Advances in Brief

Human Melanoma-specific, Noncytolytic CD8+ T Cells That Can Synthesize Type I Cytokine[1]

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Abstract

The existence of CD8+ CTLs that are capable of recognizing MHC class I-bound, human tumor-associated peptide antigens is now unequivocally documented in cancer patients. Thus far, the role of CD8+ T cells in tumor immunity has been predominantly viewed in terms of cytolytic ability as the prime mode of their function. Interestingly, it is increasingly evident that CD8+ T cells are capable of synthesizing both type I and type II cytokines. Thus, it is conceivable that tumor antigen-specific but noncytolytic CD8+ T cells might play an important role in antitumor immune response by synthesizing type I cytokine. Through such cytokines, they could provide "help" for the process of generating as well as in maintaining an effective CD8+ CTL response. In addition, they might recruit other types of effector cells (such as natural killer cells, macrophages, and others) locally at the tumor site. Either way, they could exert a profoundly positive role in cell-mediated antitumor immune response, particularly because the great majority of tumor cells express only MHC class I molecules that present peptide epitopes to CD8+ T cells. Unfortunately, tumor antigen-specific, noncytolytic but type I cytokine-secreting CD8+ T cells have not received much investigative attention. Here we show that CD8+ T cells, isolated from the tumor-infiltrating lymphocytes from human melanoma, synthesize type I cytokine (IFN-γ and tumor necrosis factor α) in a MHC class I-restricted and tumor-specific noncytolytic interaction with the autologous melanoma cells.

Introduction

It is now abundantly clear that many cancer patients do have CD8+ T cells that can recognize MHC class I-bound, tumor-associated peptide antigens, self or altered (1). Interestingly, although the precise mechanism(s) by which tumors are rejected is not quite clear, the major perception on the role of CD8+ T cells in tumor immunity is that cytolysis is the prime mode of their function. Indeed, the activation and expansion of tumor antigen-specific CTLs are thought to hold the key to effective antitumor immune response. In this context, it is generally believed that the generation of an effective CTL response to a given tumor antigen, and its maintenance, require "help," ideally, from the conventional CD4+ helper T cells (2). Although the precise mechanism(s) by which CD4+ helper T cells deliver "help" in the generation of CD8+ CTL response has not yet been clearly elucidated, the presumption is that it is carried out through certain types of cytokines, generically referred to as type I cytokines. Interestingly, it has become increasingly evident that CD8+ T cells are capable of synthesizing both type I (IFN-γ and TNF[3]) and type II (IL-4 and IL-10) cytokines. Thus, type I cytokines producing CD8+ T cells could also provide considerable help in the process of CTL genera-

tion. Furthermore, in addition to "helping" CTL generation, by recruiting other types of effector cells of the immune system (NK cells, macrophages, and others), Th1 type CD8+ T cells could play a broader role in antitumor immune response.

It has been known for some time that CD8+ Th2 type cells exist (3). However, very little information is available on CD8+ noncytolytic T cells that can synthesize Th1 type cytokines upon recognition of tumor-associated class I determinants. Here we show that CD8+ T cells that are not cytolytic yet capable of producing type I cytokines specifically upon recognition of autologous melanoma cells can be isolated from tumor-involved lymph nodes in a human melanoma system.

Materials and Methods

Tumors, Cell Lines, and Tissue Culture. The melanoma tissue under study was obtained, under informed consent, from a surgical specimen of a melanoma-involved lymph node from a patient (HLA-A1, -2; -B8, -44) who had had a number of recurrences. The melanoma cell line, JL-M, was established from this biopsy specimen in complete medium (Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum and 50 mM arginine).

In Vitro **Expansion of Tumor-involved LNLs.** The technique for expanding LNLs has been described previously (4). Briefly, the tissue was mechanically homogenized to obtain suspensions of single cells or cells in small clumps. The homogenized cells were washed, and cultures were set up in 24-well tissue culture plates in complete medium and 50 units/ml of recombinant IL-2 (Life Technologies, Inc.). The cultures were fed every third day, and the growing lymphoid cells were harvested free of tumor tissues, washed, recultured, and were split as needed. In addition, the cultures were restimulated with the autologous melanoma cells every 10 days or so at lymphocyte:tumor cell ratios of 50. Phenotypic and functional analyses were begun as soon as a sufficient number of cells were available to obtain an early idea of the nature of the effector cell populations.

Limiting Dilution Microculture. Microcultures of single LNLs were set up in 96 well microwell plates on a feeder layer of 10,000 autologous irradiated peripheral blood lymphocytes in the presence of 50 units/ml of recombinant IL-2 as described previously (4). Growing colonies were harvested, expanded in 48-well plates, and periodically stimulated with irradiated allogeneic EBV-transformed lymphoblastoid cell line and with the autologous melanoma cells.

Phenotypic Assay. Phenotypic analyses were performed by cytofluorometry as described previously (4).

Cytotoxicity Assay. The [51]CR release microcytotoxicity assay has been described previously (4). Each cytotoxicity assay was performed in the presence of unlabeled K-562 cells (unlabeled K562:labeled target, 50:1).

Cytokine Synthesis Assay. Cytokine assays for IFN-γ, IL-2, IL-4, and IL-10 were performed by ELISA (Immunotech) as per the manufacturer's instructions. Assays for TNF-α were performed in bioassay as per Mukherji *et al.* (5) and Espevic *et al.* (6).

Results

The tumor-involved lymph node cells exhibited vigorous growth upon culturing in recombinant IL-2 to allow phenotypic and functional analyses by the first week. Fig. 1 shows the phenotype and cytotoxic function of the growing population tested between days 6 and 12 of the culture. The cultured LNLs at this early stage were

A

JLLNL

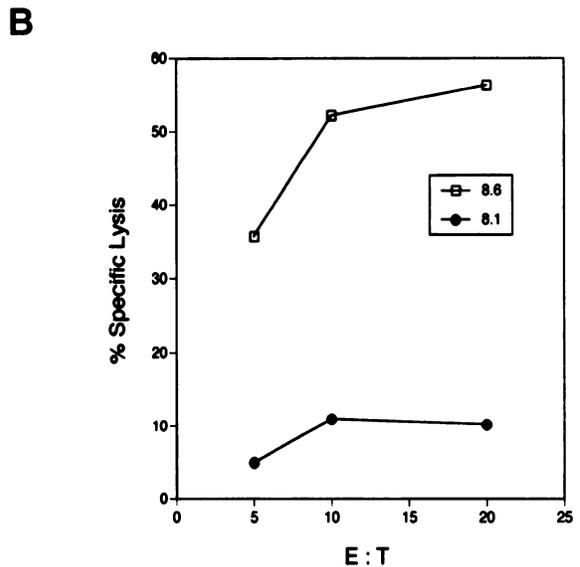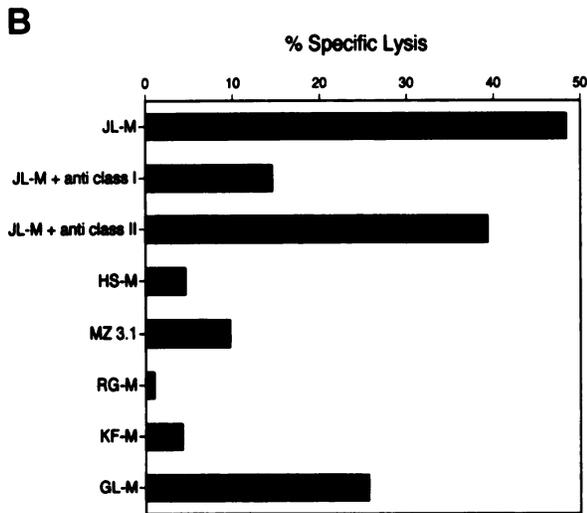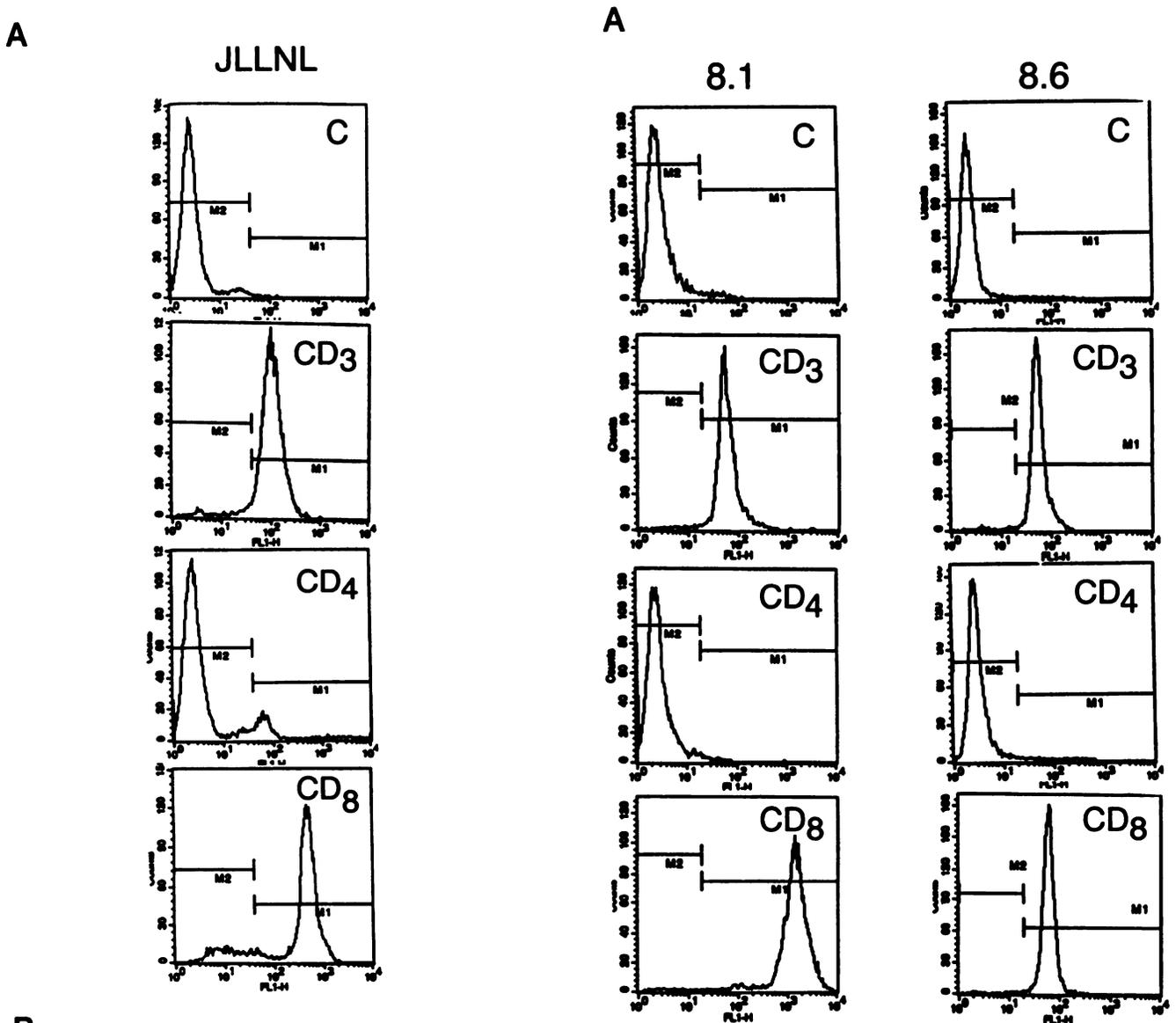**A**

8.1   8.6

B

Fig. 1. *A*, phenotype of JL-LNL cells on day 7. *B*, cytotoxicity of JL-LNL cells at E:T ratio of 40 on day 10. HS-M and MZ 3.1 are HLA-A1+ allogeneic melanoma cell lines. RG-M, KF-M, and GL-M are HLA-A2+ allogeneic melanoma cell lines. The difference in percentage of specific lysis of JL-M cells in the presence of anti-MHC class I monoclonal antibody (w6-32), when compared with the lytic activity in the presence of anti-MHC class II antibody, is significant at *P* [lteq] 0.001 by Student's *t* test. The difference in lytic activities in the absence of and in the presence of class II antibody is not statistically significant.

B

Fig. 2. *A*, phenotype of the two CD8+ T-cell lines grown from the JL-LNL cells in limiting dilution culture condition. *B*, cytotoxicity of the CD8+ T-cell lines 8.1 and 8.6 against the autologous melanoma line JL-M.
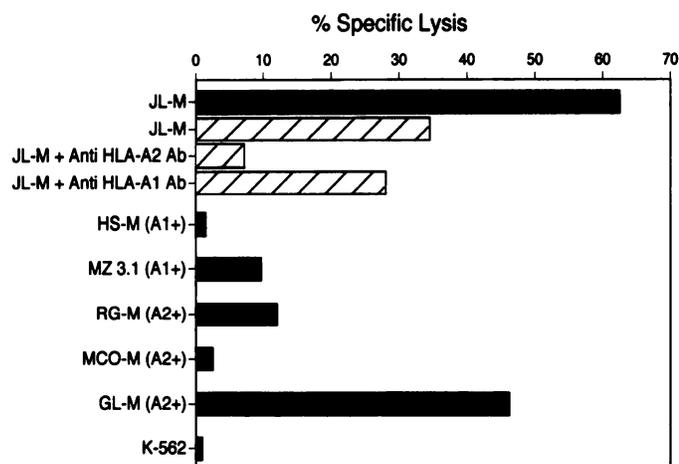
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% Specific Lysis

Fig. 3. Cytotoxicity of the CD8+ T-cell line 8.6. ■, lytic activity at E:T ratio of 40; ▨, lytic activity at E:T ratio of 10. HS-M and MZ 3.1 are HLA-A1+ allogeneic melanoma cell lines. RG-M, MCO-M, and GL-M, are HLA-A2+ allogeneic melanoma cell lines. The difference in percentage of specific lysis of JL-M cells in the presence of anti-HLA-A2 monoclonal antibody, when compared with that in the presence of anti-HLA-A1 antibody, is significant at $P \leq 0.001$ by Student's t test.

Table 1 Cytokine synthesis by the CD8+ T-cell line 8.1

Restimulation with[a]	Cytokine synthesized (pg/ml)[b]				
	IFN-γ	TNF-α	IL-10	IL-4	IL-2
None	14	0	0	0	30
JL-M	800	275	0	0	53
HS-M	10	0	0	0	NT
MZ 3.1	12	0	0	0	40
RG-M	14	0	0	0	32
MC-M	10	0	0	0	35
GL-M	14	0	0	0	30
JL-LCL	10	NT	NT	NT	NT

[a] JL-M, autologous melanoma line; JL-LCL, EBV-transformed autologous lymphoblastoid line; HS-M and MZ 3.1, HLA-A1+ allogeneic melanoma cell lines; RG-M, MC-M, and GL-M, HLA-A2+ allogeneic melanoma cell lines.
[b] Although the T cells (8.1) were washed three times and kept in IL-2-free medium for 48 h before restimulation to measure IL-2 synthesis, it is possible that the small amount of IL-2 detected in the restimulated cultures could have resulted from release of adsorbed IL-2 from the cells. However, no significant increase in IL-2 production was seen upon specific stimulation.

already predominantly CD8+ (Fig. 1A), and they exhibited MHC class I-restricted lytic activity against the autologous melanoma cells JL-M (Fig. 1B). They also lysed another HLA-A2+ allogeneic melanoma line (Fig. 1B).

The LNLs were cloned on day 14 in limiting dilution condition (1 cell/well in 96-well cluster plates). Rapidly growing wells were expanded, and the expanded populations were characterized phenotypically and functionally. Fig. 2A shows the phenotype and the cytolytic function of two lines (referred to as lines because the clonality of these populations were not completely established). As shown, lines 8.1 and 8.6 were CD8+ (Fig. 2A). Of these, only 8.6 showed cytotoxicity against the autologous melanoma cells (Fig. 2B). Line 8.1, in contrast, showed no appreciable levels of cytolytic activity against the autologous melanoma cells JL-M. Line 8.6 lysed the autologous melanoma cells in an HLA-A2-restricted manner as well as lysed another HLA-A2+ allogeneic melanoma line GL-M, although two other HLA-A2+ allogeneic melanoma lines and the NK-sensitive line K-562 cells were not lysed (Fig. 3).

In addition to the cytolytic line 8.6 and the noncytolytic line 8.1, several other CD8+ lines were also grown and tested for function and specificity. Although some of these were noncytolytic, others showed nonspecific lytic activities against various target cells, including the NK-sensitive target line K-562 (data not shown).

When some of the noncytolytic CD8+ lines were tested for their cytokine synthetic function in specific recognition of the autologous melanoma cells JL-M, the noncytolytic line 8.1 was found capable of synthesizing a large amount of IFN-γ and TNF-α but not IL-2 (Table 1). Line 8.1 did not produce IL-10 or IL-4. The cytokine production by the CD8+ T-cell line 8.1 was MHC class I restricted (Table 2). It is of considerable interest that the noncytolytic line 8.1 recognized the autologous melanoma cells, JL-M, but did not recognize the HLA-A2+ allogeneic melanoma line, GL-M, which was recognized by the cytolytic line 8.6, as shown in Fig. 2B. Thus, it is conceivable that the autologous melanoma-reactive CD8+ T-cell lines exhibiting different function recognized different epitopes. Alternatively, the cytolytic line 8.6 might contain two clones having different specificities.

In this particular system (JL), several other noncytolytic CD8+ T-cell lines (8.4, 8.5, and 8.12) were found capable of synthesizing varied concentrations of IFN-γ and TNF-α when they were stimulated by the autologous melanoma cell JL-M (data not shown).

Discussion

From an operational viewpoint of immune response against tumors, the role of CD8+ T cells is unquestionably crucial. This is due to the fundamental fact that most common types of tumors only express MHC class I molecules and that the CD8+ T cells are the major members of the cellular immune apparatus that can recognize antigenic peptides presented by class I molecules. Given the unique properties of CD8+ CTLs, cognitive specificity and cytolytic effector function, the present interest in tumor antigen-specific CTLs is understandable. Because "tumor antigen"-specific CTLs are not always deleted from the repertoire but remain in an unresponsive state, a great deal of effort has now been mounted by many, addressing the question of how these unresponsive CTLs might be activated and amplified. Much is known on the need for the obligatory signals through peptide/MHC complexes and costimulation for T-cell activation. However, very little is known on the nature of the additional "help" that seems to be needed for shaping and maintaining a full-fledged CTL response. It is believed by many that such help essentially comes from certain types of cytokines ("helper" or type 1 cytokine) elaborated by CD4+ helper cells.

In animal models, it is now fairly well established that the pattern of cytokine synthesis by CD4+ T cells "shapes" or "controls" the nature of the immune response (7, 8). Furthermore, in models of viral infections, a critical role of CD4+ T cells in the generation and maintenance of effective CTL response has also been demonstrated convincingly (9–13). The evidence of "polarization" of Th1 versus Th2 type response in humans is also beginning to emerge (14). Unfortunately, in the case of generating a CTL response to a given tumor-associated antigen, even in in vitro systems, virtually nothing is clear on how such a response is shaped. The use of cytokine mixtures in various permutations and combinations designed to optimize CTL generation, removal of CD4+ T cells from the culture, addition of "feeder cells," and others have failed to provide a satisfactory answer. Yet, the need for some form of help in the process has been generally agreed to. In this context, the logic of CD4+ T cells synthesizing a certain form of type I cytokine after getting activated through "pro-

Table 2 MHC restriction of the cytokine synthesis by the T-cell line 8.1

Restimulation with	Cytokine synthesized (pg/ml)	
	IFN-γ	TNF-α
None	35	20
JL-M[a]	670	300
JL-M + anti MHC class I Ab (W6/32)	200	100
JL-M + anti MHC class II Ab	590	280
JL-M + anti HLA-A1 Ab	620	220
JL-M + anti HLA-A2 Ab	48	35

[a] JL-M, autologous melanoma line; Ab, antibody.

1365

fessional antigen-presenting cells" presenting a class II determinant after "capturing and processing" the relevant antigen is quite plausible. Similarly, it is also possible to argue that the "helper" cytokine might be synthesized by the CD8+ T cells themselves. Thus, a great deal of attention has been focused on the questions of how CTLs against tumor cells are "primed" and how "help" is provided to the process of generating a full-fledged CTL response against tumor cells. In this context, in addition to the general agreement on the need for and the role of professional antigen-presenting cells plus CD4+ helper T cells (2), a potential role of a type I CD8+ T cells has also been envisioned (15, 16). After all, it now reasonably clear that the relevant cytokine(s) for achieving a Th1 or a Th2 effect can be made by both CD4+ and CD8+ T cells (17). The involvement of Th1 type CD8+ T cells capable of recognizing a tumor-associated epitope will be more logical because most tumor cells generally express only MHC class I molecules. Hence, the identification of Th1 type CD8+ T cells in response to tumor antigens will be useful. In this context, our observation is noteworthy.

We are not aware of many systematic searches of these types of cells in tumor immunology. We believe that they eluded discovery because attention has been focused mostly on finding tumor antigen-specific CTLs. Noncytolytic CD8+ T cells were simply discarded. Because most *in vitro* analyses of CTL response to tumor antigen as well as limiting dilution cultures have been traditionally carried out after multiple rounds of stimulation of the parent population, it can be argued that this very process may have contributed to the obscurity of CD8+ Th1 type response, which is likely to be an early event. We therefore undertook careful analyses of a broader repertoire of function (cytolytic as well as cytokine production) of CD8+ T cells emerging *in vitro* after a short-term culture of the parent population. Admittedly, it is not clear that a similar approach will be uniformly successful. Furthermore, the physiological relevance of these noncytolytic but type I cytokine-secreting CD8+ T cells remains to be elucidated with further studies. Our findings nevertheless suggest that more in-depth analyses of the entire repertoire of function of CD8+ T cells in context to tumor-associated peptide antigens will be rewarding.

Acknowledgments

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