Tumor-reactive CD8+ early effector T cells identified at tumor site in primary and metastatic melanoma

Andrea Anichini1, Alessandra Molla1, Claudia Vegetti1, Ilaria Bersani1 Roberta Zappasodi2, Flavio Arienti3, Fernando Ravagnani3, Andrea Maurichi4, Roberto Patuzzo4, Mario Santinami4, Hanspeter Pircher5, Massimo Di Nicola2, and Roberta Mortarini1

Affiliations: 1Human Tumors Immunobiology Unit, Dept. of Experimental Oncology and Molecular Medicine, 2Medical Oncology Unit 3, Dept. of Medicine, 3Immunohematology Unit, Dept. of Diagnostic Pathology, 4Melanoma and Sarcoma Unit, Department of Surgery, Fondazione IRCCS Istituto Nazionale dei Tumori, 20133 Milan, Italy and 5Department of Immunology, Institute of Medical Microbiology and Hygiene, University of Freiburg, D-79104 Freiburg, Germany.

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Requests for reprints: Andrea Anichini, Human Tumors Immunobiology Unit, Dept. of Experimental Oncology and Molecular Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, Via Venezian 1, 20133 Milan, Italy. Phone. +39-0223902817; Fax. +39-0223903237. E-mail: andrea.anichini@istitutotumori.mi.it.

R.M. and M.D.N. contributed equally to this study.

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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 to tumor-free lymph nodes (TFLN), or to peripheral blood, and in primary tumors compared to TILN. CD8\(^+\) FOXP3\(^+\) T cells did not express markers of regulatory (CTLA-4, CCL4, IL-10, TGF\(\beta\)1), 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-\(\gamma\) upon short in-vitro activation, recognized autologous tumor by CD107a mobilization and expressed Ki-67 upon 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 anti-tumor immunity even in advanced melanoma. Moreover, the CD8\(^+\) FOXP3\(^+\) “early effector” subset may be an invaluable tool for monitoring immunity at tumor site.
Introduction

Antigen-experienced, melanoma-specific effector memory (T\textsubscript{EM}) and terminally differentiated (T\textsubscript{EMRA}) CD\textsuperscript{8}\textsuperscript{+} T lymphocytes have been found at high frequencies in tumor-invaded lymph nodes (TILN) compared to tumor-free lymph nodes (TFLN) and to peripheral blood of advanced melanoma patients (see ref. 1 for review and 2-3). However, according to the models based on analysis of CD45RA, CCR7, CD27 and CD28 expression (4-5), the antigen-experienced T\textsubscript{EM} or T\textsubscript{EMRA} T cells found in the neoplastic tissues are thought to represent the late stages of CD\textsuperscript{8}\textsuperscript{+} T cell differentiation. In contrast, CD\textsuperscript{8}\textsuperscript{+} T cells at the early stages of the CD\textsuperscript{8}\textsuperscript{+} effector generation program have not been yet detected in neoplastic tissues of untreated melanoma patients.

According to several current models (6-10), mainly based on analysis of CD\textsuperscript{8}\textsuperscript{+} response to viral infection, encounter with antigen activates a differentiation program in naïve CD\textsuperscript{8}\textsuperscript{+} T cells that initially generates early effectors with a KLRG1\textsuperscript{−} CD127\textsuperscript{−} phenotype. Subsequently, these early effectors can adopt different cell fates depending on antigen density and duration of TCR stimulation, extent of IL-2 receptor signaling (11), type of antigen presenting cells and presence of inflammatory cytokines. Early effectors can in fact differentiate to CD127\textsuperscript{lo} KLRG1\textsuperscript{−} short-lived effector cells (SLEC), or to CD127\textsuperscript{hi} KLRG1\textsuperscript{−} memory precursors (MPEC). Chronic antigen stimulation can foster further effector differentiation towards 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 co-express 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 to TFLN and to periphery, and in advanced primary lesions compared to 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 retaining 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 tumor-invaded (TILN), or tumor-free (TFLN) lymph nodes of AJCC Stage IIIc and IV melanoma patients, from subcutaneous (s.c.) metastases and from advanced VGP primary melanomas (the latter lesions having Breslow depths between 5 to 10 mm) immediately after surgical removal, as described (3, 22). Written informed consent was obtained from patients and healthy donors.

**Double staining immunohistochemistry.** Two colour 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 o/n at 4 °C with a 1:250 dilution of FOXP3-specific mAb (Abcam). Slides were treated with biotin-conjugated secondary anti-mouse Ab (Dako) and then with HRP-conjugated avidin (Dako). Development was done by DAB tablets (Sigma). Sections were then stained o/n at 4 °C with a 1:15 dilution of CD8-specific mAb (Dako). After washing, polyclonal rabbit anti-mouse 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 Axiovert 100 microscope (Zeiss, Carl Zeiss, Oberkochen, Germany) equipped with a digital camera Nikon Coolpix 995.
**Antibodies and flow cytometry analysis.** Four colour stainings of lymphocytes were performed as described (22). The following mAbs were used: CD3-PE or -PerCp, CD4-PE or -PerCp, CD8-PerCp, CD45RA-PE, CD27-PerCP, CD28-FITC, CD127-FITC or -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, San Diego, CA), LAG-3-FITC (Alexis Biochemicals), CCR7-APC, CCL4-FITC, TGF-β1-APC, CTLA-4-FITC, GITR-FITC (R&D Systems), CD38-FITC (MiltenyiBiotec), FOXP3-PE (BD Biosciences) or FOXP3-FITC or –APC, T-bet-Alexa647, Ki-67-PE (eBiosciences), KLRG1-Alexa488 or -Alexa647 (23). Lymphocytes were first stained at 4 °C for 45’ 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 PE-labeled tetramers of HLA-A*0201 (ProImmune, Oxford, U.K.) containing peptides from Melan-A/Mart-126–35 (24) or gp100209-217 or from Influenza Matrix58-66 (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 1-2x10^6 lymphocytes) isolated from TILN, TFLN, s.c. metastases, primary lesions or PBL was analyzed by a dual-laser fluorescence-activated cell sorting cytofluorimeter (FACSCalibur, 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 hr with PMA (Sigma-Aldrich) plus Ionomycin (Sigma-Aldrich) as described by Ahmadzadeh et al. (26), or with immobilized anti-CD3 mAb (22) in the presence of GolgiStop reagent (BD Research.}

Biosciences). Cells were then stained for cell surface markers and, after permeabilization, for intracellular molecules as FOXP3, IL-10, TGFβ1 or IFN-γ.

**CD107a mobilization assay.** The CD107 mobilization assay was carried out as described by Betts et al. (27). Autologous melanoma cells (7.5x10^5) cells were seeded in 24 well plates and incubated overnight at 37 °C. The next day, lymphocytes (3x10^6) isolated from TILN were added. In some experiments, before adding lymphocytes, melanoma cells were pre-incubated for 30 min 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 hr at 37 °C and then monensin (Golgi-Stop, BD Biosciences) was added at 1 μl/well. Plates were incubated at 37 °C for 5 hr. Finally, cells were stained for cell surface markers and, after permeabilization, for FOXP3.

**In-vitro lymphocyte cultures and CFSE assay.** Lymphocytes from TILN were stained with 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 1x10^6/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, 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 distinct subset showing different extent of CFSE dilution.

**Data analysis and statistics.** Frequency of CD8^+ FOXP3^+ in TILN vs. TFLN or PBL of patients and of MART-1 vs. Flu-Matrix-specific T cells in TILN were compared
by paired T test. Comparison of CD8⁺ FOXP3⁺ frequency, and of CD8⁺ FOXP3⁺ to CD4⁺ FOXP3⁺ ratios, in TILN, s.c. metastases and advanced primary tumors was 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 tumor-invaded lymph nodes (TILN) of 37 patients were analyzed by four colour 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 to matched TFLN removed from the same nodal basin (see Fig. 1C, D) and to PBL of the same patients (Fig. 1E). 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 % 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.

Tissue sections from metastatic lymph nodes of ten melanoma patients were evaluated for FOXP3 and CD8 expression by double staining immunohistochemistry. In most instances the two markers were expressed by distinct cells (Supplementary Fig. S1, panels 1-5). However, some cells stained positively for both CD8 and FOXP3 in the
lesions of 8/10 patients (Supplementary Fig. S1, panels 6-12 and Supplementary Fig. S2 panels 1-14). CD8⁺ FOXP3⁺ cells could be found among intra-tumoral lymphocytes (representative results shown in Supplementary Fig. S2, panels 1-4, 6-8, 11-13), as well as among peri-tumoral lymphocytes (Supplementary Fig. S2, panels 5, 9-10, 14).

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, TGFβ1 and CCL4 (28-31). In TILN (n=5), CD8⁺ FOXP3⁺ T cells never expressed CCL4 and CTLA-4 (Supplementary Fig. S3A for representative results from 2 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 2/5 and 3/5 patients, respectively (Supplementary Fig. S3A, arrows). Freshly isolated FOXP3⁺ CD8⁺ T lymphocytes from TILN did not express IL-10, or TGF-β1 (Supplementary Fig. S3B, upper right quadrants in each dotplot). Upon 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 TGFβ1 was seen, upon 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 8 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 vs. CD28 phenotype of CD8+ FOXP3+ T cells. A predominant CD27+ CD28+ phenotype (Fig. 2D) was found that, together with the CCR7 vs 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 to CD127hi KLRG1− MPEC (6-10). Moreover, early effector T cells generated in-vivo after viral immunization transiently co-express 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 2/8 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, upper left quadrant), but the CD8+ FOXP3+ subset in these tissues was invariably KLRG1− (Fig. 3C, gated on R1, lower right quadrant). Similarly, CD8+ FOXP3+ cells from s.c. metastases and primary lesions were CD127− and KLRG1− (Fig. 3D). CD8+ FOXP3+ cells from TILN (Fig. 4A), s.c. metastases and primary lesions coexpressed HLA-DR and CD38 (Fig. 4B). CD8+ FOXP3+ cells were HLA-DR+ CD38+ CD127+, while CD8+ FOXP3− lymphocytes were HLA-DR− CD38− and CD127+ (Fig 4C). We then evaluated the expression of TBX21 (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 upper right dot plot*), in contrast with CD8⁻ CD127⁻ cells that did not express T-bet (Supplementary Fig. S4, *lower right dot plot*). We also looked at the expression of PD-1 by CD8⁺ FOXP3⁺ T cells from TILN, since 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, *lower 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, *upper 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 upon 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, *upper right quadrant in each dotplot*). Moreover, CD8⁺ FOXP3⁺ CD127⁻ lymphocytes from TILN expressed perforin (Fig. 5B, *arrows in dotplots gated on R2 in upper and middle panels*). In contrast, in the CD4⁺ CD127⁻ T cells from the same tissues, FOXP3 and perforin expression were mutually exclusive (Fig. 5B, *lower 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 15-25% of freshly isolated, unstimulated CD8+ FOXP3+ T cells from TILN of two patients (Fig. 6 A, B, upper panels), while CD4+ FOXP3+ T cells from the same tissues were CD107a+. Upon co-culture with autologous tumor cells, the percentage of CD107a+ cells increased to 40-50% in the CD8+ FOXP3+ subset, while no response was seen in the CD4+ FOXP3+ subset of TILN (Fig. 6 A, B, lower panels). Pre-incubation of autologous tumor cells with the anti-HLA Class I mAb w6/32 inhibited CD107a mobilization by CD8+ FOXP3+ cells (Supplementary Fig. S6). 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 upon ex-vivo analysis through HLA tetramer staining, were found in HLA-A2.1+ patients, but these cells did not express FOXP3 (Supplementary Fig. S7 A, regions highlighted in red, and S7 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. S7A, B). Among 15 HLA-A*0201+ patients whose TILN were investigated, the mean frequency of FOXP3+ cells in MART-1 was \( \leq 3 \) cells every 1,000 Tet+ cells (Supplementary Fig. S7 B). 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 upon 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, B, dotplots gated on R1), consistent with proliferative activity in-vivo. By CFSE dilution assays, upon culture for 4 days with autologous tumor plus low dose IL-2 or IL-15, a strong proliferative response was observed in CD8+ FOXP3+ cells from TILN (Supplementary Fig. S9B, C, region R1). No proliferation was seen in the CD8+ FOXP3+ subset in response to tumor alone (Supplementary Fig. S9A), nor in the CD8+ FOXP3- fraction in response to tumor plus cytokines (Supplementary Fig. S9B, 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, 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, C, histograms gated on R1) compared to 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. S10 C, compare histograms gated on R3 and R4 vs. 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 towards 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 at tumor site of melanoma patients, of tumor-reactive, CD8\(^+\) T lymphocytes 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, since the expression of this transcription factor is upregulated even upon 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 favour 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 (TREC) and length of telomere repeats, compared to EM2 and EM3 subsets (4). Second, FOXP3\(^+\), but not FOXP3\(^-\), CD8\(^+\) T cells co-expressed 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 and NK 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 TEM profile (2-3). However, by extensive analysis of tetramer+ 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, upon ex-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.
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References


Figure legends.

**Figure 1. CD8⁺ FOXP3⁺ T lymphocytes at tumor site of melanoma patients.** A. Lymphocytes from TILN of four patients (Pt) were evaluated by flow cytometry for FOXP3 vs. CD25 expression after gating on CD3⁺ CD4⁺ or CD3⁺ CD8⁺. Numbers in each dotplot, percentage of positive cells in each quadrant. B. Frequency of CD4⁺ FOXP3⁺ and CD8⁺ FOXP3⁺ T cells in lymphocytes from TILN of 37 melanoma patients. C. Comparison of a TILN and of a tumor-free lymph node (TFLN) removed from the same nodal basin of a melanoma patient for the presence of CD25⁺ FOXP3⁺ T cells in the CD4⁺ and CD8⁺ subsets. D. Frequency of CD8⁺ FOXP3⁺ T cells in matched pairs of TILN and TFLN from 6 patients. E. Frequency of CD8⁺ FOXP3⁺ T lymphocytes in matched pairs of TILN and PBL from 16 patients. F. Frequency of CD8⁺ FOXP3⁺ T lymphocytes in s.c. metastases (s.c. mets) and VGP primary tumors (primary me.) from 6 and 4 patients respectively. G. CD8⁺ FOXP3⁺ to CD4⁺ FOXP3⁺ cell ratios in primary lesions, s.c. metastases and lymph node metastases. Statistical analysis in panels E and F, by paired T test, in panel G by Kruskal Wallis test followed by Dunn’s multiple comparison test.

**Figure 2. CD8⁺ FOXP3⁺ T lymphocytes from tumor site express an CCR7⁻ CD45RA⁻ CD27⁺ CD28⁺ antigen experienced phenotype.** A. Four subsets (defined by regions R1 to R4) were identified in CD8⁺ T lymphocytes from TILN after staining for CCR7 vs. CD45RA. Histograms show expression of FOXP3 in each of the four subsets. B. Expression of CCR7 vs. CD45RA was evaluated in CD8⁺ lymphocytes from TILN of a melanoma patient after gating on FOXP3⁺ cells (region R1). C. CCR7 vs. CD45RA
phenotype of CD8+ FOXP3+ lymphocytes in a s.c. metastasis and in a VGP primary tumor from two patients. D. CD27 vs. CD28 phenotype of CD8+ FOXP3+ lymphocytes from TILN of two patients, from a s.c. metastasis and a VGP primary tumor. Numbers in the dotplot, % positive cells in each quadrant.

**Figure 3. CD8+ FOXP3+ lymphocytes from tumor site have an early effector phenotype.** A. Lymphocytes from TILN of two melanoma patients (*upper and lower panels*) were stained for CD8, CD127, FOXP3 and CD25. Expression of FOXP3 and CD25 was evaluated in 4 subsets defined by gating on CD8 vs. CD127 (*regions R1 to R4*). B. CD3+ CD8+ T lymphocytes from two s.c. metastases and one VGP primary tumor were evaluated for expression of FOXP3 vs CD127. C. Lymphocytes from TILN of two melanoma patients (*upper and lower panels*) were evaluated for expression of FOXP3 and KLRG1 after gating on CD3+ CD8+ (*region R1*). D. Analysis of expression of KLRG1 and CD127 in CD8+ FOXP3+ lymphocytes from a s.c. metastasis and two VGP primary tumors. Numbers in each dotplot, % positive cells in each quadrant.

**Figure 4. CD8+ FOXP3+ T cells from TILN co-express HLA-DR and CD38.** A. CD8+ lymphocytes from TILN of a melanoma patient were characterized for expression of FOXP3 and CD25. HLA-DR vs. CD38 phenotype was then evaluated after gating on FOXP3+ cells (*region R1*). B. Expression of HLA-DR vs. CD38 in CD8+ FOXP3+ lymphocytes from two s.c. metastases and one VGP primary tumor. C. HLA-DR vs. CD38 and HLA-DR vs. CD127 phenotype of FOXP3+ (*region R1*) and FOXP3- (*region...
Figure 5. CD8⁺ FOXP3⁺ lymphocytes from TILN produce IFN-γ upon activation and express perforin. A. Lymphocytes from TILN of two melanoma patients (Pt #1 and #2) and from PBL of one healthy donor were cultured for 6 hr with PMA+ionomycin or with immobilized anti-CD3 mAb and then assessed for FOXP3 expression and intracellular IFN-γ production. Rectangular gates, production of IFN-γ by FOXP3⁺ CD8⁺ lymphocytes from TILN. B. CD8⁺ Lymphocytes from TILN of two patients (Pt #1 and #2, upper and middle panel) were evaluated for expression of FOXP3 and perforin after gating on CD127⁺ (region R1) or CD127⁻ (region R2). Arrows, expression of perforin in CD8⁺ FOXP3⁺ lymphocytes. TILN from Pt #2 (lower panel) were characterized by the same approach also in the CD4⁺ subset. Numbers in each dot plot, % positive cells in the upper right and lower right quadrants.

Figure 6. CD8⁺ FOXP3⁺ T cells from TILN recognize autologous tumor cells by the CD107a degranulation assay. CD107a surface expression was assessed in lymphocytes from TILN of two melanoma patients (A, B) cultured without (-) or with (+Me) autologous tumor cells for 6 hr. Lymphocytes were also stained for CD3, CD4 and FOXP3, or CD3, CD8 and FOXP3. Numbers in each dotplot represent the percentage of all FOXP3⁺ cells that are CD107a⁺ (upper rectangular gate) or CD107a⁻ (lower rectangular gate).
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Andrea Anichini, Alessandra Molla, Claudia Vegetti, et al.

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