The Immune Signature of CD8+CCR7+ T Cells in the Peripheral Circulation Associates with Disease Recurrence in Patients with HNSCC

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Abstract

**Purpose:** Patients with cancer have an increased frequency of circulating apoptosis-sensitive CD8+ CCR7neg T cells and few CD8+ CCR7+ T cells versus normal controls. The functional and clinical significance of this imbalance was investigated using peripheral blood of patients with squamous head and neck (HNSCC).

**Experimental Design:** The frequency of circulating CD8+ T cells co-expressing CCR7, CD45RO, CD28, and Annexin V (ANXV) was evaluated in 67 patients and 57 normal controls by flow cytometry. Spearman rank correlations among immunophenotypic profiles were analyzed. Recursive partitioning classified subjects as patients or normal controls based on CD8+ CCR7+ T-cell percentages. Kaplan–Meier plots estimated disease-free survival (DFS).

**Results:** The CD8+ CCR7+ T-cell frequency was low, whereas that of total CD8+ CCR7neg and ANXV-binding CD8+ CCR7neg T cells was higher in patients with HNSCC than in normal controls (P < 0.001–0.0001). ANXV binding correlated with the absence of CCR7 on CD8+ T cells (P < 0.001). ANXV binding was negatively correlated with the CD8+CD45ROnegCCR7+ (Tn) cell frequency (P < 0.01) but positively correlated (P < 0.01) with that of CD8+CD45RO+CCR7+ (TCD4) T cells and of the two CCR7neg subsets (TnA and TnB). In recursive partitioning models, the CD8+ CCR7+ T-cell frequency of 31% distinguished patients from normal controls with 77% to 88% accuracy after cross-validation. In 25 patients tested before any therapy, the CD8+ CCR7+ T-cell frequency of less than 28% predicted disease recurrence within 4 years of definitive therapy (P < 0.0115).

**Conclusion:** The CD8+ CCR7+ T-cell frequency in HNSCC patients’ blood tested at diagnosis can discriminate them from normal controls and predicts disease recurrence. Clin Cancer Res; 19(4); 889–99. ©2012 AACR.

Introduction

One mechanism of tumor evasion in patients with cancer, including those with squamous cell carcinoma of the head and neck (HNSCC), is targeted apoptosis of activated T lymphocytes at the tumor site and in the peripheral circulation (1, 2). The proportion of CD8+ T cells undergoing spontaneous apoptosis in the blood of patients with HNSCCs is elevated relative to that in sex- or age-matched normal controls (1). CD8+ T cells are more sensitive to apoptosis than CD4+ cells, and the tumor-specific CD8+ T cells are preferentially targeted for cell death (3–5). In patients with cancer, tumor epitope–specific T cells are eliminated either directly through the Fas/FasL or TRAIL/ TRAILR pathways (6, 7) or indirectly through the release of tumor-derived exosomes carrying death receptor ligands (8–10). Antitumor functions of effector CD8+ T cells may be also suppressed in cancer through the recruitment of regulatory T cells (11, 12) or myeloid-derived suppressor cells (13).

Earlier studies showed that expression of the chemokine receptor CCR7, which binds CCL19 and CCL21, by CD8+ T cells protected them from apoptosis via Akt phosphorylation and increased Bcl-2 expression (9, 14). Although fewer CD8+ CCR7+ than CD8+ CCR7neg cells bound Annexin V (ANXV), the frequency of circulating CD8+ CCR7+ T cells was significantly reduced in patients with cancer versus normal controls, and CD8+ CCR7+ cells were replaced by an excess of apoptosis-sensitive CD8+ CCR7neg T cells (14).
Expression of CD45RA/CD45RO and CCR7 on the surface of T cells is generally used as a measure of their differentiation (15, 16). When naïve (T_N) CD8⁺ T cells (CD45RA⁺/RO⁻/CCR7⁺) interact with antigen-presenting cells (APC) in secondary lymph nodes, they undergo clonal expansion and differentiate into T central memory (T_CM) CD45RA⁻/RO⁺/CCR7⁺ and T peripheral memory (T_Pm) CD45RA⁻/RO⁻/CCR7⁻ cells, also referred to as T effector memory (T_EM) cells. The T_Pm cells migrate to peripheral tissues to mediate effector functions, whereas T_CM, which have no or little effector function, home to secondary lymphoid organs and retain the ability to proliferate and differentiate to T_Pm in response to antigen stimulation (15, 16). The co-stimulatory receptor, CD28, is also used to distinguish subsets of differentiating T cells. Similar to CCR7, it is expressed on T_N and is required for the generation of antigen-specific effector T cells through the interaction with its 2 ligands, B7.1 (CD80) and B7.2 (CD86), on APC. In contrast, antigen-experienced T_Pm downregulate CD28, and most of the memory T cells, including terminally differentiating T cells (T_DB), become CD28⁻/CD8⁺ (17).

Here, all 3 surface markers, CD45RA/RO, CCR7, and CD28, were used to evaluate the frequency of CD8⁺ T_N, T_CM, T_Pm, and T_DB cells in the blood of patients with HNSCCs. The propensity of these cells to undergo spontaneous apoptosis was evaluated by ANXV binding. ANXV preferentially binds to phosphatidylserines exposed on the lymphocyte surface, serving as a useful marker of early apoptosis. These immunologic markers were interrogated for their usefulness in discriminating patients with HNSCC from normal controls. Furthermore, by relating patient outcome after up to 4-year follow-up to the imbalance in CD8⁺ CCR7⁺ T cells at the time of diagnosis, we show that the frequency of these T cells in the patients’ peripheral blood may serve as the surrogate marker for the disease recurrence.

**Translational Relevance**

In contrast to normal subjects, patients with squamous cell carcinoma of the head and neck (HNSCC) tested at diagnosis have a low frequency of circulating CD8⁺CCR7⁺ T cells. The low frequency of circulating CD8⁺CCR7⁺ at diagnosis discriminates HNSCCs from normal subjects. Potentially, this paucity of CD8⁺ CCR7⁺ T cells could reflect their extravasation into tissues or a cancer-associated deficit in naïve or central memory CCR7⁺ T-cell subsets. The low frequency of circulating CD8⁺ CCR7⁺ T cells is a significant risk factor for disease recurrence. After up to 4 years of follow-up, disease-free survival (DFS) was found to be significantly shorter (P < 0.0115) for patients with fewer than 28% of circulating CD8⁺ CCR7⁺ T cells than in those with more than 28% of CD8⁺ CCR7⁺ T cells at diagnosis. Therefore, CD8⁺ CCR7⁻ T cells might play a role in cancer control, and a simple blood test for immune markers at diagnosis may have a prognostic value for predicting disease recurrence in HNSCCs.

**Materials and Methods**

**HNSCC patients and healthy volunteers**

Samples of venous blood were obtained from 67 patients with HNSCCs seen in the outpatient Otolaryngology Clinic of the University of Pittsburgh Cancer Institute (Pittsburgh, PA) between February 2007 and November 2008. Patients willing to participate in this study were approached by the staff for donating peripheral blood samples. Before phlebotomy, all subjects signed an informed consent approved by the Institutional Review Board (IRB# 96-82). The disease status, treatment history, and the HPV status in the tumor were recorded at the time of the blood draw. The patient cohort included 52 males and 15 females (mean age, 56.6 years). The normal control cohort included 57 age- and sex-matched volunteers (34 males and 13 females; mean age, 53 years).

**Collection of PBMC**

Venous blood (20–30mL) was drawn into heparinized tubes. The samples were hand carried to the laboratory and centrifuged on Ficoll-Hypaque gradients. Peripheral blood mononuclear cells (PBMC) were recovered, washed in RPMI medium (Invitrogen), counted in the presence of a trypan blue dye, and used for experiments.

**Antibodies and reagents**

Anti-human monoclonal antibodies (Abs) with the following specificities were used for flow cytometry: CD3-ECD, CD4-ECD, CD8-PC5, CCR7-PE, CCR7-FITC, CD45RO-ECD, CD28-PE, Fas-FITC, and fluorescein isothiocyanate (FITC) conjugates.
isothiocyanate (FITC)-conjugated ANXV. Abs and their respective isotypes were purchased from Beckman Coulter, except for anti-CCR7-FITC and anti-CCR7-PE (BD Biosciences). All Abs were titrated using normal PBMCs to establish optimal staining dilutions. All cell culture reagents were purchased from Invitrogen.

**Surface staining and flow cytometry**

PBMCs were washed twice in staining buffer (0.1% w/v bovine serum albumin and 0.1% w/v NaN₃) and stained for cell surface markers as described (11). Briefly, cells were incubated with relevant Abs for 20 minutes at room temperature in the dark, washed twice, and fixed in 1% (v/v) ethanol.

### Table 1. Clinicopathologic characteristics of the patients with HNSCCs and normal controls in the study subset (left) and the confirmatory subset (right)

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paraformaldehyde (PFA) in PBS. Four-color flow cytometry was conducted using a Beckman Coulter flow cytometer. The gate was set on lymphocytes, and at least 10^3 cells were collected for analysis. Data were analyzed using Coulter EXPO 32vl.2 analysis software.

ANXV-binding assay
ANXV binding to PBMCs was measured by flow cytometry as previously described (14). Briefly, after staining with Abs specific for surface markers, cells were incubated with FITC-conjugated ANXV for 15 minutes on ice. Staining with 7-aminoactinomycin D (7-AAD) was conducted to identify dead versus live cells and to discriminate between non-apoptotic live cells (ANXV<sup>−</sup>/7AAD<sup>−</sup>), early apoptotic cells and a higher percentage of ANXV binding CD8<sup>+</sup> T cells in tumor tissues (Supplementary Fig. S1B) and a higher percentage of ANXV binding CD8<sup>+</sup> T cells relative to normal controls (Supplementary Fig. S1B) and could be distinguished from normal control by these 2 parameters, which did not, however, correlate with each other. These correlative data imply that the low frequency of CD8<sup>+</sup>CCR7<sup>+</sup> T cells and increased ANXV binding to CD8<sup>+</sup>CCR7<sup>+</sup> cells may be independently regulated.

T-cell differentiation and apoptosis
A possibility was considered that ANXV binding to CD8<sup>+</sup> T cells could be related to their maturation stage in patients. Therefore, ANXV binding to 4 lymphocyte differentiation subsets, defined by CCR7, CD45RO/RA, and/or CD28 expression (Fig. 1A), was studied by multiparameter flow cytometry. Figure 1 shows that the higher percentages of CD8<sup>+</sup>CCR7<sup>+</sup> T cells in patients with HNSCCs than in normal controls resulted from the accumulation of terminally differentiating (T<sub>TD</sub>) T cells (P < 0.0001). In contrast, naïve (T<sub>N</sub>) cells were reduced in frequency versus normal controls (P < 0.0001; Fig. 1B). The frequency of T<sub>PM</sub> cells and T<sub>CM</sub> cells was comparable in patients and normal controls. Patients with active disease or NED had a similar frequency of these T-cell subsets (data not shown).

In patients with HNSCCs, the T<sub>TD</sub> cells contained the highest percentage of ANXV<sup>+</sup> cells (Fig. 1C), whereas the T<sub>CM</sub> and T<sub>N</sub> subsets contained fewer ANXV<sup>+</sup> T cells, albeit still higher than those in normal controls. Figure 1D
illustrates ANXV binding to T cells in each of the 4 subsets in representative normal controls and HNSCC specimens. CD8^+CCR7^- T cells, that is, the TTD and TPM subsets, contained the bulk of ANXV^+ T cells in patients and normal controls. These data clearly link ANXV binding to the T-cell maturation stage but do not explain a loss of CD8^+CCR7^+ T cells.

A distinct "immune signature" of HNSCC patients

To better define phenotypic characteristics of CD8^+ T cell subsets in the blood of patients and normal controls, multicolor flow cytometry was conducted using 20 marker combinations. The data from patients and normal controls were combined to compute Spearman correlation coefficients for all 20 phenotypes. In a heatmap (Fig. 2), the CCR7^- T cells, TPM and TTD, positively correlated with ANXV binding (red/brown), whereas among the CCR7^+ subsets, only TCM showed a weak positive correlation. Tn cells were negatively correlated with ANXV binding (green). This suggested that TPM and TTD cells are more likely to undergo apoptosis whereas Tn may be protected. In the TCM subset, this protection was less evident. The heatmap positively correlated circulating CD8^+CCR7^- T cells in patients and normal controls.

As the low frequency of CD8^+CCR7^+ T cells and the high frequency of CD8^+ANXV^+ T cells were consistently observed in patients with HNSCCs, we asked which of these characteristics would better serve as an "immune signature" for the patient population. In unsupervised hierarchical cluster analysis (Fig. 3A), including 23 normal controls and 44 patients with HNSCCs (20 active disease and 24 NED), 2 distinct clusters of interest were identified: the "control"
cluster comprised predominantly normal controls (green) and a “patient” cluster comprised active disease or NED patients. These 2 clusters were respectively characterized by high and low percentages of CD8^+ ANXV^+ T cells. The “control” and “patient” clusters also differed with respect to CCR7 expression in CD8^+ T cells and, especially, in the percentage of TN T cells (Fig. 3A). The “control” cluster contained only 6 patients with HNSCCs who, according to the clustering algorithm, were more similar to controls than to other patients. Importantly, at the time of analysis, 2 of 6 patients were NED following curative therapy. Thus, CCR7 expression and ANXV binding could discriminate patients from normal controls.

To discriminate between active disease and NED patients within the “patient” cluster based on phenotypic signatures, we constructed a heatmap where the subject classes normal controls, active disease, and NED were grouped together (Fig. 3B). We found that the immunologic markers did not clearly separate active disease from NED patients, although they discriminated patients from normal controls.

Recursive partitioning of HNSCC patients based on phenotypes

To determine whether the frequency of CD8^+ ANXV^+ T cells discriminates patients from normal controls better than that of CD8^+ CCR7^+ T cells, we used recursive partitioning methods. A series of decision tree algorithms was created to generate a simple set of rules for classifying the subjects. Initially, using the combination of only the frequency of the above listed phenotypes within the TTD subset, we partitioned subjects into normal controls or patients with the 88% cross-validated accuracy (Fig. 4A).

To validate the accuracy of the prediction models experimentally, we applied the same classification rules to the consecutively enrolled, independent confirmatory subsets of 15 normal controls and 15 patients with HNSCCs who were comparable in age, sex, and clinicopathologic parameters to the original cohort (Table 1). Furthermore, the cohorts had a similar mean apoptosis sensitivity within the cell subsets used for discrimination (Supplementary Table S1). This verification confirmed that the predictive model using only 2 phenotype profiles (as in Fig. 4A) correctly discriminated patients and controls with cross-validated accuracy of 93% (Supplementary Table S2). If CD28 is substituted for CCR7, the predictive model showed discrimination accuracy of 90% (Supplementary Table S2).

Whether using the frequency of ANXV^+ CD8^+ T cells with the T_{NP} phenotype or the T_{TD} phenotype as a differentiating factor, patients and normal controls were correctly classified (Supplementary Table S2). These results suggest that the
Figure 3. Hierarchical clustering of the 20 phenotypes in patients with HNSCCs and normal controls (NC). Clustering is based on the distance applied to subjects (rows) and the 20 phenotypes (columns) defined by flow cytometry. Percentages of positive cells were expressed as standardized values with a mean of 0 ± 1 SD. A color bar on left depicts subject groups as normal control (green), HNSCCs with active disease (yellow), and HNSCCs with NED (olive). A, unsupervised clustering revealed 2 distinct clusters: a high ANX-binding patient cluster and a low ANX-binding normal control cluster. The patient cluster also has a uniformly lower CCR7 expression and a higher percentage of CCR7<sup>+</sup>CD8<sup>+</sup>T cells than the normal control cluster. B, a heatmap of subjects (rows) and phenotypes (columns), where the subject classes normal control, active disease, and NED were grouped together. Patients with active disease do not differ from NED patients according to their CCR7 phenotypes or ANX binding. The identities of all subjects were defined by different colors in the vertical bar on the left.
predictive models based on ANXV binding to CD8^+ T cells have very high discriminating accuracy. However, measurements of ANXV binding to CD8^+ T cells require freshly harvested (2 hours) specimens and are influenced by the time elapsed from phlebotomy. In routine immune monitoring, this is an impediment. Therefore, we asked whether the use of only one phenotype, CD8^+CCR7^+T cells, could discriminate patients from normal controls. Using recursive partitioning and the CD8^+CCR7^+T-cell frequency of 31% determined by the algorithm, we showed that this phenotype discriminates patients from controls with 77% to 85% accuracy (Fig. 4B). In addition, to test whether the patients’ HPV status influenced the frequency of CD8^+CCR7^+T cells, the data available for 12 HPV^neg and 6 HPV^+ patients were compared. No significant difference in the frequency of CD8^+CCR7^+T cells was observed between these 2 cohorts (P = 0.7974).

**Frequency of circulating CD8^+CCR7^+ T cells and the tumor recurrence**

As the CD8^+CCR7^+ phenotype accurately discriminated patients from normal controls, we asked whether the frequency of circulating CD8^+CCR7^+T cells in patients with HNSCCs at diagnosis and before any curative therapy could serve as a prognostic marker for disease recurrence. Among our training set of 43 patients, 25 had active disease and donated blood just before definitive therapy with surgery, chemotherapy, radiation, or chemoradiotherapy. All were treated with curative intent and were followed for up to 4 years for disease recurrence by the standard clinical criteria.

In this cohort of 25 patients, 12 were treated with surgery and 13 were nonsurgically treated. At the median follow-up for NED patients of 25 months (range, 9–43 months), there were 11 recurrences of HNSCCs and 1 new primary of the upper aerodigestive tract (lung). The median recurrence-free survival (RFS) has not been reached. This is typical for our institution, where the median RFS occurs around 30 to 36 months. Patients with a higher percentage of CD8^+CCR7^+T cells before therapy had a decreased risk of disease recurrence. A proportional hazards model estimated the HR of 0.51 [95% confidence interval (CI), 0.27–0.96] for each 10% increase in the baseline frequency value for CD8^+CCR7^+T cells. A recursive partitioning model found a stable cutoff of 28% CD8^+CCR7^+ to discriminate patients by their risk of disease recurrence regardless of therapy subsequently received. A Kaplan–Meier plot of DFS by CD8^+CCR7^+28% is shown in Fig. 5. This discriminator identified a subgroup of 10 low-risk patients with high (>28%) CD8^+CCR7^+ cells. Only one...
The chemokine receptor, CCR7, plays a key role in migration of naïve and memory T cells as well as mature dendritic cells (DC) to lymph nodes (LN) through binding to its ligands expressed on high endothelial venules and in T-cell zones within secondary lymphoid organs (20). CCR7 also contributes to tolerance induction: the CCR7-dependent contact of T cells and DCs within LNs is essential for the induction of peripheral tolerance and the regulation of the immune response by Treg (20). In addition, CCR7 plays a role in establishing the organizational architecture of thymic and in the negative selection of self-reactive T cells (21).

Besides its functional importance for the development of immunity and tolerance, CCR7 expression on tumor cells has been positively correlated with the presence of nodal metastases and thus a poor prognosis in different types of cancer, including HNSCCs (22–24). The involvement of chemokine receptors and their ligands in tumor cell survival has been reported (23, 25). For example, CCR7 signaling protected HNSCC cells from cisplatin-induced apoptosis by engaging the Akt-dependent pathway (24). Antiapoptotic effects of CCR7 were also observed in mature DCs, where CCR7-mediated protection involved activation of the PI3K/Akt pathway and NF-κB translocation to the nucleus (26). Similar survival mechanism is present in CD8+ CCR7+ T cells but not in CD8+ CCR7neg T cells, as previously reported (14).

In patients with HNSCCs, the frequency of apoptosis-resistant CD8+ CCR7+ T cells is strikingly reduced, and these cells are replaced by the subset of CD8+ CCR7neg TTPM and TTTD cells, which are highly sensitive to apoptosis. Our results showing that these CD8+ T-cell subsets are expanded in patients with HNSCCs contradict other in vitro and in vivo studies, in which memory T cells were found to be more resistant to apoptosis than TN (27–29). These earlier studies were done in normal controls, where the relative sensitivity/resistance to apoptosis may be regulated by a differential replicative capacity of T-cell subsets. As TN and TCM cells have a high proliferation rate, whereas TTPM and TTTD replicate poorly if at all, to maintain cellular homeostasis, the former undergo apoptosis at a higher rate than the latter (28). However, in patients with cancer, the normal pattern of memory CD8+ T-cell formation, differentiation and death are altered. Chronic exposure to tumor-associated antigens tends to rapidly drive TN to TTD and ultimately to death, resulting in the exhaustion of effector cells (30). For example, repeated in vitro stimulation of gp100-specific CD8+ T cells resulted in a progressive loss of effector functions and proliferation resulting in the conversion of curative to noncurative antitumor responses (31). Also, a preferential death of CD8+ TCM and CD8+ TEM has been reported under oxidative stress, a condition common in cancer (32). Thus, in cancer, chronic antigenic stimulation and oxidative stress drive the rapid recruitment and turnover of differentiating T cells and culminate in their accelerated death.

While the shift in CCR7 expression on T cells in cancer could be rationally explained by their differentiation status and rapid turnover, its clinical significance remains unclear (33). Nevertheless, it has been suggested that the rapid demise of CD8+ effector T cells in cancer contributes to poor antitumor responses often seen in patients with...
HNSCCs (1–3). An alternative possibility is that CD8+ CCR7+ effector T cells migrate to the tumor and their depletion from the periphery corresponds with the accumulation in situ.

Correale and colleagues reported that the tumor infiltration by CCR7+ T cells was predictive of favorable outcome in patients with advanced colon carcinoma enrolled in the GOLFIG-2 phase III trial (34). A high content of CD8+ CCR7+ tumor-infiltrating lymphocytes (TIL) in tumor samples obtained at baseline, that is, before any therapy, significantly correlated with a prolonged overall survival (OS) and prolonged DFS after front-line chemotherapy (34). This suggests that CD8+ CCR7+ T cells accumulating at tumor sites play a critical role in immunosurveillance and thus patients’ survival, as proposed by Zitvogel and colleagues (35). It also provides an independent confirmation for the potential prognostic importance CD8+ CCR7+ T cell infiltrations in patients with cancer at diagnosis.

To test the possibility that a loss of CCR7 expression on CD8+ T cells in the periphery might be important for cancer prognosis, we simultaneously evaluated 2 phenotypic characteristics of these cells: ANXV binding and CCR7 expression. Aside from discriminating patients with cancer from normal controls based on ANXV binding to CD8+ cells, the mere presence or absence of CCR7 on the surface of CD8+ T cells was shown to be sufficient for discrimination of patients from normal controls and also to serve as a useful surrogate marker of DFS. In a small cohort of patients whose blood was sampled for lymphocyte phenotyping before any therapy, the frequency of CD8+ CCR7+ T cells appears to accurately predict who is at high risk of recurrence regardless of the type of definitive therapy received. Given a small number of patients involved in the study, this is a surprising and unexpected finding. This is also the first report of surrogate immune marker measured in the blood with a promising clinical application in HNSCCs. The disease recurrence after definitive curative therapy is a major clinical problem in this and other malignancies. The ability to predict recurrence at the time of diagnosis before any therapy with a simple flow cytometry-based blood test offers a possibility for the selection of more aggressive therapy for patients in the high-recurrence cohort. The predictive value of this test will need to be confirmed in much larger studies involving a careful patient follow-up over an extended period of several years and its relationship to existing predictive markers such as smoking and HPV status analyzed. Nevertheless, the assay is simple and reliable, uses peripheral blood, and is required only at one time point, before curative therapies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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