Tumor-Reactive CD8+ Early Effector T Cells Identified at Tumor Site in Primary and Metastatic Melanoma

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Abstract

CD8+ T cells at the earliest stage of effector generation have not been identified at tumor site of melanoma patients. Such early effectors, if present, should be characterized by a specific phenotype, distinct from that expressed at later stages of the antigen-induced differentiation program, by short-lived effector cells, memory precursors, and terminal effectors. Here, we show that neoplastic tissues from primary and metastatic lesions of melanoma patients contain a subset of CD8+ T cells expressing FOXP3. CD8+ FOXP3+ CD25+ T lymphocytes were found in tumor-invaded lymph nodes (TILN), s.c. metastases, and advanced primary lesions. Their frequency was significantly higher in TILN compared with tumor-free lymph nodes or with peripheral blood and in primary tumors compared with TILN. CD8+ FOXP3+ T cells did not express markers of regulatory [CTLA-4, CCL4, interleukin-10 (IL-10), transforming growth factor-β1], exhausted (PD-1), or senescent (CD57) CD8+ T lymphocytes. Instead, this subset showed an antigen-experienced "EM1" phenotype (CCR7+ CD45RA− CD28+ CD27+) and exhibited a CD127−, KLRG1+, HLA-DR−, CD38−, T-bet+, perforin− “early effector” profile predicted by current models. CD8+ FOXP3+ T cells produced IFN-γ on short in vitro activation, recognized autologous tumor by CD107a mobilization, and expressed Ki-67 on ex vivo analysis. In response to autologous tumor plus IL-2/IL-15, the CD8+ FOXP3+ T cells proliferated promptly and showed competence for differentiation (downregulation of CD27 and upregulation of T-bet). These results suggest development of early phases of antitumor immunity even in advanced melanoma. Moreover, the CD8+ FOXP3+ “early effector” subset may be an invaluable tool for monitoring immunity at tumor site. Cancer Res; 70(21); 8378–87. ©2010 AACR.

Introduction

Antigen-experienced, melanoma-specific effector memory (TEM) and terminally differentiated (TEMRA) CD8+ T lymphocytes have been found at high frequencies in tumor-invaded lymph nodes (TILN) compared with tumor-free lymph nodes (TFLN) and with peripheral blood of advanced melanoma patients (see ref. 1 for review and refs. 2, 3). However, according to several current models (6–10), mainly based on analysis of CD8+ response to viral infection, an encounter with antigen activates a differentiation program in naïve CD8+ T cells that initially generates early effectors with a KLRG1− CD127− phenotype. Subsequently, these early effectors can adopt different cell fates, depending on antigen density and duration of TCR stimulation, extent of interleukin-2 (IL-2) receptor signaling (11), type of antigen presenting cells, and presence of inflammatory cytokines. Early effectors can in fact differentiate to CD127hi KLRG1− short-lived effector cells (SLEC) or to CD127hi KLRG1− memory precursors (MPEC). Chronic antigen stimulation can foster further effector differentiation toward exhaustion, marked by PD-1 expression (12, 13), or senescence, associated with expression of KLRG1 and CD57 upregulation (14, 15). Early effectors generated in human subjects after vaccination against smallpox and yellow fever have also been shown to coexpress HLA-DR and CD38 at the peak of the primary response (16).

In this study, with the aim to assess whether tumor-reactive CD8+ T cells with an “early effector” phenotype...
and function can be found at tumor site of melanoma patients, we looked for the presence in such tissues of CD8+ T lymphocytes expressing the forkhead/winged helix transcription factor FOXP3. In fact, although FOXP3 marks subsets of CD4+ and CD8+ T lymphocytes with regulatory/suppressive function (see refs. 17, 18, for review), it is also expressed by recently activated T cells that acquire effector functions (19–21). We found a CD8+ FOXP3+ T-cell subset enriched in TILN compared with TFLN and periphery and in advanced primary lesions compared with TILN. Extensive profiling indicated that CD8+ FOXP3+ T cells from tumor site were functional, tumor-reactive lymphocytes at the earliest stage of effector generation and retain strong proliferative potential and competence for further differentiation.

Materials and Methods

Lymphocytes from patients and healthy donors

Lymphocytes were isolated by Ficoll density gradient centrifugation (Ficoll-Paque, Amersham Biosciences) from peripheral blood (PBL) of healthy donors and of melanoma patients. Lymphocytes were also isolated from TILNs or TFLNs of American Joint Committee on Cancer Stage IIIc and IV melanoma patients from s.c. metastases and from advanced vertical growth phase primary melanomas (the latter lesions having Breslow depths between 5 and 10 mm) immediately after surgical removal, as described (3, 22). Written informed consent was obtained from patients and healthy donors.

Double staining immunohistochemistry

Two-color immunohistochemistry was performed with formalin-fixed, paraffin-embedded tissues. Deparaffinized sections were treated with 3% hydrogen peroxide and then subjected to heat-induced epitope retrieval. After blocking slides with normal goat serum (Dako), sections were incubated overnight at 4°C with a 1:250 dilution of FOXP3-specific monoclonal antibody (mAb; Abcam). Slides were treated with biotin-conjugated secondary antimouse antibody (Dako) and then with horseradish peroxidase–conjugated avidin (Dako). Development was done by 3,3′-diaminobenzidine tablets (Sigma). Sections were then stained overnight at 4°C with a 1:15 dilution of CD8-specific mAb (Dako). After washing, polyclonal rabbit antimouse immunoglobulins were added (Dako), followed by tertiary APAAP mAb (Dako). The latter treatment was repeated twice, and then development was done with Fast Red Substrate System (Dako). Images were acquired with an Axiosview 100 microscope (Zeiss, Carl Zeiss) equipped with a digital camera Nikon Coolpix 995.

Antibodies and flow cytometry analysis

Four color stainings of lymphocytes were described as described (22). The following mAbs were used: CD3-PE or CD3-PerCp, CD4-PE or CD4-PerCp, CD8-PerCp, CD45RA-PE, CD27-PerCp, CD28-FITC, CD127-FITC or CD127-PE, CD25-FITC, CD57-FITC, IL-10-APC, IFN-γ-APC, HLA-DR-PE or -APC, PD-1-PE, CD107a-PE, perforin-FITC (BD Biosciences), LAG-3-FITC (Alexis Biochemicals), CCR7-APC, CCL4-FITC, transforming growth factor-β1 (TGF-β1)-APC, CTLA-4-FITC, GITR-FITC (R&D Systems), CD38-FITC (MiltenyiBiotec), FOXP3-PE (BD Biosciences) or FOXP3-FITC or FOXP3-APC, T-bet-Alexa647, Ki-67-PE (eBiosciences), and KLRG1-Alexa488 or KLRG1-Alexa647 (23). Lymphocytes were first stained at 4°C for 45 minutes with mAbs to cell surface markers. Staining for FOXP3 and for other intracellular molecules was carried out as described (22). In some experiments, lymphocytes from HLA-A*0201+ patients were initially stained as described (22) with phycoerythrin-labeled tetramers of HLA-A*0201 (ProImmune) containing peptides from Melan-A/Mart-126–35 (24) or gp100209–217 or from Influenza Matrix39–46 (25). Cells were then stained with mAbs to cell surface antigens (such as CD8 and CD25) and, after permeabilization, with FOXP3 mAb.

Each sample (at least 1–2 × 10^6 lymphocytes) isolated from TILN, TFLN, s.c. metastases, primary lesions, or PBL was analyzed by a dual-laser fluorescence-activated cell sorting cytometer (FACS Calibur, BD Biosciences) using CellQuest software (BD Biosciences).

Intracellular cytokine production assays

Lymphocytes from TILN of melanoma patients or PBL of healthy donors were stimulated for 6 hours with phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) plus ionomycin (Sigma-Aldrich) as described by Ahmadzadeh and colleagues (26) or with immobilized anti-CD3 mAb (22) in the presence of GolgiStop reagent (BD Biosciences). Cells were then stained for cell surface markers and, after permeabilization, for intracellular molecules as FOXP3, IL-10, TGF-β1, or IFN-γ.

CD107a mobilization assay

The CD107a mobilization assay was carried out as described by Betts and colleagues (27). Autologous melanoma cells (7.5 × 10^5) were seeded in 24-well plates and incubated overnight at 37°C. The next day, lymphocytes (3 × 10^6) isolated from TILN were added. In some experiments, before adding lymphocytes, melanoma cells were preincubated for 30 minutes with 10 μg/ml of anti-HLA class I mAb w6/32. Control wells contained lymphocytes alone. After CD107a-PE mAb (BD Biosciences) addition, plates were incubated for 1 hour at 37°C and then monensin (Golgi-Stop, BD Biosciences) was added at 1 μl/well. Plates were incubated at 37°C for 5 hours. Finally, cells were stained for cell surface markers and, after permeabilization, for FOXP3.

In vitro lymphocyte cultures and carboxyfluorescein succinimidyl ester assay

Lymphocytes from TILN were stained with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes), as described (22), and then cultured with autologous tumor cells (at a lymphocyte-to-tumor ratio of 5:1) with/without IL-2 (Chiron, 15 ng/ml) or IL-15 (PeproTech, 5 ng/ml) at 1 × 10^5/ml in RPMI 1640 containing 10% pooled human serum from healthy donors. After 4 to 6 days, cells were stained with mAbs to cell surface antigens (such as CD8, CD127, KLRG1,
and CD27) and, after permeabilization, with FOXP3 mAb and, in some experiments, with T-bet mAb. Cells were then evaluated by flow cytometry for the phenotype of a distinct subset showing a different extent of CFSE dilution.

Data analysis and statistics

Frequency of CD8+ FOXP3+ in TILN versus TFLN or PBL of patients and of MART-1 versus Flu-Matrix–specific T cells in TILN were compared by paired t test. Comparisons of CD8+ FOXP3+ frequency and CD8+ FOXP3+ to CD4+ FOXP3+ ratios in TILN, s.c. metastases, and advanced primary tumors were carried out by Kruskal-Wallis test followed by Dunn’s multiple comparison test.

Results

CD8+ FOXP3+ T lymphocytes at tumor site in primary and metastatic melanoma

Freshly isolated lymphocytes from TILNs of 37 patients were analyzed by four-color flow cytometry. TILN from most patients contained not only CD3+ CD4+ FOXP3+ T cells but even a small fraction of CD3+ CD8+ FOXP3+ lymphocytes (Fig. 1A and B; mean, 1.6%/CD8+; range, 0.2–5.2%/CD8+; n = 37). CD8+ FOXP3+ cells frequently expressed CD25 (Fig. 1A). CD8+ FOXP3+ T cells were significantly more frequent in TILN compared with matched TFLN removed from the same nodal basin (see Fig. 1C and D) and to
Furthermore, CD8+ FOXP3+ were also found in s.c. metastases (n = 6, mean 7.3%/CD8+) and in VGP advanced primary tumors (n = 4; mean, 11.7%/CD8+; Fig. 1F). CD8+ FOXP3+ frequency was significantly higher in these tissues than in TILN (by Kruskal-Wallis test followed by Dunn’s multiple comparison test, P = 0.01), but not in s.c. metastases. Frequencies of CD8+ and CD4+ lymphocytes expressing FOXP3 (normalized as percentage of all CD3+ cells in each tissue sample) were then used to obtain a CD8+ FOXP3+-to-CD4+ FOXP3+ ratio. The mean ratio was 0.086 ± 0.068 (Fig. 1G) in TILN of 37 patients, but 0.29 ± 0.25 in s.c. metastases (n = 6) and 1.82 ± 1.63 in primary tumors (n = 4). These results suggest that an inversion of the CD8+FOXP3+/-to-CD4+FOXP3+ ratio takes place along with tumor progression.

Taken together, these results indicate that a subset of CD8+ FOXP3+ T cells is present at tumor site in advanced melanoma.

**CD8+ FOXP3+ T cells in TILN do not express a regulatory CD8+ T-cell phenotype**

Suppressive CD8+ T cells have been shown to express CTLA-4 and, in some instances, LAG-3 and GITR, and to produce IL-10, TGF-β1, and CCL4 (28–31). In TILN (n = 5), CD8+ FOXP3+ T cells never expressed CCL4 and CTLA-4 (see Supplementary Fig. S3A for representative results from two patients). LAG-3 and GITR, which can be expressed also by activated T cells (32, 33), were found only on rare cells among the FOXP3+ CD8+ cells in two and three of five patients.
respectively (Supplementary Fig. S3A, arrows). Freshly isolated FOXP3+ CD8+ T lymphocytes from TILN did not express IL-10 or TGF-\(\beta\)1 (Supplementary Fig. S3B, top right quadrants in each dotplot). On activation with PMA + ionomycin or with immobilized anti-CD3 mAb expression of IL-10 was upregulated in CD4+ FOXP3+ cells from TILN (arrows, Supplementary Fig. S3C), but not in the CD8+ FOXP3+ cells from the same tissue. Similarly, no expression of TGF-\(\beta\)1 was seen, on activation, in the CD8+ FOXP3+ cells from TILN (data not shown). Taken together, the phenotypic and cytokine profile of these cells did not support a CD8+ Treg cell phenotype.

**CD8+ FOXP3+ T cells at tumor site have an “EM1” antigen–experienced phenotype**

In CD8+ cells from TILN, FOXP3+ cells were mostly found in the CCR7\(^{-}\)CD45RA\(^{-}\) subset (Fig. 2A, arrow in histogram gated on R3 region). In TILN from a different melanoma patient, after gating on CD8+ FOXP3+ cells, we confirmed that these lymphocytes were mostly CCR7\(^{-}\)CD45RA\(^{-}\) (Fig. 2B, region R1, representative of eight patients investigated). The same predominant CCR7\(^{-}\)CD45RA\(^{-}\) phenotype was found in CD8+ FOXP3+ lymphocytes from s.c. metastases and primary tumors (Fig. 2C). We then looked at the CD27 versus

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CD28 phenotype of CD8+ FOXP3+ T cells. A predominant CD27+ CD28+ phenotype (Fig. 2D) was found that, together with the CCR7 versus CD45RA phenotype, was consistent with an “EM1” maturation stage for these T cells (4).

CD8+ FOXP3+ T cells at tumor site have an early effector profile

Early effectors are expected to have a CD127− KLRG1− profile, in contrast to CD127lo KLRG1+ SLEC or CD127hi KLRG1− MPEC (6–10). Moreover, early effector T cells generated in vivo after viral immunization transiently coexpress HLA-DR and CD38 (16). In TILN, most of the FOXP3+ CD25+ cells were in the CD127− fraction of CD8+ cells (Fig. 3A, dotplots gated on R1 region, showing representative results from two of eight patients). Similarly, CD8+ FOXP3+ T cells from s.c. metastases and primary tumors were CD127− (Fig. 3B). KLRG1 could be expressed by some CD8+ cells from TILN (Fig. 3C, gated on R1, top left quadrant), but the CD8+ FOXP3+ subset in these tissues was invariably KLRG1− (Fig. 3C, gated on R1, bottom right quadrant). Similarly, CD8+ FOXP3+ cells from s.c. metastases and primary lesions were CD127− and KLRG1− (Fig. 3D). CD8+ FOXP3+ cells were HLA-DR+ CD38+ CD127−, whereas CD8+ FOXP3− lymphocytes were HLA-DR− CD38− and CD127− (Fig. 4C). We then evaluated the expression of T-BX21 (T-bet), one of the transcription factors induced when T cells differentiate to effector stage (9, 34). CD8+ FOXP3+ CD127− T cells were mostly T-bet positive (Supplementary Fig. S4, red arrow in top right dotplot), in contrast with CD8+ CD127+ cells that did not express T-bet (Supplementary Fig. S4, bottom right dot plot). We also looked at the expression of PD-1 by CD8+ FOXP3+ T cells from TILN, because CD8+ HLA-DR+ CD127− PD-1+ functionally impaired T cells have been described at tumor site of melanoma patients (13). Most CD8+ FOXP3+ T cells from TILN of these patients did not express PD-1 (Supplementary Fig. S5B, bottom right quadrant in dotplots gated on R1), although some CD8+ PD-1+ FOXP3+ T cells were found (range, 0.9–7.9%/CD8+; n = 5; Supplementary Fig. S5B, top left quadrant in dotplots gated on R1). Finally, CD8+ FOXP3+ cells from TILN did not express CD57 (Supplementary Fig. S5C), a marker of senescent T cells (15).

CD8+ FOXP3+ T cells from TILN produce IFN-γ on activation, express perforin, and recognize autologous tumor cells

Intracellular IFN-γ production in FOXP3+ and FOXP3− CD8+ T cells from TILN was evaluated after stimulation with PMA + ionomycin or with anti-CD3 mAb. FOXP3 expression on T cells was not modified by these stimuli, and most lymphocytes from patients and from a healthy donor remained FOXP3 negative (Fig. 5A). However, all the CD3+ CD8+ FOXP3+ cells from TILN produced IFN-γ in response to either PMA + ionomycin or to CD3 ligation (Fig. 5A, top right quadrant in each dotplot). Moreover, CD8+ FOXP3+ CD127− lymphocytes from TILN expressed perforin (Fig. 5B, arrows in dotplots gated on R2 in top and middle). In contrast, in the CD4+ CD127+ T cells from the same tissues, FOXP3 and perforin expressions were mutually

Figure 4. CD8+ FOXP3+ T cells from TILN coexpress HLA-DR and CD38. A, CD8+ lymphocytes from TILN of a melanoma patient were characterized for expression of FOXP3 and CD25. HLA-DR versus CD38 phenotype was then evaluated after gating on FOXP3+ cells (region R1). B, expression of HLA-DR versus CD38 in CD8+ FOXP3+ lymphocytes from two s.c. metastases and one VGP primary tumor. C, HLA-DR versus CD38 and HLA-DR versus CD127 phenotype of FOXP3+ (region R1) and FOXP3− (region R2) CD8+ lymphocytes from TILN of a melanoma patient. Numbers in each dotplot, percentage of positive cells in each quadrant.
exclusive (Fig. 5B, bottom panel, gated on R2). To assess whether CD8+ FOXP3+ T cells from TILN could recognize autologous tumor cells, we used the CD107a mobilization assay (27). Surface expression of CD107a was found in 15% to 25% of freshly isolated, unstimulated CD8+ FOXP3+ T cells from TILN of two patients (Fig. 6A and B, top), whereas CD4+ FOXP3+ T cells from the same tissues were CD107a−. On coculture with autologous tumor cells, the percentage of CD107a+ cells increased to 40% to 50% in the CD8+ FOXP3+ subset, whereas no response was seen in the CD4+ FOXP3+ subset of TILN (Fig. 6A and B, bottom). Preincubation of autologous tumor cells with the anti-HLA
Class I mAb w6/32 inhibited CD107a mobilization by CD8+ FOXP3+ cells (Supplementary Fig. S5). These results indicated that CD8+ FOXP3+ T cells from tumor site are directed to melanoma-associated antigens expressed by autologous tumor. However, CD8+ FOXP3+ T cells from TILN were not directed to the immunogenic and shared melanocyte lineage–specific epitopes as Melan-A/MART-1 or gp100. In fact, Melan-A/Mart-1–specific T cells, identified on ex vivo analysis through HLA tetramer staining, were found in HLA-A2.1+ patients, but these cells did not express FOXP3 (Supplementary Fig. S7A, regions highlighted in red, and B, for results from 15 patients). Lack of FOXP3+ cells was found even among gp100–specific (data not shown) and Flu–matrix–specific T cells from the same tissues (Supplementary Fig. S8A and B). Among 15 HLA-A*0201 patients whose TILN were investigated, the mean frequency of FOXP3+ cells in MART-1 was <3 cells every 1,000 Tet+ cells (Supplementary Fig. S7B). Thus, the early effectors are directed to autologous neoplastic cells, but the antigenic determinants being recognized by these lymphocytes remain to be identified.

Proliferative response and differentiation of CD8+ FOXP3+ T cells from TILN on stimulation with autologous tumor cells and cytokines

Current models predict that CD8+ early effector cells should have a high proliferative potential (35). Accordingly, freshly isolated CD8+ FOXP3+ KLRG1+ lymphocytes from TILN contained a high fraction (~40–70%) of Ki-67+ cells (Supplementary Fig. S8A and B, dotplots gated on R1), consistent with proliferative activity in vivo. By CFSE dilution assays, on culture for 4 days with autologous tumor plus low dose of IL-2 or IL-15, a strong proliferative response was observed in CD8+ FOXP3+ cells from TILN (Supplementary Fig. S9B and C, region R1). No proliferation was seen in the CD8+ FOXP3+ subset in response to tumor alone (Supplementary Fig. S9A) or in the CD8+ FOXP3+ fraction in response to tumor plus cytokines (Supplementary Fig. S9B and C, region R3). Then, to assess whether the CD8+ FOXP3+ early effectors were competent for differentiation, we compared the expression of KLRG1, CD127, CD27, and T-bet (8, 35–37) on such cells after culture with tumor with/without IL-2 or IL-15. CD8+ FOXP3+ cells that proliferated to tumor plus IL-2 or plus IL-5 retained a predominant KLRG1+CD127– early effector phenotype (Supplementary Fig. S9B and C, histograms gated on R1 region) as did the CD8+ FOXP3+ lymphocytes kept with tumor alone. However, the proliferating CD8+ FOXP3+ lymphocytes showed a partial loss of CD27 (highlighted by red arrows in Supplementary Fig. S9 B and C, histograms gated on R1) compared with the phenotype of CD8+ FOXP3+ cells kept with tumor alone (Supplementary Fig. S9A). Further experiments showed that CD8+ FOXP3+ cells showed a progressive loss of CD27, associated with a gradual upregulation of T-bet fluorescence intensity, along with the proliferative response to tumor plus IL-2 (Supplementary Fig. S10C, compare histograms gated on R3 and R4 versus those gated on R2 and R1). Similar results were obtained at day 6 of culture (data not shown).

Both CD27 downregulation and T-bet upregulation are consistent with the expected action of cytokines as IL-2 in promoting early effector cell commitment toward SLEC fate (8, 35–37).

Taken together, these results indicate that CD8+ FOXP3+ T cells from TILN are uncommitted early effectors that retain competence for prompt proliferative response and for further differentiation.

Discussion

This study provides evidence for the existence of tumor-reactive, CD8+ T lymphocytes at tumor site of melanoma patients at the earliest effector stage, before embracing subsequent cell fates (such as SLECs or MPECs). We used FOXP3 as an initial marker to identify early effectors, because the expression of this transcription factor is upregulated even on CD8+ T-cell activation (19–21). Expression of FOXP3 has been frequently reported to mark even CD8+ regulatory T lymphocytes found in cancer patients (28–31, 38–40). However, TILN–associated CD8+ FOXP3+ T lymphocytes described in this study did not express phenotypic and functional features of CD8+ Tregs found in patients or in thymus and tonsils (41, 42) or generated in vitro (29–31). Assessment of CD8+ FOXP3+–to–CD4+ FOXP3+ ratio in different tissue samples indicated that CD8+ FOXP3+ cells were more frequent than CD4+ FOXP3+ Tregs in primary lesions, but the ratio reversed in favor of Tregs in s.c. metastases and lymph node metastases. Thus, the phenotype and functional features of the CD8+ FOXP3+ T lymphocyte subset suggests that the neoplastic tissue is involved in the generation of early effectors. However, immune suppression may become dominant in advanced disease.

The identification of the CD8+ subset described in this study as “early effectors” was corroborated by several lines of evidence. First, the EM1 differentiation phenotype has been shown to be associated with a short replicative history, as defined by a high level of TCR excision circles and length of telomere repeats, compared with EM2 and EM3 subsets (4). Second, FOXP3+, but not FOXP3–, CD8+ T cells coexpressed HLA-DR and CD38, two markers that characterize effector CD8+ T cells at the peak of the primary response (16). Third, CD8+ FOXP3+ T cells from tumor site were KLRG1+CD127–; thus, these cells likely reflect an early differentiation stage before the emergence of KLRG1–CD127+ SLEC and KLRG1+CD127+ MPEC (8). Moreover, CD8+ FOXP3+ T cells did not express CD57, a marker of proliferative inability, of history of more cell divisions, and of short telomeres in T cells and natural killer cells (14, 43). Fourth, the CD8+ FOXP3+ T cells were functionally competent, as they did not express the exhausted T-cell marker PD-1, expressed perforin, and were efficient producers of IFN-γ. Most importantly, CD8+ FOXP3+ T cells from tumor site recognized the autologous tumor as documented by the CD107a degranulation assay. Fifth, in agreement with the expected proliferative ability and differentiation potential of early effectors (6–10, 35–37), the CD8+ FOXP3+ cells from TILN expressed Ki-67, proliferated extensively in
response to autologous tumor plus low dose of IL-2 or IL-15, and, along with proliferation, downregulated CD27 and upregulated T-bet. Taken together, these results support the notion that the CD8\(^+\) FOXP3\(^+\) cells from TILN are uncommitted early effectors with marked proliferative potential and competence for further differentiation.

TCR-dependent priming of T cells in the neoplastic tissue is a likely mechanism explaining generation of CD8\(^+\) FOXP3\(^+\) T cells with early effector phenotype. An alternative hypothesis is that these early effectors may be generated elsewhere but are then recruited at tumor site. Recently, CD8\(^+\) effectors lacking the lymph node homing receptor CCR7 (as the CD8\(^+\) T-cell subset described in this study) have been shown to be recruited to reactive lymph nodes through the chemokine receptor CXCR3 (44). Moreover, recent studies have indicated that presence of T lymphocytes in melanoma metastases correlates with expression, in neoplastic cells, of several chemokine genes, including those encoding CXCL9 and CXCL10, two CXCR3 ligands (45). Phenotypic analysis of the CD8\(^+\) FOXP3\(^+\) cells from TILN indicated that they indeed express CXCR3 (data not shown).

In the attempt to identify the melanoma-associated epitopes being recognized by the CD8\(^+\) FOXP3\(^+\) T cells, the immunogenic epitopes encoded by differentiation antigens as Melan-A/MART-1 and gp100 were the most likely candidates. In fact, T cells directed to these epitopes can be found in TILN at frequencies even higher than 1%/CD8\(^+\) (3), indicating selective expansion/accumulation. Second, such antigen-specific T cells frequently show an antigen-experienced T\(_{EM}\) profile (2, 3). However, by extensive analysis of tetramer\(^\text{T}\) cells from TILN of a panel of HLA-A2.1\(^+\) patients, we found that differentiation-specific T cells were negative for expression of FOXP3 on \textit{in vivo} analysis. This suggests that the early effectors recognize a different class of melanoma-associated antigens. For instance, the immunogenic epitopes recognized by CD8\(^+\) FOXP3\(^+\) early effectors at tumor site may be the class of unique antigens generated by mutations and described even in human melanoma (46).

In conclusion, we have shown that tumor site of melanoma patients contains a subset of tumor-reactive CD8\(^+\) T cells that, by extensive phenotypic and functional analysis, can be classified as representing the earliest stages of effector generation.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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