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Aberrant Expression of MHC Class II in Melanoma Attracts Inflammatory Tumor-Specific CD4$^+$ T-Cells, Which Dampen CD8$^+$ T-cell Antitumor Reactivity

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Abstract

In the absence of a local inflammatory response, expression of MHC class II molecules is restricted mainly to hematopoietic cells and thymus epithelium. However, certain tumors, such as melanoma, may acquire aberrant constitutive expression of MHC class II. In a set of primary melanoma cell populations and correspondingly expanded autologous tumor-infiltrating lymphocytes (TIL), we show how MHC class II expression on melanoma cells associates with strong MHC class II–restricted CD4$^+$ T-cell responses that are specific for tumors. Notably, we found that tumor-specific CD4$^+$ T-cell responses were dominated by TNF production. TNF reduced CD8$^+$ T-cell activation in IFNγ-rich environments resembling a tumor site. Conversely, direct CD4$^+$ T-cell responses had no influence on either the proliferation or viability of melanoma cells. Taken together, our results illustrate a novel immune escape mechanism that can be activated by aberrant expression of MHC class II molecules, which by attracting tumor-specific CD4$^+$ T cells elicit a local inflammatory response dominated by TNF that, in turn, inhibits cytotoxic CD8$^+$ T-cell responses *Cancer Res; 75(18); 3747–59. ©2015 AACR.*

Introduction

In the absence of a local inflammatory response, MHC class II expression is mainly restricted to hematopoietic cells and thymus epithelium (1–3). However, certain types of solid tumors, including a subset of melanomas, *de novo* constitutively express MHC class II molecules (1, 3). Furthermore, MHC class II can be induced in several cell types, including tumor cells, by exposure to cytokines such as IFNγ (1, 4).

MHC class II expression in melanoma has been previously associated with both shorter and longer survival (5–7). Indeed, MHC class II expression can make these tumors directly detectable by tumor-antigen-specific CD4$^+$ T cells that we and others previously have shown to be capable of generating Th1 responses in response to autologous tumor antigens (8–11). In contrast, recent studies proposed that engagement of MHC class II by lymphocyte-activation gene 3 (LAG3), expressed respectively on tumor cells and tumor-infiltrating immune cells, may trigger prosurvival signals and tumor cell resistance to apoptosis (12). In addition, LAG3 has been characterized as an immune inhibitory receptor, and its engagement on T cells may mediate downregulation of immune responses in the tumor microenvironment during priming but not effector phase (13, 14).

In order to clarify the association of MHC class II expression with CD4$^+$ T-cell responses and CD4$^+$ T-cell functional patterns in melanoma, we conducted a multifunctional analysis of expanded tumor-infiltrating lymphocyte (TIL) direct recognition of a panel of 38 autologous-matched melanoma cell lines generated from individual patients with advanced disease. Our results shed light on a novel mechanism of inflammatory CD4$^+$ T-cell attraction to the tumor microenvironment by primary MHC class II expression on melanomas, which dampen CD8$^+$ T-cell responses via TNF-induced counteraction of IFNγ-mediated local amplification of immune responses.

Materials and Methods

Patients and samples

All the procedures were approved by the Scientific Ethics Committee for the Capital Region of Denmark. Written informed consent was obtained from patients before any procedure according to the Declaration of Helsinki. All patients were diagnosed with histologically confirmed advanced melanoma, AJCC stage IV ($n = 32$) or IIIB ($n = 1$, completely resected), IIIC ($n = 5$, two of those were completely resected).

Tumor cell lines from primary melanomas WM-115, FM-55-P, FM-55-M1, and FM-55-M2 were obtained from ESTDAB (http://www.ebi.ac.uk/ipd/estdab/). The characteristics of WM-115, WM-75, WM-793, and WM-266-4 have been described elsewhere (15).

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Antibodies for flow cytometry

Antibody panels used for flow cytometry were:

- Multifunctional characterization of $CD4^+$ and $CD8^+$ T-cell responses: CD4 QDOT705 (Life Technologies), CD8 QDOT 605 (Life Technologies), Live/Dead Fixable Dead Cell Stain Near-IR (Life Technologies), MIP-1α FITC (eBioscience), MIP-1β PerCP-eFluor 710 (eBioscience), CD107a Brilliant Violet 421, IFNγ PE-Cy7, TNF-APC, IL2 Brilliant Violet 650 (BioLegend), IL17A Brilliant Violet 510.
- All other tumor-T cell coculture experiments: CD4 FITC, CD8 PerCP, Fixable Viability Dye eFluor 450 (eBioscience), IFNγ PE-Cy7, TNF-APC, CD107a PE.
- MHC class I characterization: anti-HLA-ABC APC, 7-AAD
- MHC Class II characterization: anti-HLA-DP, DR, DQ FITC, 7-AAD

Generation of TILs and melanoma cell lines

TILs used in this study were generated with a protocol extensively described in other studies (16). Briefly, TILs were initially isolated and minimally expanded in high doses of IL2 (6,000 IU/mL IL2; Proleukin from Novartis) from surgically resected melanoma tumors, which were cut in 1- to 2-mm^3 fragments under sterile conditions. When a minimum of 50×10^6 TILs were obtained (typically about 14–28 days after surgical resection—the product at this stage is named "minimally cultured TILs"; ref. 17), expansion was further achieved by a standard 14-days rapid expansion protocol (REP), in which TILs are unspecifically expanded with a 200-fold excess of allogeneic irradiated peripheral blood mononuclear cells (PBMC) from at least three different healthy donors, 30 ng/mL anti-CD3 antibodies (OKT3, from Janssen-Cilag or Miltenyi Biotec). All through this article, this TIL product is named "expanded TILs" or "REP-TILs." From some patients, "uncultured (or fresh) TILs" were obtained from tumor fragments that were digested overnight in the presence of 1 mg/mL collagenase type IV (Sigma-Aldrich) and 0.0125 mg/mL dornase alpha (Pulmozyme, Roche) and immediately cryopreserved.

Pure $CD8^+$ or $CD4^+$ T-cell cultures—for the indicated experiments in which sorted T-cell subpopulations were used—were generated by positive magnetical selection, respectively, with CD8 or CD4 microbeads (Miltenyi Biotec), according to the manufacturer's instructions, from unselected TILs before REP. Subsequently, sorted subpopulations were expanded with REPs separately. Only cultures with frequency of over 95% of either $CD8^+$ or $CD4^+$ T cells were used for the indicated experiments.

Autologous melanoma cell lines were generated separately from TILs either from tumor fragments or from a combination of cells recovered from suspension in transport medium or after mincing, as previously described (16, 18).

Analysis of T-cell responses by flow cytometry

Evaluation of T-cell responses was performed as previously described (10, 16).

Briefly, TILs were thawed and rested for 3 days (for multifunctional characterization of REP TILs), 2 days (minimally cultured TILs), or overnight (in all other cases) in RPMI-1640 (Life Technologies) supplemented with 10% AB human serum (Sigma-Aldrich), thereafter washed twice and cocultured with autologous short-term cultured melanoma cell lines. Tumor reactivity was evaluated by assessing the amount of T cells previously gated as CD4 or CD8 T cells expressing cytokines (IFNγ and TNF) or CD107a.

For screening of $CD8^+$ and $CD4^+$ T-cell responses to autologous tumor antigens, autologous tumors in log-phase of growth were incubated with 100 IU/mL IFNγ (Imukin, Boehringer-Ingelheim) for 72 hours and thereafter extensively washed and added to the cocultures. This was done to maximize the possibility to detect antitumor responses of low frequency to autologous tumors with MHC downregulation or with no constitutive MHC class II expression. A response was defined as the presence of a minimum of 0.5% responding cells (expressing at least one of the following T-cell functions: TNF, IFNγ, or CD107a) in the relative $CD8^+$ or $CD4^+$ T-cell subpopulation, with a minimum of 50 positive events acquired and a minimum of a T-cell function positive cell frequency of three times the background (i.e., unstimulated samples). The frequency of tumor-reactive cells in stimulated samples was subtracted from unstimulated samples. 0.5% was used as limit of significance.

For MHC class II blocking experiments, tumor cells were incubated for 30 minutes at 37°C with 20 μg/mL of anti-HLA DR, DP, DQ antibody (clone TÜ39, from BioLegend) or with relevant isotype control. Thereafter, tumor cells were added to cocultures with TILs without additional washing (final concentration in the T-cell stimulation cocktail was around 0.5 μg/mL).

Combined tetramer (peptide–MHC multimer PE conjugated and produced in-house, HLA-A2–restricted MART-1/Melan-A–derived peptide ELAGIGILTV) and intracellular cytokine staining (CD107a PE was in this case switched with CD107a FITC) was performed to assess the frequency of MART-1–specific T cells producing functional responses, as previously described (10).

In multifunctional characterization experiments (seven T-cell function characterization), incubation time in coculture with autologous tumor cells was extended to 12 hours in order to be able to detect both early and late cytokine production.

Where indicated, cancer cells were treated for 72 hours with 1,000 IU/mL TNF (CellGenix), 100 IU/mL IFNγ or both. Because of the high sensitivity of assessment of simultaneous positivity of multiple T-cell functions by $CD8^+$ T cells, cells expressing simultaneously at least two functions (double positive cells) among TNF, IFNγ, and CD107a were chosen as a measure of the overall CD8 tumor reactivity in order to evaluate the effects of TNF on $CD8^+$ T-cell recognition.

Cells were acquired with a BD FACSCanto II flow cytometer or, for multifunctional characterization of T-cell responses, with a 5 lasers BD LSR II. Flow cytometers were equipped with FACS Diva Software 6.3 (BD).

ELISPOT and cytotoxicity assays

IFNγ ELISPOT experiments were conducted as previously described (16). A total of 3×10^4 TILs (respectively 3×10^4 $CD8^+$ T cells, 3×10^4 $CD4^+$ T cells, or 1.5×10^4 $CD8^+$ plus 1.5×10^4 $CD4^+$ T cells, so that the total amount of TILs was identical in each well) and 3×10^3 cancer cells were added in each well. Triplicate wells were analyzed. Results are presented as number of IFNγ spots in stimulated wells minus background.

Conventional ^{51}Cr-release assays for CTL-mediated cytotoxicity were carried out as described elsewhere (19).

Analysis of cancer cells

Analysis of MHC expression. Semiquantitative MHC class I or II expression of cancer cells was assessed by standard staining of

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freshly detached cancer cells with anti-HLA-ABC or HLA-DP, DR, DQ antibodies or isotype controls, washed, and 2 μL of 7-AAD was added to each sample 5 minutes before acquisition. Cells were acquired with a BD FACSCanto II flow cytometer, and given the different autofluorescence of individual cell lines voltage parameters were adjusted for each cell line in order to obtain an APC mean fluorescence intensity (MFI) of 275 ± 15 and a FITC MFI of 65 ± 5.

Given the nonhomogeneous MHC class II staining of several cancer cell lines in our panel, melanomas were identified as MHC class II–positive when the MFI of the antibody-stained sample under study exceeded at least four times the isotype-control stained.

Analysis of cell proliferation. Cell proliferation was evaluated using a flow cytometry–based counting method, as previously described (10). Briefly, after standard trypsinization, at day −1, melanoma cells were seeded at 5 to 8 × 10^4/well into 24-well plates and grown for 24 hours into standard medium. Thereafter, medium was exchanged with fresh standard medium ± indicated dilutions of supernatants from activated $CD4^+$ T cells from corresponding patients for a final concentration of 0.5 mL/well. Baseline control wells were trypsinized at day 0 with 50 μL of trypsin solution per well and 250 μL of standard medium with 0.05 μg/mL of propidium iodide (PI; from Sigma-Aldrich) was added into each well to exclude dead cells, and the obtained suspension was counted under a standard rate for a constant amount of time (for 90 seconds at high flow rate) in a BD FACSCanto II flow cytometer equipped with BD FACS Loader carousel. After 72 hours of drug exposure, the other wells were trypsinized and the cells were counted after identical working conditions of the baseline control wells. Growth inhibition was calculated using the following formula: (T72 − T0)/(K72 − T0) × 100, where T72 is the cell count after 72 hours, T0 is the cell count of the control well at time zero, and K72 is the cell count of the control well (medium) after 72 hours. Control values were arbitrarily set to 100. Values below 0 indicated net cell loss while values between 0 and 100 indicated growth inhibition.

Supernatants from activated $CD4^+$ T cells were obtained by incubating 5 × 10^6 sorted $CD4^+$ T cells from individual patients with 5 × 10^5 autologous IFNγ-treated (72 hours) tumor cells in wells of a 24-well plate in a total volume of 1 mL. After 24 hours, supernatants were collected, cells were washed out by two centrifugation steps at 1,500 rpm/5 minutes, and supernatants were subsequently cryopreserved at −80°C for later use.

RT-PCR. Total RNA was extracted from samples using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription reactions were performed using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Real-time PCR (qPCR; for IDO-1, PDL-1, MLANA, TYR, PMEL, TAPBP, PSMB9, and GAPDH) analyses were carried out using in-house designed primers and a LightCycler Nano instrument (Roche).

Statistical analysis

The D'Agostino-Pearson normality test was performed to check for normal distribution of the values, and F tests to check whether populations had equal variances. Decisions on whether to perform parametric or nonparametric tests in relevant datasets were made on the basis of results from tests discussed above. Results were compared between different groups with two-tailed tests (either paired *t* tests, unpaired *t* tests, Mann–Whitney test, or Wilcoxon matched pairs tests). Survival analyses were conducted with log-rank test. Qualitative data were compared with the Fisher exact test.

Logarithmically transformed data were used to compare differences between groups for analysis of relative quantitative capacity to express single T-cell functions. For qPCR data, ΔC_t were obtained by subtracting GAPDH C_t to target gene C_t. Wilcoxon matched-pairs signed-ranks test was used to compare differences between IFNγ-treated cells and medium-, TNF-, and TNF + IFNγ-treated cells. Data are shown as fold change relative to IFNγ-treated cells, which were set arbitrarily to 1.

In multifunctional characterization experiments (seven T-cell function characterization), flow cytometry data were initially processed with the FlowJo 9 (TreeStar Inc.) using a sequential gating strategy until identification of $CD4^+$ and $CD8^+$ T cells that were subcategorized in T-cell function–positive cells with parallel Boolean gating (gates drawn solely around responsive cells). Data were exported into Pestle 1.7 (courtesy of Dr. Mario Roederer, ImmunoTechnology Section, VRC/NIAID/NIH, Bethesda, MD) and properly formatted, according to Pestle instructions. Analysis and presentation of distributions was performed using SPICE version 5.2, downloaded from http://exon.niaid.nih.gov (20). In the analysis of REP TILs and minimally cultured TILs, background subtraction of unstimulated samples was performed with Pestle 1.7, according to the manufacturer's instructions. In contrast, in the analysis of uncultured TILs, background samples could not be subtracted because tumor digests unavoidably also contain uncultured tumor cells that can stimulate TILs during the 12 hours of incubation (data not shown). A threshold of at least 1% responding cells in $CD4^+$ or $CD8^+$ T-cell subsets was accepted, thus the impact of noise from background T-cell function expression was not more than approximately 30% to 40% (value estimated from background staining of Young TILs or REP TIL samples). Although with these thresholds, the contribution of background events to the overall response may be relatively high (especially for $CD107a^+$ cells, where a proportion of positive cells was observed in unstimulated samples in minimally cultured TILs and REP TILs). The analysis was possible only with these conditions because the frequency of tumor-reactive cells in uncultured TILs was generally low (of five samples analyzed from patient (Pt.) 11, 15, 19, 24, and 25, the average frequency of positive cells was 2.2% ± 1.6% for $CD4^+$ T cells, and 5.3% ± 5.1% for $CD8^+$ T cells).

In SPICE, thresholds were set at 0.1 for analysis of REP TILs, while in all the other analyses, thresholds were set at 0.02. Comparison of distributions was performed using a Student *t* test and a partial permutation test as described previously (20). Other statistical analyses were performed with GraphPad Prism 5 (GraphPad Software).

Results

Screening of $CD8^+$ and $CD4^+$ T-cell responses to autologous tumor antigens

Despite the fact that *in vivo* tumor heterogeneity may not be fully reflected by short-term cultured autologous melanoma cell lines, coculture assays of TILs/autologous tumor cell lines currently represents a gold standard as they allow the identification of

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T-cell responses directed against many, if not all, possible patient relevant tumor antigens. In order to obtain maximal T-cell responses, autologous tumors were pretreated with a low-dose IFNγ, which we have previously shown to increase TIL recognition and reactivity. Thereafter, tumors were exposed to autologous TILs (10).

Screening of 38 *in vitro* expanded TILs/autologous tumor pairs identified detectable direct $CD8^+$ and $CD4^+$ T-cell responses, respectively, in 34 (89%) and 18 (53%) patients (Fig. 1A; $P = 0.001$). The magnitude of responses, measured as the frequency of TILs expressing either TNF, IFNγ, or CD107a, was respectively 18% ± 18% (mean values, while the median was 10%) for $CD8^+$ and 4.0% ± 9% (mean values, while the median was 0.66%) for $CD4^+$ T cells (Fig. 1B; $P < 0.0001$). Thus, $CD8^+$ T-cell responses appeared both more frequent and stronger in magnitude than $CD4^+$ T-cell responses.

Blockade of MHC class II on tumor cells significantly reduced $CD4^+$ T-cell responses in 6 of 6 samples analyzed (samples from 6 patients with high $CD4^+$ T-cell responses were selected), confirming class II dependent recognition (Fig. 1C and D).

The presence of high $CD4^+$ T-cell responses (14 patients, identified with the presence of at least 2% cells in the whole $CD4^+$ T-cell subpopulation responding to autologous tumor) was not associated with a higher frequency of $CD4^+$ T cells among TILs (Fig. 1E). This may indicate that the magnitude of *in vivo* $CD4^+$ T-cell infiltration, which is likely to be reflected in the CD4/CD8 T-cell ratio of expanded TIL cultures, is not associated with the presence of $CD4^+$ T-cell responses but rather the majority of $CD4^+$ TILs represents non-tumor relevant immune cells. Notably, we have previously shown the presence of high frequencies of non-tumor–related virus-specific $CD8^+$ T cells in the tumor microenvironment (21).

In addition, the characteristics of $CD8^+$ T-cell responses of patients with high $CD4^+$ T-cell responses did not significantly differ from patients with no or low $CD4^+$ T-cell responses (Fig. 1F and Supplementary Fig. S1).

Analysis of FoxP3 expression on expanded TILs from patients with high $CD4^+$ T-cell reactivity (using flow cytometry) showed a positive staining on a relatively high fraction of both $CD4^+$ and $CD8^+$ TILs immediately after thawing. However, complete loss of FoxP3 expression 7 days after resting in IL2-free media was observed (data not shown). Therefore, we interpreted this temporary FoxP3 expression as an effect of high T-cell activation caused by *in vitro* culture conditions, and not associated with classical regulatory T-cell functions.

Expression of MHC class II in melanoma cell lines

Previous studies have shown constitutive expression of MHC class II molecules in 43% of melanoma cell lines, but also that the vast majority of melanomas (>70%) expressed class II upon exposure to IFN-γ—a cytokine that is presumably present at high levels in the tumor microenvironment, in close association with $CD8^+$ T-cell antitumor responses (1).

Nineteen out of 38 melanomas (50%) expressed constitutively MHC class II molecules (Table 1 and Fig. 2A). Relative fold increase of MHC class II expression after IFNγ was similar in both MHC class II constitutively positive and negative tumors (data not shown) and with high or no/low $CD4^+$ T-cell responses (Supplementary Fig. S2A). Only three out o19 constitutively

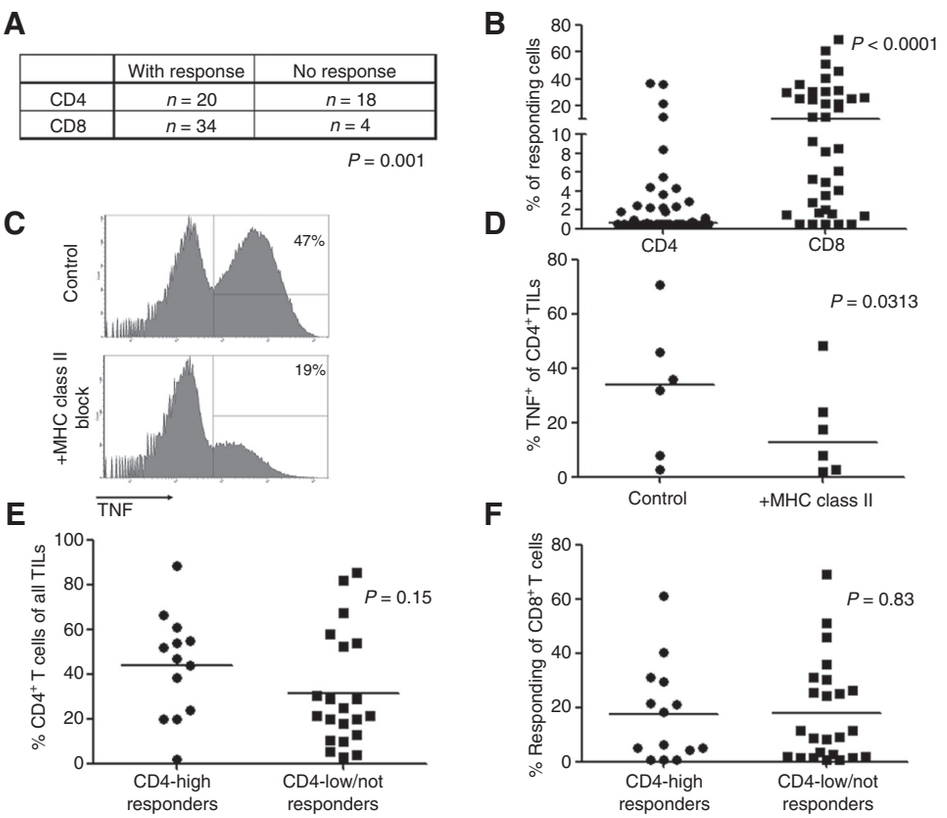

Figure 1.
$CD4^+$ T-cell responses against melanoma. A, frequency of patients with $CD4^+$ or $CD8^+$ TIL responses against autologous melanoma cell lines. B, frequency of tumor-responding $CD8^+$ or $CD4^+$ TILs against autologous melanoma cell lines. Tumor cells were pretreated with 100 IU/mL IFNγ before coculture with TILs, as indicated in Materials and Methods. Tumor-responding cells expressing at least one of the following T-cell functions: TNF, IFNγ, or CD107a. Lines show median values. C and D, production of TNF from $CD4^+$ T cells after MHC class II blocking on target tumor cells. C, one representative sample of six with similar results, gated on $CD4^+$ T cells. Lines in D show median values. E, frequency of $CD4^+$ T cells of all TILs in patients with high or no/low $CD4^+$ T-cell responses. Lines show mean values. F, frequency of tumor-responding $CD8^+$ T cells in patients with high or no/low $CD4^+$ T-cell responses. Lines show mean values.

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Table 1. Characteristics of TILs and melanoma cell lines

	Disease stage	MHC class II	CD8 T cells			CD4 T cells			
Patient no	AJCC stage	Constitutive MHC class II	% CD8	% Responder CD8	CD8 response	% CD4	% Responder CD4	CD4 response	High CD4 response
1	IIIC	YES	58.7	21.20	YES	38.2	36.98	YES	YES
2	IIIB	YES	97	35.79	YES	3	0.94	YES	NO
3	IIIC	NO	68	51.18	YES	30.7	0.50	NO	NO
4	IV	NO	81	68.91	YES	18	0.50	NO	NO
5	IV	YES	77	18.21	YES	10	11.77	YES	YES
6	IV	NO	17.3	1.52	YES	82.1	0.50	NO	NO
7	IV	NO	3.5	2.77	YES	5.1	0.50	NO	NO
8	IV	NO	80	24.43	YES	20	1.77	YES	NO
9	IV	YES	52.5	0.50	NO	46.8	2.38	YES	YES
10	IV	NO	78.2	8.23	YES	21.4	0.50	NO	NO
11	IV	YES	15	61.11	YES	82	21.48	YES	YES
12	IIIC	NO	12.8	3.51	YES	85.1	0.50	NO	NO
13	IV	YES	77.8	30.97	YES	21.5	0.50	YES	NO
14	IIIC	YES	10.4	0.50	NO	88.3	4.45	YES	YES
15	IV	YES	64	4.12	YES	33	2.46	YES	YES
16	IV	NO	69.6	9.22	YES	29	0.64	YES	NO
17	IIIC	YES	97	40.46	YES	1	4.27	YES	YES
18	IV	YES	47.5	21.38	YES	51.8	36.05	YES	YES
19	IV	YES	73	29.66	YES	24	3.61	YES	YES
20	IV	NO	92.2	45.89	YES	4	0.50	NO	NO
21	IV	NO	49.9	30.42	YES	28.9	0.50	NO	NO
22	IV	YES	37.3	1.57	YES	10.1	0.50	NO	NO
23	IV	NO	41.3	0.51	YES	56.7	0.50	NO	NO
24	IV	NO	60	6.10	YES	31	5.47	YES	YES
25	IV	NO	19.8	4.90	YES	66.5	2.27	YES	YES
26	IV	YES	57.7	8.50	YES	37.7	0.50	NO	NO
27	IV	YES	84	11.45	YES	14	0.50	NO	NO
28	IV	YES	25.1	0.50	NO	72.6	2.27	YES	YES
29	IV	YES	71	1.74	YES	27	0.67	YES	NO
30[a]	IV	YES	76	26.22	YES	23	0.50	NO	NO
31	IV	NO	77.9	25.41	YES	20.2	0.50	NO	NO
32	IV	NO	45	0.50	NO	40	0.50	NO	NO
33	IV	NO	83	25.00	YES	10	0.50	NO	NO
34	IV	YES	34.1	5.25	YES	61	2.91	YES	YES
35[a]	IV	YES	96.7	30.87	YES	2.5	8.44	YES	YES
36	IV	NO	34.3	1.38	YES	64.9	1.11	YES	NO
37	IV	NO	80	11.62	YES	18	1.76	YES	NO
38	IV	NO	5.1	2.00	YES	91	0.50	NO	NO

[a]Pt 30 and Pt 35 represent two spatially and temporally distinct metastases obtained from the same patient.

class II–negative tumors did not express class II molecules after IFNγ exposure (data not shown).

In order to clarify whether the aberrant expression of MHC class II was an exclusive event of metastatic melanomas, two cell lines originated from primary melanomas (WM-115 and FM-55-P) were characterized for MHC expression. Both cell lines showed a similar pattern of MHC class I expression compared with cells from metastatic origin (constitutive expression, increased expression after IFNγ). WM-115 was constitutively positive for MHC class II while FM-55-P was negative, and both stained positive (WM-115 with increased expression) after exposure to IFNγ (data not shown). Two additional cell lines, obtained from separate metastatic lesions of the same patient FM-55 (FM-55-P representing the primary melanoma), namely FM-55-M1 and FM-55-M2, were analyzed. Both showed no constitutive expression of MHC class II (but positive after IFNγ) exactly as their primary counterpart.

To extend the analysis and confirm our results, the ESTDAB database (http://www.ebi.ac.uk/ipd/estdab/) was examined. Indeed, our data on constitutive expression of WM-115 and FM-55-P matched those reported at ESTDAB. In addition, two out of two other cell lines derived from primary melanoma (WM-75 and WM-793) were reported to constitutively express at least one MHC class II isoform, exactly as the WM-266-4 that is a cell line originating from a metastatic lesion diagnosed 18 months after establishment of the primary cell line (WM-115) of one of the patients that we analyzed. These results confirm previous *in situ* data from Bröcker and colleagues (22), and indicate that activation of constitutive MHC class II expression may be a very early event in melanoma.

Association of $CD4^+$ T-cell responses and constitutive expression of MHC class II

In order to characterize whether constitutive tumor expression of MHC class II is associated with increased frequency of $CD4^+$ tumor-specific T cells in TILs, we examined $CD4^+$ T-cell responses in matched samples (expanded TILs).

A higher frequency of tumor specific $CD4^+$ T-cell responses were detected in TILs from tumors expressing MHC class II constitutively (mean 7.5% ± 11% for class II constitutively positive vs. 1% ± 1% for class II constitutively negative; $P = 0.002$; high $CD4^+$ T-cell responses in 12 of 19 class II constitutively positive tumors vs. 2 of 19 class II constitutively negative tumors; $P = 0.002$; Fig. 2B; Table 1). In all cases, tumor recognition assays were performed after IFNγ pretreatment, allowing eventual detection of T-cell responses if tumor-specific $CD4^+$ T

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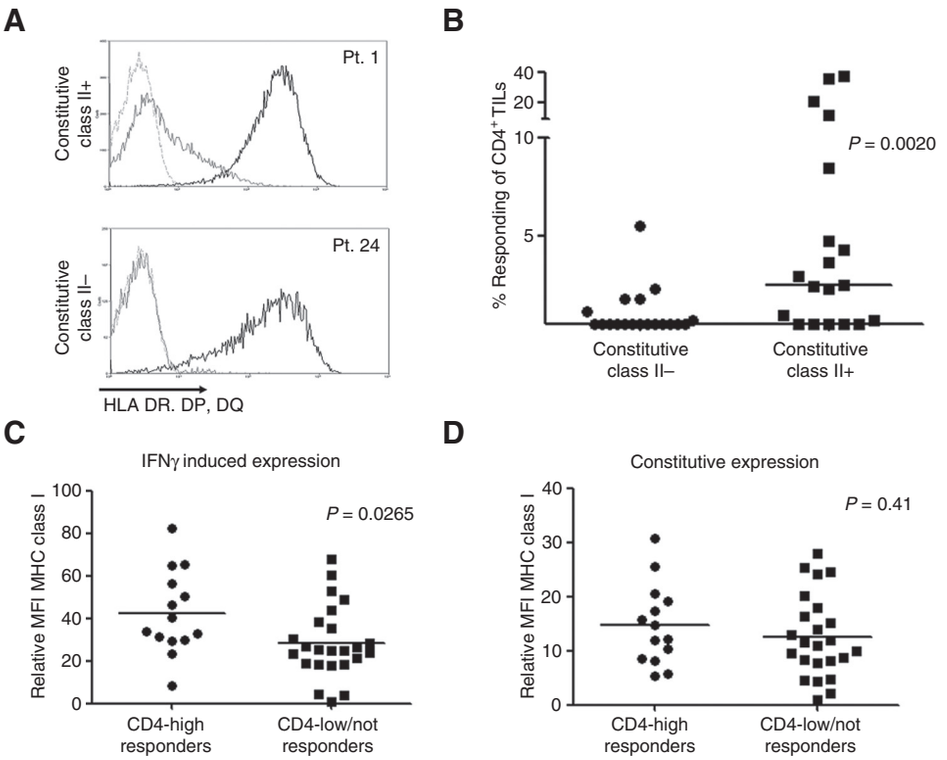

Figure 2.
MHC class I and II expression in melanoma cells and association with $CD4^+$ T-cell responses. A, expression of MHC class II molecules (HLA DP, DR, DQ) in two representative patients in either a class II constitutively positive melanoma or a MHC constitutively negative melanoma. Dotted line, isotype control; solid gray solid line, constitutive expression; solid black line, expression after pretreatment with IFNγ 100 IU/mL for 72 hours. B, frequency of tumor-responding $CD4^+$ T cells in patients with constitutive expression of MHC class II molecules. Lines show median values. C and D, relative constitutive or IFNγ-induced class I expression of melanoma cell lines from patients with high or no/low $CD4^+$ T-cell responses. Lines show mean values.

cells were present, because the vast majority of tumors expressed high levels of MHC class II molecules regardless of constitutive positivity (see above).

Surprisingly, further quantitative analyses revealed that melanomas associated with a high $CD4^+$ T-cell response upregulated surface MHC class I expression to a significantly higher extent upon IFNγ exposure compared with melanomas with low/no $CD4^+$ T-cell responses (relative MFI 42 ± 19 vs. 29 ± 17, $P = 0.026$; relative fold increase 3.0 ± 0.8 vs. 2.4 ± 0.9, $P = 0.047$; Fig. 2C; Supplementary Fig. S2B). However, both melanomas had similar constitutive expression of MHC class I (relative MFI 15 ± 7 vs. 13 ± 8; $P = 0.41$; Fig. 2D).

These data indicate that class II expression is an early event that is frequently followed by infiltration of tumor antigen–specific $CD4^+$ T cells. However, this is not an absolute requirement as the absence of strong $CD4^+$ T-cell responses in some melanomas with constitutive class II expression (7 out of 19` 37%) indicate that this is not sufficient, and the presence of high-frequency $CD4^+$ T-cell responses in two melanomas with no constitutive class II expression (Pt. 24 and Pt. 25; 2 out of 19; 10.5%) indicate that this is not mandatory.

Of particular interest, two samples of our cohort (Pt. 30 and Pt. 35) were obtained sequentially from the same patient (Table 1). The first metastasis (named Pt. 30) was resected for inclusion in a TIL trial (clinicaltrials.gov identifier: NCT00937625). Shortly after, the patient was infused with the expanded TILs and this treatment resulted in >80% regression of all the preexistent tumor lesions, but one new metastatic lesion appeared at first evaluation (data not shown). This lesion continued to grow, and was resected approximately 6 months after infusion of TILs (named Pt. 35). As shown in Table 1, both cell lines were expressing MHC class II constitutively, but only the treatment-refractory and progressive metastatic lesion was infiltrated with tumor-specific $CD4^+$ T cells, at high frequencies (Table 1). This is an interesting anecdotal observation indicating that the infiltration of tumor-specific $CD4^+$ T cells can be subjected to a spatiotemporal dynamic regulation, and can be associated with disease progression without obvious changes in MHC class II expression on tumor cells.

Multifunctional characterization of $CD4^+$ and $CD8^+$ T-cell responses to melanomas

In order to determine potential functional patterns of $CD4^+$ and $CD8^+$ T-cell responses, we selected expanded TILs from 8 patients with strong responses in both subsets (>2% of $CD4^+$ or $CD8^+$ gated TILs simultaneously expressing TNF and IFNγ, and a subpopulation frequency of at least 10% $CD4^+$ and/or $CD8^+$ T cells; from Pts. 1, 5, 11, 15, 18, 19, 24, and 25) and performed a multifunctional analysis of their effector functions based on seven different known antitumor activities, in order to determine intrapatient variability of responses related to T-cell subsets.

To determine the relative quantitative capacity to express single T-cell functions, we compared the MFI of tumor-reactive cells. Tumor-reactive cells were identified by strict gating strategies of $CD4^+$ and $CD8^+$ T cells, gated solely on responsive (T cell-function positive) T cells.

These analyses revealed a higher intrinsic ability, on a single cell basis, of $CD4^+$ T cells to produce TNF (Supplementary Fig. S3) and IL2, but comparable IFNγ, MIP-1α, and MIP-1β production (Fig. 3A). On the other hand, IL17A production was detected in $CD4^+$ T-cell populations from only 1 patient and never in $CD8^+$ T cells, while CD107a mobilization was much more associated with $CD8^+$ T-cell activity (Fig. 3A). To summarize, a single TNF-producing $CD4^+$ T-cell produces, on average, much more TNF

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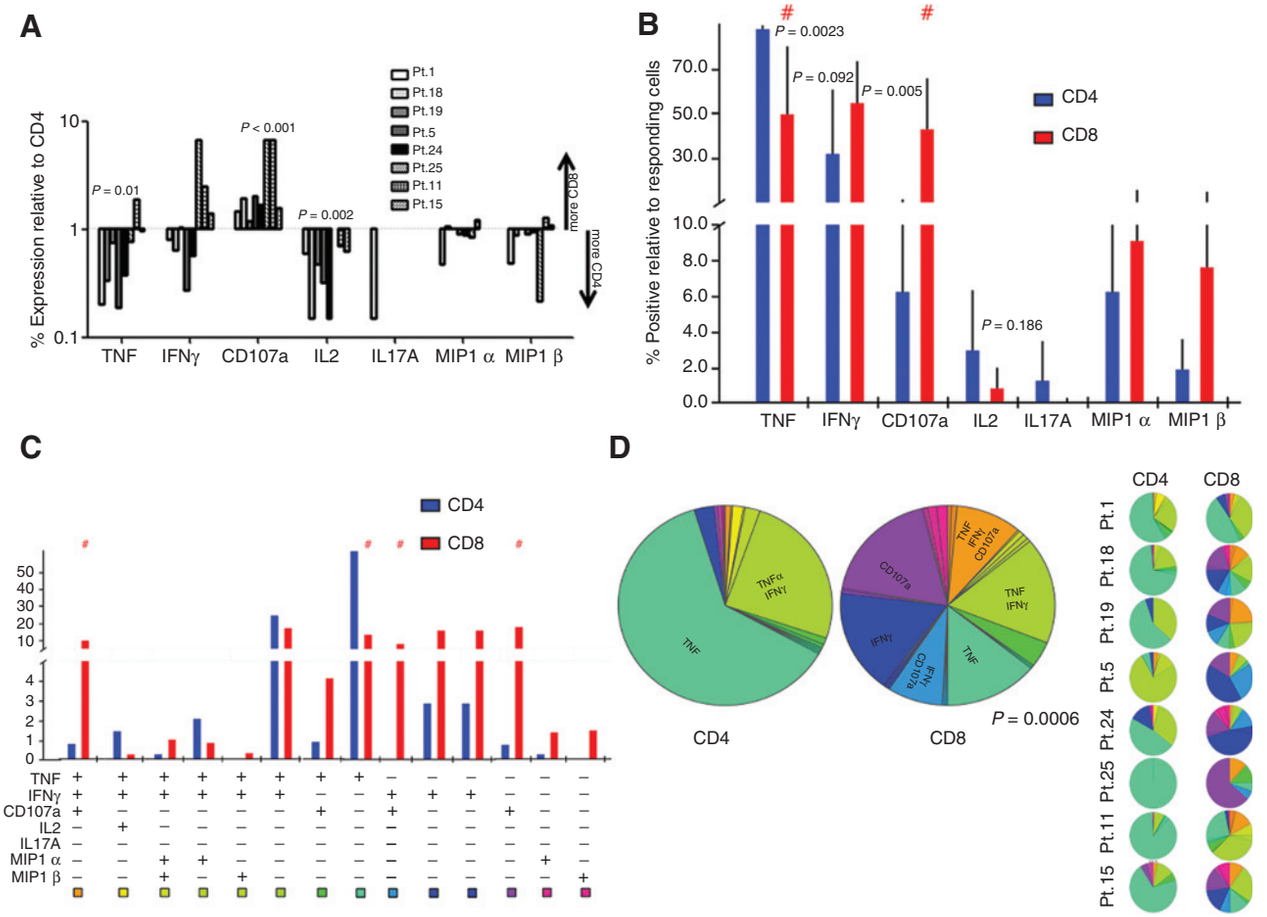

Figure 3.

Multifunctional characterization of $CD4^+$ and $CD8^+$ T-cell responses to melanoma. A, semiquantitative comparison of the expression of seven individual T-cell functions from tumor-reactive $CD4^+$ and $CD8^+$ T cells. For any individual T-cell function, the relative expression value showed in the bar chart was obtained with the following formula: 100/[(MFI of all $CD4^+$ T cells expressing the individual function in the stimulated sample/MFI of unstimulated $CD4^+$ T cells)/(MFI of all $CD8^+$ T cells expressing the individual function in the stimulated sample/MFI of unstimulated $CD8^+$ T cells)]. Values over 1, the individual T-cell function is predominantly expressed by $CD8^+$ T cells (e.g., in the case of a cytokine, cytokine-producing $CD8^+$ T cells produce on a single cell basis more cytokine than cytokine-producing $CD4^+$ T cells). Values below 1, the individual T-cell function is predominantly expressed by $CD4^+$ T cells. B, CD4 or CD8 T-cell subpopulations were gated on cells expressing at least one of the seven T-cell functions analyzed, and the frequency of cells expressing any of the seven cell functions was assessed. Figure shows that the vast majority of tumor-responding $CD4^+$ T cells (i.e., expressing at least one of the seven T-cell functions) also produce TNF. Columns show the average values ± error bars. C and D, SPICE T-cell function pattern analysis of cells expressing at least one of the seven T-cell functions shows very different functional patterns of $CD4^+$ and $CD8^+$ T cells. #, $P < 0.05$.

than a TNF-producing $CD8^+$ T cell. Similar results were obtained with IL2 production but, as expected, opposite results with CD107a mobilization.

Subsequently, selective analysis of relative proportions of cells generating at least one of the seven T-cell functions revealed a significant skewing of $CD4^+$ T cells toward production of TNF (Fig. 3B). Indeed, over 85% of $CD4^+$ T cells generating at least one function were positive for TNF production. Thus, production of TNF seems a condition *sine qua non* that $CD4^+$ T cells have to satisfy, in order to generate direct responses to melanomas. This is very different from what was observed for $CD8^+$ T cells in the same TIL products, as only about 50% of responding cells were positive for TNF (Fig. 3B). Other T-cell functions, except for IFNγ production from $CD8^+$ T cells (only a trend was observed), mirrored results of quantitative expression assessments. Indeed, similar frequency of MIP-1α- and MIP-1β-producing cells, slightly higher proportion of $CD4^+$ T cells expressing IL2, but significantly more $CD8^+$ T cells mobilizing CD107a were detected (Fig. 3B). Globally, these results indicate that regardless which function is expressed by $CD4^+$ T cells, TNF production seems to be universal.

Simplified Presentation of Incredibly Complex Evaluations (SPICE) is a novel bioinformatics tool that allows in-depth dissection of diverse "patterns" of T-cell effector functions, with potential identification of a multitude of distinct cell populations (not evidenced with classical sequential gating strategies) by combinatorial Boolean gating and complex algorithms of data analysis (20, 23). To this end, we have subjected our samples to supervised analysis of T-cell response quality with SPICE.

Analysis of complex functional patterns revealed strong intrapatient differences between expanded $CD4^+$ and $CD8^+$ T cells reacting to autologous melanomas ($P = 0.0006$). Again confirming a significant $CD4^+$ T-cell skewing toward TNF

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production—in particular, over 50% of $CD4^+$ T cells generating detectable responses were producing only TNF—while $CD8^+$ T-cell responses appeared much more complex, multifunctional and mostly based on IFNγ production or CD107a mobilization (Fig. 3C and D). Supplementary Fig. S4 shows a graphical representation of SPICE data analysis displaying all possible combinations of T-cell functions ($n = 128$) in a bar chart (Supplementary Fig. S4A), NPlot for $CD4^+$ (Supplementary Fig. S4B), or $CD8^+$ (Supplementary Fig. S4C) T cells or CoolPlot (Supplementary Fig. S4D).

Because of the technical complexity and the availability of large TIL samples required for the experiments described, expanded TILs were used as previously indicated. However, it is not known whether the observed functional patterns are maintained during several logs expansion. Thus, we conducted similar analyses with minimally expanded TILs as well as uncultured TILs, and compared the results with expanded TILs obtained from the same patients. We conducted seven T-cell function experiments, but as with expanded TILs, only a minority of T cells expressed IL2, IL17A, MIP-1α, or MIP-1β (data not shown). Therefore, only three functions (TNF, IFNγ, and CD107a) were analyzed.

Keeping in mind potential technical pitfalls in the analysis of uncultured TILs (see Materials and Methods/Statistical Analysis), this analysis showed that similar patterns were observed with all TILs analyzed. Close similarities with previous multifunctional result from expanded TILs only where obtained. Indeed, when comparing $CD4^+$ with $CD8^+$ T-cell subsets within each type of TIL culture, a higher proportion of TNF-producing cells was observed in $CD4^+$ T cells (Supplementary Fig. S5A). In addition, $CD4^+$ T cells and $CD8^+$ T cells patterns were similar in different types of TIL cultures (Supplementary Fig. S5A and S5B), Supplementary Fig. S6 shows the T-cell functional patterns for each individual patient, again showing close intrapatient similarities between types of TIL culture. Thus, we concluded that the main functional differences between $CD4^+$ and $CD8^+$ T-cell subsets observed in expanded TILs were stable during *in vitro* expansion, thus mirrored T-cell responses in the tumor microenvironment.

On a different note, it seemed that a higher proportion of $CD107a^+$ cells along with a lower proportion of $IFN\gamma^+$ cells were present in uncultured TILs (Supplementary Fig. S5A and S5B). However, given the small number of patients analyzed as well as technical issues in the analysis of uncultured TILs (see Materials and Methods/Statistical Analysis), this incidental observation will need further investigation, but it does not change the overall conclusion that $CD4^+$ T cells produce more TNF also in uncultured TILs.

Effects of TNF on $CD8^+$ T-cell recognition

Because our data indicated that TNF production was the main effector function of melanoma-specific $CD4^+$ T cells, we asked whether exposure of autologous melanoma to TNF could influence recognition by expanded CD8 TILs. To this end, melanoma cells were treated with TNF, IFNγ, or both TNF and IFNγ simultaneously. This was chosen on the basis of previous SPICE analyses, in order to reproduce a tumor microenvironment with strong $CD4^+$ T-cell responses, $CD8^+$ T-cell responses or both.

Landsberg and colleagues (24) has previously demonstrated that TNF induces melanoma reversible dedifferentiation with reduced T-cell recognition of melanoma differentiation antigens (MDA). Analysis of T-cell responses revealed, as expected, that TNF significantly reduced recognition of MDAs (Fig. 4A and Supplementary Fig. S7—figures show a representative patient, experiments were conducted with samples from three different patients and similar results were obtained; data not shown). However, TNF did not significantly influence global $CD8^+$ T-cell reactivity (Fig. 4A and B). As expected, IFNγ significantly increased both global $CD8^+$ and $CD4^+$ T-cell reactivity (Fig. 4B–D and Supplementary Fig. S8) as previously demonstrated by our group (10). Of note, it has similarly been shown that IFNγ neither increases or reduces autologous or allogeneic tumor recognition by MDA-specific cells, such as $CD8^+$ T cells recognizing MART-1_{EAA} or gp-100_{YLE} peptides (10)—although in this case, it is not fully elucidated whether the effects are antigen class–specific or rather peptide-specific because of the IFNγ-induced shift from proteasome to immunoproteasome (25, 26).

Surprisingly, exposure to TNF significantly reduced the well-known IFNγ-mediated increase of $CD8^+$ T-cell responses to melanoma (Fig. 4B and Supplementary Fig. S8). On the other hand, TNF increased (although not significantly) $CD4^+$ T-cell responses compared with untreated tumors (Fig. 4C and D).

These data indicate that on one hand TNF tends to marginally amplify itself with a positive feedback mechanism (tumor exposure to TNF increase TNF production from $CD4^+$ T cells), but on the other hand, it dampens $CD8^+$ T-cell reactivity in an IFNγ-rich tumor microenvironment, as it occurs when strong $CD8^+$ T-cell responses are present simultaneously.

Effects of TNF on gene expression of melanoma cells

To clarify whether the observed influences on tumor recognition by $CD8^+$ T cells were associated with changes in gene expression, a panel of genes involved in immune recognition was analyzed by qPCR in eight cell lines (from Pts. 1, 5, 11, 13, 15, 17, 18, and 19). Besides the well-known effects of IFNγ to increase the expression of immunosuppressive genes, the addition of TNF increased expression of genes such as indoleamine 2,3 dioxygenase-1 (IDO-1) or programmed death ligand-1 (PD-L1) by about 5- to 10-fold (Supplementary Fig. S9A). In addition, a trend toward reduced expression of MDAs was evidenced (Supplementary Fig. S9B). On the other hand, a trend toward increase in expression of the MHC class I processing and presentation pathway was observed with the addition of TNF by 2- to 5-folds, but notably IFNγ itself increased gene expression by about 10 folds relative to control (Supplementary Fig. S9C).

These data may suggest that TNF reduces melanoma immunesensitivity in an IFNγ-rich tumor microenvironment through augmentation of tumor cell immunosuppressive capacity.

Effects of tumor-specific $CD4^+$ T cells on melanoma cell proliferation and viability or on short-term CD8 IFNγ responses

In order to characterize whether $CD4^+$ T-cell responses could influence melanoma cell proliferation or viability, we conducted additional proliferation and cytotoxicity assays.

Autologous tumors were incubated for 72 hours with supernatants from sorted $CD4^+$ T-cell cultures from 6 patients with a high level of $CD4^+$ T-cell reactivity (Pts. 1, 5, 11, 15, 18, and 19) as described above (activated with autologous tumors), and cell proliferation was assessed with a standard flow cytometry–based counting assay as previously described (10). Meaningful antiproliferative properties of $CD4^+$ T-cell supernatants were evident in only 2 out of 6 melanoma cell lines (Fig. 5A).

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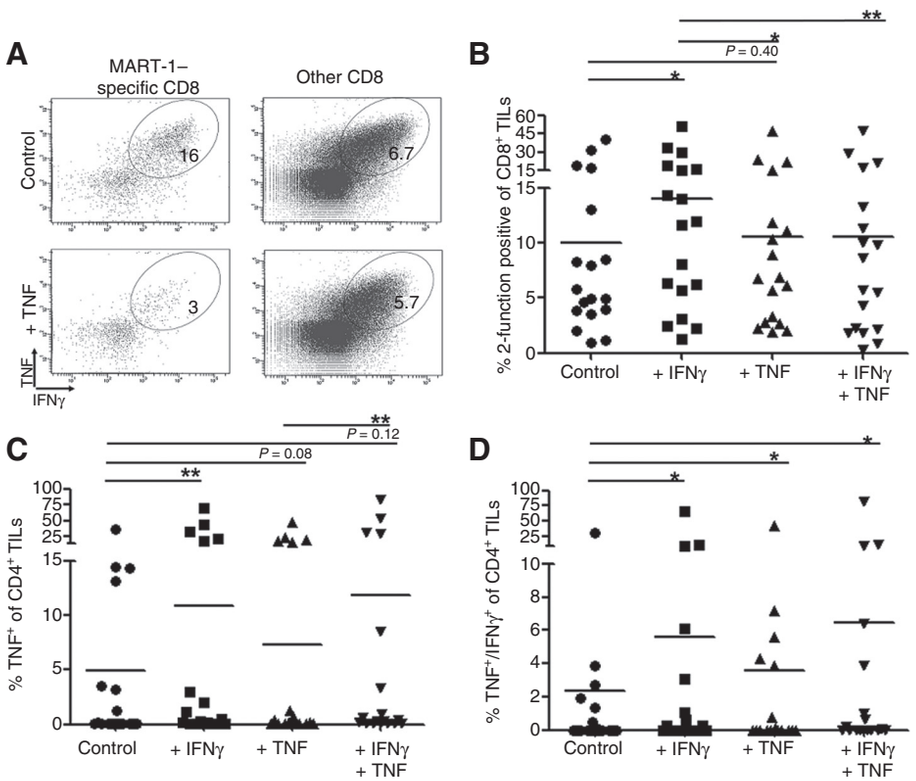

Figure 4.
Effects of cytokines on tumor recognition of $CD4^+$ and $CD8^+$ TILs. A, TNF reduces tumor recognition of MART-1–specific $CD8^+$ TILs without significantly modifying tumor recognition of other-specific $CD8^+$ TILs. FACS plots of cytokine expression from a representative patient of three tested with similar results are shown in the figure. B, effects of IFNγ, TNF, or simultaneous exposure of TNF and IFNγ on tumor recognition of bulk $CD8^+$ TILs. Lines show mean values of the frequency of $CD8^+$ TILs expressing at least two functions simultaneously among TNF, IFNγ, and CD107a. C and D, effects of IFNγ, TNF, or simultaneous exposure of TNF and IFNγ on tumor recognition of bulk $CD4^+$ TILs. Lines show mean values *, $P < 0.05$; **, $P < 0.01$.

$CD4^+$ T cells with direct cytotoxic activity to melanomas have been previously shown in mice. In order to assess whether tumor cytotoxic $CD4^+$ T cells could be detected in humans, a standard 4-hour cytotoxicity assay was performed with sorted $CD4^+$ or $CD8^+$ T cells from 5 patients (Pts. 1, 5, 11, 15, and 19) with high $CD4^+$ T-cell responses. $CD8^+$ T cells displayed the ability to kill autologous tumors (>10% lysis at a effector:target ratio of 90:1) in 4 out of 5 cases, while $CD4^+$ T cells did not in any of the cases (Fig. 5B).

These results indicate that $CD4^+$ T-cell responses do not seem to have strong direct antitumor activities in the effector phase of the immune response.

To assess whether production of IFNγ from $CD4^+$ and $CD8^+$ T cells would be influenced by the presence of the other cell subset and/or whether the presence of $CD4^+$ T cells may enhance CD8 responses, ELISPOT assays with the addition of either $CD4^+$, $CD8^+$ or $CD4^+$, and $CD8^+$ T cells from 6 individual patients (Pts. 1, 5, 11, 15, 18, and 19) with high $CD4^+$ T-cell reactivity were conducted. The number of IFNγ specific spots obtained with the addition of both cell subsets was consistently around the average of the number of spots obtained with either $CD4^+$ or $CD8^+$ T cells alone and no multiplicative effects were observed (Fig. 5C and D). Therefore, we concluded that $CD4^+$ and $CD8^+$ T cells do not seem to interact or influence one another in short-term assays in regard to tumor recognition and relative IFNγ production.

Outcome of patients with constitutive and high $CD4^+$ T-cell responses

Finally, we attempted to assess whether the presence of high $CD4^+$ T-cell responses, constitutive class II positivity, or high CD4/CD8 T-cell ratios in expanded TILs (this latter with a larger group of patients) were associated with known prognostic factors in melanoma or patient survival. Plasma lactate dehydrogenase (LDH) was chosen as it currently represents the most accurate prognostic circulating marker in metastatic melanoma.

Strong differences in plasma LDH levels were not observed (Supplementary Fig. S10A and S10E), except for a higher LDH value in patients with MHC class II constitutively negative tumor (Supplementary Fig. S10C). No differences in overall survival were observed between patient groups (Supplementary Fig. S10B, S10D, and S10F). Keeping in mind the relatively small number of patients, these data suggest that the factors examined are not strong independent prognostic indicators in melanoma.

Discussion

Anecdotal cases of tumor regression mediated exclusively by $CD4^+$ T-cell responses have been reported in humans with metastatic melanoma (8, 9, 27). However, little is known regarding how natural effector $CD4^+$ T-cell responses are generated, and how $CD4^+$ T cells influence $CD8^+$ T-cell responses in the tumor microenvironment.

Constitutive expression of MHC class II is usually restricted to professional antigen-presenting cells (APC; ref. 28). However, previous studies have shown that constitutive expression of MHC class II in melanoma via abnormal transcription of class II transactivator (CIITA) might not be a random event, but rather linked to neoplastic transformation (29). CIITA activation was not involved *per se* in tumor progression (30), suggesting that its abnormal induction is initiated and maintained by more complex networks dependent on MHC class II expression.

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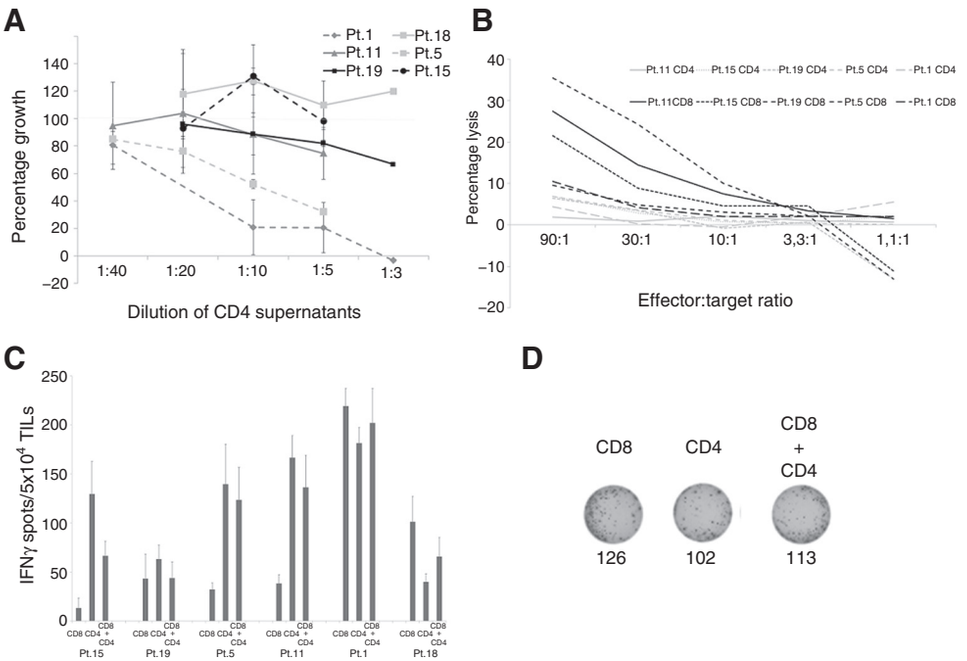

Figure 5.
Effects of tumor-responding CD4$^+$ T cells on tumor cells or CD8 IFNγ responses. A, effects of supernatants of activated CD4$^+$ T cells from patients with high CD4$^+$ T-cell responses on proliferation of autologous melanoma cells. Dots show average values with error bars. B, autologous tumor cytotoxicity of CD8$^+$ or CD4$^+$ TILs from patients with high CD4$^+$ T-cell responses. CD4$^+$ T-cell cultures are shown in gray while CD8$^+$ T cells are shown in black. C and D, IFNγ ELISPOT analysis of selected CD8$^+$, CD4$^+$, or combined CD8$^+$ and CD4$^+$ TILs. C and D, results from individual patients (average of triplicate observations and error bars; C), while D shows ELISPOT wells from a representative patient.

In this study, we have shown that, despite not being necessary or sufficient, *de novo* constitutive expression of MHC class II in melanoma cells is a powerful stimulus to promote accumulation of tumor-specific CD4$^+$ T cells in the tumor microenvironment. Our data indicate that, presumably, this event already occurs at an early stage—that is, localized melanoma. CD4$^+$ T cells directly recognize tumor antigens presented in association with MHC class II molecules on the surface of melanomas, and exert effector functions dominated by TNF production. Indeed, endogenous proteins can be presented on class II via autophagy and/or nonclassical antigen processing (31, 32).

We have shown that the effector functions generated by tumor-specific CD4$^+$ T cells do not significantly influence melanoma proliferation or viability, with parallel effective cytotoxicity of CD8$^+$ T cells. This implies that, under standard conditions of cell expansion, direct antitumor activities of tumor-specific CD4$^+$ T cells are not very much effective in limiting the growth of melanoma *in vitro*, at least in the majority of cases. This may reflect an *in vivo* condition where the majority of tumor-specific CD4$^+$ T cells are not able to mediate powerful direct antitumor effects.

Previous studies have shown the deleterious effects of chronic exposure of melanoma tumors to TNF. Landsberg and colleagues (24) have shown that a TNF-rich inflammatory environment can reduce the immunogenicity and promote immune escape of melanoma by inducing reversible dedifferentiation. In this study, we have confirmed that chronic exposure of melanoma tumors to local TNF downregulate recognition of MDA-specific CD8$^+$ T cells. However, when using bulk TIL cultures, it was interesting to see that preexposure to TNF did not significantly influence the global CD8$^+$ T-cell reactivity. This may indicate that the contribution of MDA-specific T cells to the overall CD8$^+$ T-cell reactivity is relatively small.

In contrast, exposure to TNF significantly reduced the well-known IFNγ-mediated increase of CD8$^+$ T-cell responses to melanoma. This suggests that in an IFNγ-rich environment, such as a tumor microenvironment containing IFNγ-producing CD8$^+$ T cells, TNF from tumor-specific CD4$^+$ T cells (or other sources) may reduce CD8$^+$ T-cell reactivity and, in parallel, increase CD4$^+$ T-cell responses through a positive feedback, thereby amplifying local TNF signaling.

In an attempt to characterize the molecular events leading to the observed phenomena, we analyzed the expression of a set of genes capable of influencing immune sensitivity by melanoma. Although the addition of TNF further increased the immunosuppressive capacity of tumor cells as evidenced by induction of IDO-1 and PD-L1 and reduced the expression of MDA genes, other genes associated with MHC class I antigen processing and presentation pathway seemed to be induced in parallel. Despite difficulties to quantify the relative contribution of different immune-activating/immune-suppressive pathways, an increased immunosuppressive capacity of tumor cells may explain the reduced immune sensitivity observed in this setting.

Interestingly, these data support a model of tumor progression where some melanomas, in order to escape effective CD8$^+$ T-cell responses, activate CIITA and consequently express MHC class II constitutively. This recruits tumor-specific CD4$^+$ T cells in the tumor microenvironment, which, in turn, dampen CD8$^+$ T-cell responses via production of TNF.

Notably, MHC class II has several features in common with other tumor-associated immunosuppressive molecules such as, for instance, IDO and PD-L1. In fact, as shown in this and several other studies, MHC class II is aberrantly activated in some melanomas, and exactly as IDO and PD-L1 (33, 34), it is upregulated by IFNγ-mediated immune responses. Thus, *in situ* detection of MHC class II in melanoma may represent a constitutive expression in melanoma cells or induced by the presence of IFNγ-secreting cells (e.g., tumor antigen–specific CD8$^+$ T cells), or both.

Several other mechanisms to evade CD8$^+$ T-cell responses have been characterized so far (35). CD8$^+$ T cells recognize tumor antigens expressed on the surface of tumor cells in association with MHC class I molecules. Therefore, defects in the class I antigen processing and presentation pathway, as well as downregulation of

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MHC class I molecules may *per se* dampen $CD8^+$ T-cell responses. Indeed, several studies have demonstrated that reduced expression of class I molecules is associated with a worse prognosis in several types of solid tumors, including melanoma (5).

Interestingly, in this study those melanomas that did not attract strong $CD4^+$ T-cell responses displayed a defective upregulation of MHC class I in response to IFNγ. This may indeed be interpreted as an additional immune escape mechanism, thereby suggesting the existence of two different subsets of tumors: (i) melanomas with high $CD4^+$ T-cell responses (attracted in most cases by *de novo* constitutive expression of class II) and normal class I upregulation after exposure to IFNγ; (ii) melanomas with defective class I upregulation in response to IFNγ, which do not attract strong $CD4^+$ T-cell responses. According to this model, both melanomas efficiently downregulate $CD8^+$ T-cell responses either indirectly through attraction of inflammatory $CD4^+$ T cells (melanoma subset i), or directly through defective upregulation of MHC Class I (melanoma subset ii).

As an indirect confirmation of this theory, patients with strong $CD4^+$ T-cell responses (or with constitutive MHC class II–positive tumors) did not seem to have a worse prognosis than patients without $CD4^+$ T-cell responses—with the latter displaying on average a defective upregulation of MHC class I molecules.

The vast majority of patients in our study (85%) were diagnosed with AJCC stage IV melanoma (distant metastases). However, we detected strong $CD4^+$ T-cell responses also in 3 out of 6 patients with earlier disease stage (IIIB/IIIC). As indicated by a recent study (36), in those cases where prompt and complete resection of locoregional melanoma is performed, the impact of local immune responses may be irrelevant in regard to the clinical outcome. In contrast tumor-intrinsic features may dictate whether the patient will develop distant metastases shortly. Thus, it is more likely that our observations can be applied to a disease stage where complete resection is not feasible.

It should be highlighted that, despite the natural *in situ* responses to melanomas from $CD4^+$ T cells and the associated chronic exposure to TNF may reduce antitumor $CD8^+$ T-cell responses in the majority of cases (i.e., most tumor-specific $CD4^+$ T cells), this does not exclude a potential beneficial role of selected $CD4^+$ T-cell subpopulations or the possibility to take advantage of these responses therapeutically, as recently suggested by Tran and colleagues (37) in colangiocarcinoma (i.e., polyfunctional mutated-antigen specific $CD4^+$ T cells) or by previous single-case reports (9, 27). On this note, Linnemann and colleagues (11) have recently characterized mutated (neo-) antigen-specific $CD4^+$ T cells in melanoma. In their study, the authors describe in 4 out of 5 patients a (relatively) small subset of $CD4^+$ T cells that produce IFNγ, but not other cytokines, upon exposure to neo-antigens. This is particularly intriguing, as our data show instead that the vast majority of tumor-specific $CD4^+$ T cells in melanoma produce TNF and not IFNγ, but also that a very small subset of melanoma-specific $CD4^+$ T cells produce only IFNγ (Fig. 3C and D). It is anyway difficult to draw firm conclusions, because most T-cell recognition analyses in Linnemann and colleagues (11) were performed with autologous B cells (presenting neo-antigens) as targets and not tumor cells. It is indeed known from previous studies that several factors can affect T-cell functionality, including antigen density (38, 39) that may of course be different in peptide-loaded APCs and tumor cells. Future studies will determine whether functional differences exist between $CD4^+$ T-cell subsets specific to different classes of tumor antigens.

In addition, the role of tumor antigen–specific $CD4^+$ T cells is certainly not limited to direct recognition of tumor cells. For instance, tumor antigen–specific $CD4^+$ T cells may promote the cross-priming of tumor antigen–specific $CD8^+$ T cells through CD40-ligand mediated activation of antigen presenting cells (40). Recently, it was shown in three independent murine models that, unexpectedly, the majority of the immunogenic mutanome is recognized by $CD4^+$ T cells (41). If recognition of tumor antigens by $CD4^+$ T cells is so common in cancer, it could be speculated that during tumor progression, cancer cells are shaped in order to take advantage of, or at least not suffer from, such features of the immune system. Our results suggest that this may be realized through generation of local conditions (e.g., aberrant expression of MHC class II) inducing such $CD4^+$ T cells to accumulate in the tumor microenvironment in order to support, instead of inhibiting, tumor growth.

In conclusion, several mechanisms of immune escape have been identified and strategies to counteract tumor immune inhibition show impressive results in the clinical setting (42–45). In this study, we have identified a potential mechanism by which *de novo* aberrant expression of MHC class II on melanomas may contribute to tumor escape via recruitment of inflammatory tumor antigen–specific $CD4^+$ T cells. However, the fundamental role of MHC class II molecules in the initiation and maintenance of immune responses does not immediately warrant strategies directly counteracting this pathway, and future studies must address this issue.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Donia, F. Nicoletti, P. Thor Straten, I.M. Svane
Development of methodology: M. Donia, P. Fagone, S. Munir
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Donia, R. Andersen, I.M. Svane
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Donia, R. Andersen, P. Fagone
Writing, review, and/or revision of the manuscript: M. Donia, R. Andersen, P. Fagone, F. Nicoletti, M.H. Andersen, I.M. Svane
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Donia, J.W. Kjeldsen, I.M. Svane
Study supervision: M. Donia, I.M. Svane

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