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HLA class II-restricted recognition of common tumor epitopes on human melanoma cells by CD4⁺ melanoma-infiltrating lymphocytes

CD4⁺ T cell clones derived from lymphocytes infiltrating four human melanomas specifically recognized melanoma-derived tumor epitopes as shown by secretion of tumor necrosis factor (TNF) *in vitro* upon interaction with autologous melanoma cells, whereas they did not recognize HLA class II-expressing autologous lymphoblasts or HLA class II mismatched allogeneic melanoma cells. Specificity was further established by demonstrating that TNF responses to tumor cells were inhibited by HLA-DR or HLA-DQ monoclonal antibodies. Most of these clones cross-reacted with allogeneic melanoma cells expressing a potentially restricting HLA allele or a structurally similar one. These data show that shared epitopes of human melanoma cells presented on HLA class II molecules are frequently recognized by autologous CD4⁺ T lymphocytes.

1 Introduction

Although human melanoma tumor-infiltrating lymphocytes (TIL) frequently develop tumor-specific cytotoxic activity *in vitro* [1–3], it remains unclear whether these cells can also express their cytolytic potential *in vivo* or whether they remain as precytotoxic precursors whose differentiation is artificially induced *in vivo* by addition of exogenous IL-2. Cytotoxic T lymphocyte priming, differentiation and function classically depend on lymphokine-mediated T helper cell function. Therefore, an important issue concerning the anti-tumor function of melanoma-specific CTL *in vivo* is to ascertain whether some CD4⁺ TIL specifically recognize autologous human melanoma cells, which should depend at least on the capacity of these cells to express endogenous melanoma-restricted epitopes in association with HLA class II molecules. Only a few studies have reported HLA class II-restricted responses of CD4⁺ T lymphocytes to melanoma cell or melanoma-derived antigens presented by EBV-transformed B cells [4–7]. To address this issue, we looked for specific recognition of four HLA class II-expressing melanoma lines by autologous CD4⁺ TIL clones.

The data reported here show that all CD4⁺ TIL clones analyzed exhibited HLA class II-dependent responses to autologous melanoma cells, but not to autologous HLA class II-expressing lymphoblasts. This suggests that recruitment of melanoma-reactive CD4⁺ T lymphocytes within HLA class II-expressing metastatic melanomas is a fre-

quent, if not systematic event, although it has rarely been considered in the literature. This study also established that some of the antigens recognized by these CD4⁺ TIL are commonly expressed by allogeneic melanoma cells.

2 Materials and methods

2.1 Cell lines and TIL clones

Melanoma cell lines M were established from metastatic tumor fragments obtained from different patients and cultured in RPMI 1640 containing 10% FCS. Other melanoma lines used were DEFR, GERL, MZ2 and MAMO (gift from T. Boon, Ludwig Institute for Cancer Research, Brussels, Belgium); DAU, Mel 4 (gift from J. F. Doré, INSERM U218, Lyon, France); and IPC200/B6, IPC205/14 and IPC277/5 (gift from C. Aubert, INSERM U119, Marseille, France). EBV-transformed B cell lines were cultured in RPMI 1640 containing 8% human AB serum (CTS, Nantes, France). The B17 cell line was established from PBL of patient M17 according to a classical protocol [3]. BH was a DRB1*07 cell line from the panel of the New York HLA workshop (1987). Normal autologous stimulator cells were T lymphoblasts obtained by stimulating autologous CD8⁺ TIL clones with PHA-P (Difco, Detroit, MI) (1:1000) for 3 days. The allogeneic EBV-transformed B cell line LAZ 388 used for TIL cloning was a gift from T. Hercend. Mouse fibrosarcoma WEHI 164 clone 13 used for TNF production was obtained from T. Boon. TIL were obtained from four different patients by culturing metastatic melanoma fragments in RPMI 1640 containing 150 U/ml of recombinant IL-2 (gift from Rousset Uclaff, Romainville, France) and antibiotics. Cloning was done by limiting-dilution culture as described [8]. Briefly, different numbers of TIL were seeded in microplates with irradiated feeder and stimulator cells (2 × 10⁴ LAZ cells and 1.5 × 10³ autologous melanoma cells) in 200 µl rIL-2 medium containing PHA-P (1:1000). Microcultures showing greater than 80% probability of monoclonality according to the single-hit Poisson law were transferred into new plates with freshly irradiated feeder and stimulator cells. CD4⁺ clones were expanded by a similar transfer of 5 × 10³ lymphocytes/well every 3–4 weeks.

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Abbreviation: TIL: Tumor-infiltrating lymphocytes CaI: Calcium ionophore

Key words: Human melanoma tumor antigen / CD4⁺ tumor-infiltrating lymphocyte / T cell clone / HLA class II restriction

Table 1. TNF production (pg/ml) by melanoma-reactive CD4⁺ TIL clones^{a)}

Stimulation	CD4 ⁺ clones								
	M6-T	M17-137	M17-311	M74-6	M74-33	M74-94	M77-180	M77-181	M77-184
Medium	0	12	44	8	6	8	5	0	1
PMA/CaI	356	1533	1700	515	182	185	956	101	119
Autologous melanoma	33	1020	1400	219	27	19	1091	358	66
Allogeneic melanoma ^{b)}	0	15	31	6	6	5	1	0	0
Autologous blasts ^{c)}	0	5	35	0	5	8	1	0	0
EBV-BH transformed B cells ^{c)}	ND	ND	ND	ND	ND	ND	0	0	0
EBV-B17 transformed B cells ^{c)}	ND	91	273	ND	ND	ND	0	0	0

a) TNF was determined in the supernatant of clones ($2.5 \cdot 10^3$ /well) stimulated either by melanoma cells (2×10^4 /well) or by a combination of PMA-CaI as a polyclonal activator, as described in Sect. 2.3, using the biological assay on the TNF-sensitive Wehi cell line. Values are from one representative experiment out of three or more.

b) Allogeneic melanoma cells used here did not share a common HLA allele with the donor.

c) Since these cells produced TNF alone, we subtracted this baseline production.

2.2 mAb and cell staining

Mouse mAb were: IOT4 (anti-CD4) and anti- α/β T cell receptor from Immunotech (Marseille, France); W6/32 (anti-HLA class I) from ATCC; 206 (anti-HLA class II), B7-21 (anti-HLA DP) and D1-12 (anti-HLA DR), a gift from D. Charron (Institut des Cordeliers, Paris, France); and IOT2d (anti-HLA DQ) from Immunotech. All anti-HLA antibodies except IOT2d were used as diluted and filtered ascites fluids. Others were purified antibodies. Cells were labeled by indirect immunofluorescence. Briefly, cells were treated at 4°C for 30 min with mouse or rat mAb, washed in PBS and incubated with F(ab')₂ fragments of goat anti-mouse or anti-rat immunoglobulin for 30 min. Cells were analyzed on a FACScan (Becton Dickinson, Grenoble, France). Background fluorescence was estimated using isotype-matched irrelevant antibodies.

2.3 TNF detection assay

At least 10 days after the last stimulation, T cell clones were washed three times to remove IL-2 and conditioned medium and then stimulated in triplicate cultures either by melanoma cells or autologous lymphoblasts or by a combination of phorbol 12-myristate 13-acetate (PMA) and 4-bromo-calcium ionophore A23187 (CaI) (Sigma, l'Isle d'Abeau, Chesne, France) using 10 ng/ml and 4 μ M, respectively. The T cell/stimulator cell ratio was $2.5 \times 10^3/2 \times 10^4$ (up to 10^5 stimulating autologous lymphoblasts were also used) in 100 μ l medium. After 20 h of incubation, supernatants were harvested and stored at -20°C. Controls were supernatants of T cells and stimulating cells cultured with medium alone. TNF determination was done by a biological assay based on measurement of WEHI 164 clone 13 cell lysis by clone supernatants, in comparison with a standard curve obtained with rTNF- β (Genzyme), as described [9].

2.4 Inhibition of cytokine secretion with mAb

Target cells or TIL clones were pretreated with anti-MHC class I and II antibodies and anti-human TcR α/β , respectively. Cells were incubated with serial dilutions of the mAb in 50 μ l for 1 h at 37°C before TIL and tumor cells were mixed for TNF production, as described above.

2.5 HLA class II typing

HLA class II polymorphism of DR, DP and DQ tumor cell loci was studied by reverse dot-blot hybridization [10] (Innolipa tests, Innogenetics, Belgium). Briefly, tumor DNA samples were amplified using DRB1, DRB3, DRB4 and DRB5 primer mix and a pair of DQB1 and DPB1 primers. The second exon was specifically amplified, and biotinylated nucleotides were incorporated into the amplified DNA during PCR. PCR product was then hybridized with specific oligonucleotide probes immobilized for 30 min as parallel lines on membrane-based strips. After washing, alkaline phosphatase-labeled streptavidin was added. Incubation with BCIP/NBT (bromochloroindolyl phosphate and nitroblue tetrazolium in dimethyl formamide) chromogen resulted in a visually interpretable precipitate. Positive probe numbers were collected using a transparent reading chart, and interpretation was performed by reference to an HLA typing list or with a genotyping program. Cell surface expression of HLA-DR, -DQ and -DP was assessed by indirect immunofluorescence using appropriate mAb or performed by the CRTS HLA Laboratory (Dr. Bignon, CTS, Nantes, France) using a standard microcytotoxicity procedure.

3 Results

3.1 HLA class II-restricted TNF production by CD4⁺ TIL clones in response to autologous melanoma cells

We show here that all CD4⁺ TIL clones tested were induced to secrete TNF by autologous melanoma cells, but not by autologous lymphoblasts expressing a good level of HLA class II antigens (data not shown) or by fully mismatched allogeneic melanoma cells (Table 1). Since fetal calf serum had been used to culture tumor cells, whereas lymphocytes were cultured with human serum, it was possible that responses to melanoma cells were directed to a calf serum-derived peptide. This possibility was ruled out by the fact that tumor cells established in culture with human serum induced TNF production by the clones (data not shown). Data in Table 2 suggest that TNF production was restricted by HLA class II since it was significantly inhibited, according to clone, by treatment of tumor cells with anti-HLA-DR or anti-HLA-DQ mAb, neither of

Table 2. HLA class II restriction of TIL clones: inhibition of TNF production^{a)}

Monoclonal antibodies	CD8 ⁺ clone		CD4 ⁺ clones					
	M6-E	M6-T	M17-137	M17-311	M74-94	M77-180	M77-181	M77-184
none	1112	106	1445	86	12	224	335	1515
anti-HLA-DR	948	107	800	22	14	84	38	20
anti-HLA-DQ	1745	43	1552	41	7	486	350	1500
anti-HLA-DP	1124	109	1515	91	11	297	592	1520
anti-HLA class I	69	78	1515	86	13	291	566	1500

a) TNF was determined in the supernatant of clones (2.5×10^3 lymphocytes/well) stimulated by autologous melanoma cells (2×10^4 well) previously incubated or not with anti-HLA mAb. Values are from one representative experiment out of three at mAb dilution 1:200.

Table 3. Cross-reactivity of CD4⁺ TIL clones with autologous and allogeneic melanoma lines, identified on the basis of TNF production^{a)}

Stimulating melanoma lines	CD4 ⁺ clones									
	M6-T	M17-137	M17-311	M74-6	M74-94	M77-59	M77-152	M77-180	M77-181	M77-184
M6	++	-	+	-	-	-	-	-	-	+
M17	-	++	++	+	+	-	-	-	-	++
M74	-	-	-	+	+	-	-	-	-	-
M77	-	-	+	-	-	+	++	+++	+++	++
M47	-	-	-	-	+	-	-	-	-	-
M60	-	-	-	-	-	-	-	++	+	-
M67	-	+	-	++	+	-	++	-	-	-
GERL	-	-	++	+	+	-	-	-	-	-
MZ2-MEL2-2	-	-	+++	+	++	-	-	-	-	-
MAMO	-	-	++	-	-	-	-	-	-	-
IPC-277/5	-	-	-	+	+	-	-	-	-	-
IPC-205/14	-	-	-	-	-	++	-	-	+++	-
IPC-200B/6	-	+	-	-	-	-	++	+++	+++	-
DAUDEL	-	-	-	-	-	-	++	+++	-	-
MEL-4	-	-	-	-	-	-	-	+++	-	-

a) TNF production was determined as in Table 1. TNF productions are indicated by: (-) = no production, (+) = 10 to 100 pg/ml, (++) = 100 to 500 pg/ml, (+++) = > 500 pg/ml. These productions were determined as the mean of three independent experiments.

which inhibited TNF production by a CD8⁺ clone. Moreover, data in Table 1 show that M77 clones did not recognize EBV-transformed B cells B17 and BH, which share all the potentially restricting DR alleles for these clones, whereas M17 clones recognized the autologous EBV-transformed B cell line. EBV-transformed B cell recognition could not be tested for M6 and M74 clones because of a lack of the appropriate cell lines required to check recognition of epitopes on all potentially restricting HLA molecules.

3.2 Stimulation of TNF production by allogeneic melanoma cells

As shown in Table 3, all CD4⁺ clones, with the exception of the HLA-DQ-restricted clone M6-T, were stimulated to produce TNF not only by autologous melanoma cells but also by a few allogeneic melanoma lines, most of which shared at least one of the restricting isotype alleles expressed by autologous cells. In some cases, higher amounts of TNF were induced by allogeneic than by autologous melanoma cells.

3.3 Cross-reactivity of CD4⁺ TIL clones suggests that their restriction pattern may be monogamous or promiscuous

As the restricting HLA isotype of the clones was shown by inhibition experiments, we tried to identify fine HLA restriction by determining the HLA phenotype of allogeneic melanoma cells recognized by these clones. Table 4 shows the alleles found at the DNA level in autologous and allogeneic cell lines recognized by HLA-DR-restricted clones. Expression at the surface of these cells of HLA-DRB1, -DRB3, -DRB4 and DRB5 cells was further checked by immunofluorescence or microcytotoxicity. These data showed that two DR alleles, DRB1*07 and DRB4*0101, were shared by all cell lines recognized by clones M77-180. All cell lines recognized by clone M77-181 shared DRB4*0101. In addition, either DRB1*1601 or the structurally similar DRB1*1501 allele was present in all cell lines recognized by clones M17-137, M77-181 and M77-184. These alleles thus represent two potential restricting elements for clones M17-137 and M77-184 as well as for clone M77-181.

Table 4. DR subtypes of melanoma lines inducing TNF production by DR-restricted clones^{a)}

Clones	Tumor cell lines recognized	DRB1	DRB1	DRB3	DRB4	DRB5
M17-137	M17	0801	1601	–	–	02
	M67	13	15	ND	–	ND
	IPC200B/6	07	15	–	0101	0101
	M6	0101	15	–	–	ND
M17-311	M17	0801	1601	–	–	02
	M77	07	1601	–	0101	02
	GERL	0101	1302	0101	–	–
	MZ2	0101	0101	–	–	–
	MAMO	11	11	–	–	–
M77-180	M77	07	1601	–	0101	02
	M60	07	1601	–	0101	02
	IPC200B/6	07	15	–	0101	0101
	DAUDEL	07	11	0202	0101	–
	MEL4	07	04	–	0101	–
M77-181	M77	07	1601	–	0101	02
	M60	07	1601	–	0101	02
	IPC205/14	0404	15	–	0101	0101
	IPC200B/6	07	15	–	0101	0101
M77-184	M6	0101	15	–	–	ND
	M17	0801	1601	–	–	02
	M77	07	1601	–	0101	02

a) HLA class II typing was done by reverse dot-blot hybridization after second exon amplification by PCR, as described in Sect. 2.5.

4 Discussion

This study shows that CD4⁺ TIL clones from four human melanoma tumors exhibited an HLA class II-restricted TNF response to melanoma cells, but not to autologous T lymphoblasts. Possible recognition of serum determinants, already described for CD4⁺ clones in culture, was ruled out. Since the expression of the HLA class II-restricting isotype by T lymphoblasts was determined for each assay and shown to be generally similar to that expressed by autologous tumor cells, our data suggest that CD4⁺ TIL clones recognize tumor-restricted antigens. Additional data obtained using EBV-transformed B cell lines show that some, but not all, melanoma antigens recognized by CD4⁺ clones were shared by EBV-transformed B cells.

We also established that the epitopes recognized by DR-restricted TIL were shared by several melanoma lines. In contrast, one DQ-restricted clone, M6-T, did not cross-react with any melanoma line sharing one of the two potentially restricting DQ elements. This may have been due to recognition by this clone of a rare or mutated antigen, or to the presentation of a common melanoma antigen by a rare DQ molecule expressed on M6 tumor cells, such as the DQB1*0504 allele found at the DNA level in this cell line (data not shown), which is expressed in only 4 out of 26 ethnic groups analyzed and at very low frequencies [11], or a hybrid molecule either inter- (DQ/DR) or intra-isotypic [12, 13].

Data from HLA typing of melanoma lines suggest that the antigen-presenting allele to clone M77-180 was either DRB1*07 or DRB4*0101. Since all the cells recognized by clones M17-137 and M77-184 expressed one of the two very structurally similar alleles DRB1*1601 or *1501, it is

possible that these clones recognized the antigen on these two alleles indifferently. The same conclusion holds true for clone M77-181. However, the DRB4*0101 allele shared by all melanoma lines which this clone recognized was also a potentially restricting allele. Promiscuous antigen binding to several HLA-DR alleles has already been reported [14]. Our recent demonstration that M17-311 is in fact a mixture of two CD4⁺ clones (Pannetier et al., unpublished), probably accounts for the cross-reactions of this clone with melanoma lines of quite distinct DR phenotypes.

Since all the CD4⁺ clones analyzed here reacted against the autologous tumor, it appears that the development or recruitment of melanoma-reactive CD4⁺ lymphocytes within human metastatic melanomas is a frequent event, although probably limited to HLA class II-expressing tumors. This argues for a common presentation of endogenous melanoma epitopes on HLA class II molecules and an effective recognition of these complexes by autologous CD4⁺ T lymphocytes. However, it is noteworthy that most CD4⁺ clones used here produced TNF, but no detectable IL-2 in response to antigen presentation by melanoma cells *in vitro*, despite the production of significant amounts of IL-2 in response to stimulation by anti-CD3 or PMA-CaI [15]. If a similar limitation of CD4⁺ TIL responses to melanoma antigens also exists *in vivo*, the ability of these cells to exert a significant function is questionable. Limited responses of TIL could result from anergy induction or other improper T cell triggering related to HLA or antigen density, or to antigen conformation.

Taken together, our data confirm and extend previous reports about melanoma antigen recognition by CD4⁺ T cells [4–7]. In one of these studies [7] it was established

that a product of the tyrosinase gene is recognized by CD4⁺ TIL on EBV-transformed B cells pulsed with melanoma cell extracts. In the other two studies, as in the present one, the nature of the target antigens was not indicated. Epitopes recognized by our DR- or DQ-restricted clones may belong to one of two types of shared melanoma antigens recognized by cytotoxic CD8⁺ lymphocytes: either melanocytic differentiation antigens or genes, such as MAGE 1, of unknown function but restrictively expressed by tumor tissues (see [16] for a review). The antigens recognized by M17 clones and expressed by EBV-transformed B cells would either belong to the latter type or be classified as autologous antigens not expressed or not efficiently processed by HLA class II-expressing T lymphoblasts. On the other hand, CD4⁺ clones lacking cross-reactivity with allogeneic melanoma lines may recognize mutated protein products.

The recent identification of peptide recognized by CD8⁺ lymphocytes on melanoma cells has encouraged cancer therapists to devise methods to exploit these peptides as vaccines to induce cytotoxic T cell responses [16]. However, animal models have shown that T helper cells are often crucial agents of tumor rejection, either through induction of CTL-mediated activities or as direct effectors by lymphokine production [17]. If some of the epitopes recognized on melanoma cells by CD4⁺ T lymphocytes are expressed restrictively on tumor cells, they will represent new targets against which to induce or stimulate anti-tumor responses. This indicates the need to identify HLA class II-restricted tumor epitopes, whose existence is suggested here, to facilitate the development of alternative vaccination therapies involving T helper cells. This could prove more effective than therapies already under way that seek to develop CTL activities.

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