healthy individuals. CD4+CD161+ cells used in these experiments were not treated with mitomycin C because the upper sections containing the CD4+CD161+ cells were removed before harvesting for [3H]thymidine uptake measurement. At this point, it is worth mentioning that mitomycin C-treated CD4+CD161+ cells produce lower amounts of cytokines (almost 8-fold less) compared with untreated CD4+CD161+ cells. Hence, MLR assays done using transwell inserts were more suitable for estimating the effect of soluble factors produced by CD4+CD161+ cells on PBMC alloresponses. As shown in Fig. 6A, 72% suppression of normal donors' PBMC proliferation is observed when CD4+CD161+ cells are added to transwell inserts and not treated with mitomycin C, compared with 26% with mitomycin C-treated CD4+CD161+ cells (Fig. 5C).

The effect of IL-10, IL-4, and TGF-β on PBMC proliferation was estimated by adding neutralizing mAbs to investigate whether neutralization of these cytokines restores proliferation of PBMC. Neutralization of IL-10, IL-4, and TGF-β reduced the percentage inhibition to 58%, 66%, and 42%, respectively. The percentage suppression of patient PBMC proliferation was 79% when ME CD4+CD161+ cells were added (Fig. 6B). Neutralization of cytokines decreased this inhibition to 55%, 52%, and 45% when mAbs specific for IL-10, IL-4, and TGF-β were added in the MLR. When all neutralizing mAbs were added, inhibition was reduced to 26% in the normal donor (Fig. 6A) and 16% in the cancer patient (Fig. 6B). These results indicate that the suppressive effect of CD4+CD161+ cells on autologous PBMC proliferation is dependent on soluble factors, mainly IL-10, IL-4, and TGF-β.

**Fig. 6.** Suppressive activity of CD4+CD161+ cells is mostly due to soluble factors, including IL-10, IL-4, and TGF-β. Healthy donors' or cancer patients' PBMC were cocultured with irradiated allogeneic LCL in the lower section of transwell systems. Autologous to PBMC, CD4+CD161+ cells, previously stimulated for 3 days with high concentration (four beads per cell) of CD3/CD28 beads in the presence of 100 IU/mL IL-2, were added to the transwell inserts. For neutralization of IL-10, IL-4 mAbs and anti-IL-10 mAb were added. Anti-IL-4 or anti-TGF-β mAbs (1 mg/mL) were used for IL-4 and TGF-β neutralization. Proliferation of PBMC was estimated after 4 days by [3H]thymidine incorporation. Columns, mean of triplicate cultures from a representative healthy donor (out of two tested; A) and a cancer patient (out of two tested; B); bars, SD.

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donors and cancer patients. Increase of CD4+ cells expressing CD161 in cancer patients may be due to previous immune stimulation by tumor-associated antigens and acquisition of memory phenotype. However, the issue of tumor specificity of these cells remains to be elucidated. Peripheral blood CD4+CD161+ cells tested \textit{ex vivo} were characterized by low expression of activation markers (CD25, CD38, HLA-DR), implying that they do not represent a currently activated T-cell population in \textit{vivo}. On the contrary, CD4+CD161+ cells in ME, as well as CD4+CD161+, were found to comprise more activated cells, because both populations exhibited higher HLA-DR expression.

Production of both Th1 and Th2 cytokines is also in agreement with the memory characteristics of these CD4+CD161+ cells. Furthermore, costimulation through CD28 is essential for proliferation and cytokine secretion of CD4+CD161+ cells. In the absence of costimulation, proliferation of CD4+CD161+ cells is almost undetectable, unless IL-2 or a strong TCR signal is provided.

The increase of CD4+ cells expressing CD161 in cancer patients prompted us to examine whether these cells are capable of exerting suppressive function and, therefore, may contribute to possible tumor escape from immune surveillance. In this respect, we found that only CD3/CD28-stimulated cells were capable of significantly inhibiting autologous PBMC responses. Additionally, the suppressive capacity of CD28- costimulated CD4+CD161+ cells did not require cell-to-cell contact, but was attributed to soluble factors, mainly IL-10, IL-4, and TGF-β. The above observations indicate that CD4+CD161+ cells may exert their immunoregulatory role mainly through cytokine secretion, depending on the microenvironment in which they develop.

The natural ligand of human CD161 has been recently defined (24, 25). Interaction of the human CD161 receptor with lectin-like transcript 1 triggers opposing signals on NK and T cells. Namely, CD161 ligation with lectin-like transcript 1 inhibits cytotoxicity and IFN-γ production by NK cells, whereas simultaneous engagement of CD3 and CD161 on T cells enhances IFN-γ production (24). Lectin-like transcript 1 is expressed on a variety of cell types (monocytes, B cells, IL-2-activated NK or T cells, osteoclasts) and is considered the orthologue of murine CIR family genes, which are recognized by Nkr-p1 receptors (26, 27).

Apart from the potential suppressive effect of CD4+CD161+ cells through cytokine production, interactions of this population with other cells of the immune system, through lectin-like transcript 1, would be interesting to be elucidated.

Because the increase in CD4+CD161+ cells is observed in all cancer patients independently of the chemotherapeutic scheme they receive, \textsuperscript{4} it can be speculated that they may directly correlate with the clinical status of the patient and possibly their tumor burden. Nevertheless, apart from the biological function of CD4+CD161+ cells and the role they might play in tumor immunity, the fact that this cell population increases in cancer implies that determination of CD4+CD161+ frequency in peripheral blood might be used as a marker for the follow-up evaluation of cancer patients.

\textsuperscript{4} Unpublished data.

\textbf{References}

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