Paucity of Functional T-Cell Memory to Melanoma Antigens in Healthy Donors and Melanoma Patients

Madhav V. Dhodapkar, James W. Young, Paul B. Chapman, William I. Cox, Jean Francois Fonteneau, Sebastian Amigorena, Alan N. Houghton, Ralph M. Steinman, and Nina Bhardwaj


ABSTRACT

The functional characteristics of CD8+ T cells specific for melanoma antigens (MAs) have often been defined after in vitro culture using nonprofessional antigen-presenting cells. We have examined CD8+ T-cell immunity to MAs and a viral antigen (influenza) in uncultured T cells of healthy donors and melanoma patients using autologous, mature, monocyte-derived dendritic cells (DCs) pulsed with peptide antigens and viral vectors. Antigen-specific IFN-γ-producing T cells reactive with HLA-A*0201-restricted peptides from four melanoma antigens (MelanA/MART-1, MAGE-3, tyrosinase, and gp100) were detected only at low frequencies (<30 per 2 × 10⁵ peripheral blood mononuclear cells for each of the MAs) from HLA-A2.1-positive healthy donors (n = 12) and patients with stages III/IV melanoma (n = 8). Detection of MA-specific, but not influenza matrix peptide (Flu-MP)-specific, T cells required a high concentration (10 μg/ml) of the peptide in this assay. Furthermore, these T cells did not recognize endogenously processed antigen on tumor cell lines or cells infected with viral vectors capable of expressing MAs. The use of autologous, mature DCs led to a significant increase in the number of Flu-MP, but not MA-specific, T cells in 16-h ELISPOT assays for both melanoma patients and healthy donors. In 1-week cocultures with DCs pulsed with 10 μg/ml peptide, MelanA/MART-1-specific T cells did not readily proliferate or differentiate into lytic effectors, in contrast to strong influenza-specific lytic responses. Therefore, despite distinct memory responses to influenza antigens, melanoma patients and healthy controls have a paucity of MA-reactive memory T cells, failing to rapidly generate IFN-γ-secreting lytic effectors in short-term assays, even when stimulated by DCs.

INTRODUCTION

Over the past decade, several MAs have been identified that serve as targets of tumor-infiltrating lymphocytes in the laboratory (1). These antigens are, therefore, important potential targets for immunotherapy of melanoma. However, nearly all defined MAs are nonmutated “self” antigens expressed not only by tumors but also by normal tissues (e.g., melanocytes; Ref. 2). Whether immune recognition and functional T-cell memory to these antigens are present in healthy individuals and melanoma patients is therefore a central question in the immunotherapy of melanoma. This is because the immune system could be tolerized to these “self” antigens, or these antigens may be ignored because of inadequate costimulation (2, 3).

Several studies have examined functional immune reactivity to MAs in the blood from healthy donors and melanoma patients (4). However, in most studies, functional aspects have been examined only after prolonged in vitro culture, with repeated antigen restimulation and exogenous cytokines. Therefore, the in situ biology and function of MA-specific T cells are difficult to ascertain, and the possibility of inadvertently priming responses in vitro during prolonged cultures cannot be excluded. In studies where sensitive and quantitative functional assays such as ELISPOT have been used to quantify MA-specific T cells in freshly isolated cells (5–10), potent APCs like mature DCs have not been used, which may be essential for the analysis of T-cell function in vitro (11, 12).

DCs are specialized to initiate T-cell immunity (13). Maturation of DCs leads to a severalfold enhancement of their APC function in vitro (13). We have shown recently that mature DCs are potent immune adjuvants in humans (14). DCs pulsed with several forms of melanoma antigens (peptides, protein, DNA, or viral vectors) have been shown to elicit MA-specific T cells in vitro (15–19). Therefore, DCs are now being actively pursued for immunotherapy of melanoma, with promising initial results (20, 21). Surprisingly, however, the use of DCs to evaluate T-cell memory to melanoma antigens has not been reported, as has been done with viral antigens (11, 12, 22).

The abbreviations used are: MA, melanoma antigen; DC, dendritic cell; APC, antigen-presenting cell; PBMC, peripheral blood mononuclear cell; IL, interleukin; MOI, multiplicity of infection; SFC, spot-forming cell; Flu-MP, influenza matrix peptide.
As a prelude to our studies of DC-mediated immunotherapy of melanoma, we examined functional T-cell immunity to a panel of MAs (MAGE-3, MelanA/MART-1, tyrosinase, and gp100) in a cohort of healthy volunteers and patients with stages III/IV melanoma. As a positive control for CD8+ T-cell memory, we compared melanoma antigens with Flu-MP, to which most individuals are primed. We used autologous, monocyte-derived, mature DCs to quantify antigen-specific, functional T cells in 1-day and 1-week recall assays (14). We found that only low frequencies of circulating MA-specific functional T cells can be detected in melanoma patients and healthy controls, even after stimulation with antigen-bearing DCs. In contrast to Flu-MP-specific memory T cells from the same donor, these MA-reactive T cells require higher doses of peptides for cytokine production, do not recognize endogenously processed antigen on tumor cell lines or cells infected with recombinant viral vectors carrying melanoma antigens, and do not proliferate and differentiate into lytic effectors after 1 week of coculture with antigen-bearing DCs.

**MATERIALS AND METHODS**

**Peptides, Cell Lines, and Clones.** HLA-A*0201-restricted melanoma, influenza matrix, and HIV gag peptides were synthesized by Biosynthesis, Inc. (Lewisville, TX). All peptides were >95% pure as determined by high-performance liquid chromatography and mass spectrometry. The following HLA-A*0201-restricted peptides were used: MAGE-3 (271–279), FLWGPRALV; MelanA/MART-1 (27–35), AAGIGITV; tyrosinase (386–376, 370D), YMDGTSQV; gp100 (209–217, 210M), IMDQPFSV; Flu-MP (58–66), GILGFVFVTL; and as a negative control, HIV gag peptide, SLYNTVATL.

Human melanoma cell lines G-Mel and SK-MEL-29 (both HLA A*0201 positive and known to express several MAs, including MelanA/MART-1) were used as targets and in some experiments, as APCs. An HLA-A*0201-restricted MelanA/MART-1-specific clone, LT-12, was kindly provided by Dr. Florence Faure (Institut Curie, Paris, France). The clone was cultured in DMEM with 10% FCS and restimulated weekly with T2 cells pulsed with 5 μM MelanA/MART-1 peptide, together with allogeneic PBMCs as feeders and 20 units/ml IL-2 (as Lymphocult). The clone was used for experiments 5–8 days after stimulation.

**Viral Vectors.** Recombinant poxvirus vectors expressing MAs (Virogenetics Corp., Troy, NY) included the vaccinia (recombinant vaccinia virus) vectors WR-gp100 (vP1513) with WR-eco-gpt as control and canarypox (rALVAC)-based vectors carrying MelanA/MART-1 (rALVAC-MART-1; vP1473), gp100 (rALVAC-gp100; vP1465), or parental control (rALVAC).

**Human Subjects.** Leukocyte-enriched buffy coats from anonymous blood donors were obtained from The Greater New York Blood Center (New York, NY). All buffy coats were serotyped for HLA-A2 status by staining with a monoclonal antibody (BB7.2, HB-82; American Type Culture Collection, Rockville, MD). Melanoma patient blood samples were obtained after written informed consent, in accordance with an Institutional Review Board-approved protocol at the Memorial Sloan Kettering Cancer Center. All patients were HLA-A*0201 positive by DNA typing.

**Isolation of Mononuclear Cells.** PBMCs were isolated by density gradient centrifugation on Ficoll-Hypaque. These PBMCs were either tested directly in the ELISPOT assay, used for generation of DCs, or cryopreserved in DMSO with 5% FCS. For some experiments, CD4 and CD8 subsets were selectively depleted using magnetic beads (MACS; Miltenyi Biotec, Auburn, CA). Efficiency of depletion was monitored by flow cytometry.

**Generation of Mature DCs.** DCs were generated from plastic adherent blood mononuclear cells as described previously (14), using granulocyte/macrophage-colony stimulating factor and IL-4. Nonadherent cells were used as sources of T cells in recall assays as described later. On day 6 or 7 of culture, DCs were matured by culturing 2 more days in 50% vol/vol monocyte conditioned medium as described (23).

**Viral Infection.** Infection of PBMCs or DCs with vaccinia or ALVAC viruses was carried out at 37°C in 1% autologous plasma at a MOI of 2 for vaccinia or 10 for ALVAC vectors, as described (24). Cells were then washed twice, before use in ELISPOT assays.

**ELISPOT Assay for IFN-γ Release from Single Antigen-Specific T Cells.** Two sources of antigen were used, peptide antigens and recombinant poxvirus vectors. As described previously (14, 24–26), PBMCs (2 × 10^7 cells/well) were added to plates precoated with 10 μg/ml of a primary anti-IFN-γ monoclonal antibody (Mabtech, Stockholm, Sweden) in the presence or absence of graded doses (10 to 0.01 μg/ml) of peptide antigens to stimulate IFN-γ secretion. Pox vectors were also used to target MAs to PBMCs, which were infected with the vectors as described above. The plates were incubated overnight (14–18 h) at 37°C and washed, and then the wells were incubated with biotin-conjugated, anti-IFN-γ antibody (1 μg/ml; Mabtech). After staining with Vectastain Elite kit (Vector Laboratories, Burlingame, CA), colored spots with fuzzy borders indicated the IFN-γ-secreting cells (SFCs). Responses in the peptide ELISPOT were considered positive if a minimum of 10 SFCs per 2 × 10^5 cells were detected after the control had been subtracted (24). Because of higher background with control vectors, responses in the viral vector ELISPOTs were considered positive only above a threshold (mean ± 3 SD) of 30 SFCs per 2 × 10^5 cells. All assays included a positive control in the form of staphylococcal enterotoxin A (SEA). For some assays, 10^4 LT-12 cells (HLA-A*0201-positive MelanA/MART-1-specific clone) per well were added to serve as a positive control to ensure that this antigen was being presented by APCs. For some experiments, irradiated G-Mel cells were added as APCs at a PBMC:cell ratio of 30:1. For other experiments, mature DCs were pulsed with peptides (10 μg/ml) or viral vectors (MOI 2 for vaccinia and MOI 10 for canarypox), washed, and then added to PBMCs at a ratio of 1:30. A Melan A/MART-1-specific clone (LT-12; 10^5 cells/well) was used, where possible, as an internal control in the ELISPOT to verify the presentation of the relevant antigen (MelanA/MART-1) by DCs or PBMCs.

**Antigen-Specific Recall T-Cell Responses.** To evaluate the ability of tumor- or viral antigen-specific T cells to proliferate and differentiate in culture, T cells (2 × 10^5 cells/well) were cocultured with DCs pulsed with 0.1–10 μg/ml peptides for 7 days at a T:DC ratio of 30:1, as described (14). After 7 days, the presence of antigen-specific T cells was quantified by...
either an ELISPOT or CTL assay. Restimulation by specific peptide at day 7 was essential to detect antigen-specific IFN-γ production (27). For the ELISPOT assay, cells were transferred to an ELISPOT plate and cultured overnight with or without specific antigen. Antigen-specific, IFN-γ-producing cells were quantified as described above. For CTL assays, T cells were added to labeled targets at varying E:T ratios, and CTL activity was measured in a standard 5-h 51Cr release assay. Targets consisted of labeled T2 cells with or without specific peptide and with 80-fold excess of unlabeled K562 cells as cold targets to reduce background attributable to natural killer-mediated lysis. Labeled melanoma cell lines known to express melanoma differentiation antigens (SK-MEL-29 and G-Mel) were also used as targets. The percentage of specific lysis was calculated after subtracting the lysis against unpulsed T2 cells or control unpulsed DCs (14).

**RESULTS**

**Low Frequency of MA-specific T Cells Using ELISPOT Assay.** We first determined whether antigen-specific, IFN-γ-producing T cells to a panel of HLA-A*0201-restricted melanoma peptides (MAGE-3, MelanA/MART-1, tyrosinase, and gp100) and Flu-MP could be detected in freshly sampled PBMCs of 14 healthy donors (B1–B14; all except B4 and B12 were HLA-A2 positive) and 8 melanoma patients (M1–M8; all were HLA-A*0201 positive) using ELISPOT assays with 10 μg/ml peptide. Clinical characteristics of melanoma patients are shown in Table 1. Background reactivity to no peptide or control HIV gag peptide was generally <5 SFCs per 2 × 10^5 cells. Using a cutoff of >10 SFCs per 2 × 10^5 cells, the presence of antigen-specific T cells to MelanA/MART-1, tyrosinase, and gp100 peptides at 10 μg/ml was demonstrated in 6, 4, and 3 of 12 HLA-A2-positive healthy donors and 2, 3, and 3 of 8 melanoma patients, respectively (Table 2). Flu-MP-specific T cells were detected in 7 of 10 healthy donors and 5 of 7 melanoma patients, whereas MAGE-3-specific T cells were detected in none of the subjects. Frequencies of both MA- or Flu-MP-specific T cells were not significantly different in the healthy cohorts or melanoma patients. No reactivity to any of the peptides was detected in the two HLA-A2.1-negative donors.

**MA-specific CD8+ T Cells Require Higher Peptide Doses for IFN-γ Production and Do Not Recognize Tumor Cell Lines.** The reactivity to MAs was highly dependent on the peptide dose and was rapidly lost when the dose was reduced from 10 to 1 μg/ml (Fig. 1, A and B). In contrast, reactivity to Flu-MP, when detected, was well maintained at peptide dose of 0.1 μg/ml. Thus, the mean percentage of decline in reactivity at 0.1 μg/ml peptide dose for MelanA/MART-1, tyrosinase, and gp100 in melanoma patients was 88, 80, and 80%, respectively, whereas the reactivity for Flu-MP declined by only 36% at this dose (P < 0.01 by Student’s t test for all comparisons between Flu-MP and MA). Addition of Lymphocult with 50 units/ml IL-2 to the peptide ELISPOT led to an increase in background but no net change in the detection of MA-specific T cells in three separate experiments (data not shown). As shown previously for MP-reactive cells in this assay (14, 24), the reactivity to the 10 μg/ml dose of melanoma peptides was lost after the depletion of CD8 but not CD4 T cells, demonstrating the CD8+ nature of the response (Fig. 1C).

Three melanoma patients (M4, M5, and M6), including two with detectable antigen-specific T cells for tyrosinase, gp100 (M4), or MelanA/MART-1 (M6) using the peptide ELISPOT, were also tested for recognition of HLA-A*0201-positive melanoma cell lines (G-Mel and SK-MEL-29) known to express melanocyte differentiation antigens. T cells from these patients failed to recognize these cell lines (<10 SFFCs per 2 × 10^5 cells; data not shown).

**Lack of Significant Reactivity to Pox Vectors Carrying MelanA/MART-1 and gp100 Genes.** Next, we evaluated immunity to MelanA/MART-1 and gp100 in uncultured T cells using recombinant viral vectors carrying these constructs as a source of antigen. Use of these vectors allows evaluation of immune response to endogenously processed antigen and is

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**Table 1** Melanoma patient characteristics

<table>
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<th>AJCCa stage</th>
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<td>78</td>
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a AJCC, American Joint Committee on Cancer.

**Table 2** Melanoma peptide-reactive T cellsa are infrequent in the PBMCs of healthy subjects and stages III/IV melanoma patients

<table>
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<tr>
<th>ID</th>
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<th>Tyrosinase</th>
<th>gp100</th>
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<td>39</td>
<td>3</td>
<td>5</td>
<td>6</td>
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a Data represent the number of SFCs per 2 × 10^5 cells at the highest peptide dose (10 μg/ml) after subtracting the data with control peptide (HIV-gag peptide). Data shown are the means of duplicates. SE for all measurements was <20%. Numbers in bold reflect the positive assays (>10 SFCs per 2 × 10^5 cells). B4 and B12 were HLA-A*0201-negative healthy controls that yielded no reactivity to any of these peptides.

b ND, not done.
not restricted by HLA haplotype. For this, we modified the ELISPOT assay for the use of these vectors, as described recently for HIV and EBV antigens (22, 24, 26). The ability of infected PBMCs to present MelanA/MART-1 under these experimental conditions was first verified by using a MelanA/MART-1-specific clone in the ELISPOT assay (data not shown). MelanA/MART-1- and gp100-specific T cells were calculated by subtracting data using control vectors from those expressing MelanA/MART-1 or gp100. Use of pox vectors in this assay leads to a higher background (mean ± SD, 10 ± 7 SFCs per 2 × 10^5 cells) than the use of peptides. Thus, the threshold for detection was higher and set at 30 SFCs per 2 × 10^5 cells (mean ± 3 SD) after subtracting the control data. Using this limit, MelanA/MART-1-specific T cells were detected in none of the 7 healthy donors or melanoma patients tested (data not shown). Similarly, reactivity to gp100 above the threshold was seen in only 1 of 7 melanoma patients and none of the healthy donors.

**Paucity of MA-specific T-Cell Responses in Uncultured T Cells Using Antigen-bearing Mature, Monocyte-derived DCs.** We have shown recently that the use of peptide-pulsed, mature DCs as APCs in the ELISPOT assay leads to an increase in the number of antigen-specific T cells to EBV antigens, as compared with that using PBMCs alone (26). Concurrent with these data, when mature DCs from healthy donors or melanoma patients were pulsed with Flu-MP and used as APCs in the ELISPOT assay, there was a significant increase in the number of Flu-MP-specific, IFN-γ SFCs and an increase in the size of spots (indicating greater cytokine production/cell; Fig. 2, A and B). In contrast, there was no significant increase in either the spot number or spot size (indicative of cytokine production/cell) when DCs pulsed with melanoma peptide antigens were used as APCs in a 16-h ELISPOT assay. Data from 3 melanoma patients tested are shown in Fig. 2A. Similar data were observed with 8 healthy donors tested (Fig. 2B). With 5 of these donors who had detectable MP-specific T cells using resident APCs in PBMCs, greater numbers of MP-specific T cells were seen with the use of mature DCs. When DCs were infected with viral vectors carrying MAs, again there was no boosting of responses in this short-term assay (data not shown). We confirmed the ability of peptide-pulsed and viral vector-infected mature DCs to present MelanA/MART-1 under our experimental conditions by using a MelanA/MART-1-specific clone (LT-12; Fig. 2C). We conclude that the use of DCs to present MAs to uncultured T cells does not lead to the detection of high numbers of MA-specific, IFN-γ-secreting T cells when using a short-term (16-h) cytokine production assay and uncultured PBMCs from either healthy donors or melanoma patients.

**Mature DCs Induce Only a Modest Expansion of MA-specific T-Cell Responses in Short-Term DC:T-Cell Cultures.** A cardinal feature of memory T cells is their ability to proliferate in response to antigen and to differentiate into lytic effectors during culture. In prior experiments, we have observed that antigen-pulsed mature DCs (but not immature DCs or monocytes) could readily elicit influenza virus-specific lytic effectors in 7-day cocultures, without the need for exogenous IL-2 (12). Therefore, we compared the ability of Flu-MP-specific versus MelanA/MART-1-specific T cells from both healthy donors and melanoma patients to proliferate in 7-day
Fig. 2  A and B, use of peptide pulsed mature DCs as APCs in 16-h ELISPOT assay. PBMCs were cocultured overnight in the presence of 10 μM peptide alone or with autologous monocyte-derived mature DCs (matured using monocyte conditioned medium) pulsed with 10 μM peptide (DC:T ratio, 1:30), and the presence of antigen-specific, IFN-γ-producing cells was quantified using an ELISPOT assay.

A, data for melanoma patients (n = 3). B, data for healthy donors (n = 7). SE for all measurements was <25%. MART, MelanA/MART-1; MP, Flu-MP; Tyr, tyrosinase.

C, DCs pulsed with MelanA/MART-1 peptide or viral vectors carrying MelanA/MART-1 stimulate MelanA/MART-1-specific CD8+ T-cell clones in vitro. DCs matured using monocyte conditioned medium were pulsed with melanoma (MelanA/MART-1) or control Flu-MP (MP) or infected with canarypox (ALVAC) vectors (MOI 10) carrying MelanA/MART-1 and incubated with 10^5 MelanA/MART-1-specific clone cells (LT-12; DC:clone ratio, 1:30) for 16 h. Antigen-specific, IFN-γ-producing cells were quantified using an ELISPOT assay. MART, MelanA/MART-1; MP, Flu-MP.
cific lysis of MelanA/MART-1-pulsed T2 targets or melanoma cell lines was observed. Similar data demonstrating <2–3-fold increase in antigen-specific T cells after 1 week of culture with DCs pulsed with gp100, tyrosinase, MAGE-3, or MelanA/MART-1 have been observed in preimmunization specimens from four additional melanoma patients from an ongoing DC vaccination trial (data not shown).

DISCUSSION

In this study, we have compared the antigen-dependent, cytokine-secreting function of T cells of healthy donors and patients with stages III/IV melanoma using autologous PBMCs and monocyte-derived, mature DCs as APCs. In all cases, freshly isolated circulating T cells were tested using either unfractionated PBMCs or autologous, monocyte-derived, mature DCs as APCs. The antigens were HLA-A*0201-restricted peptides for MAs (MAGE-3, tyrosinase, MelanA/MART-1, and gp100) and as a positive control, Flu-MP. Two sources of antigen were examined: HLA-A*0201-restricted peptides or recombinant pox vectors carrying MA (gp100 and MelanA/MART-1). We have found that only low frequencies of MA-specific, IFN-γ-producing T cells are detected in the circulation of both healthy donors and melanoma patients, even with the use of autologous mature DCs as APCs.

When the quality of the MA-specific T-cell response was examined, the cells were functionally inferior to virus-specific cells in four respects: (a) MA-specific T cells required higher peptide doses for cytokine production under comparable conditions in vitro, as compared with influenza-specific memory T cells as a control; (b) consistent with these observations, T cells from individuals with detectable MA-specific T cells did not recognize antigen-bearing melanoma cell lines. Similarly, endogenously processed antigens on PBMCs infected with viral vectors were not efficiently recognized after adjusting for the background response to control vectors; (c) the use of antigen-bearing DCs did not improve the detection of MA-specific T cells in the ELISPOT assay; and (d) MA-specific T cells lacked another characteristic feature of virus-specific memory T cells, the ability to proliferate and differentiate rapidly into lytic effectors in short-term DC cocultures without exogenous cytokines (12, 28, 29).

Other investigators have also used the ELISPOT assay for the detection of immunity to melanoma peptides (5–10). However, there are several methodological differences. Most studies have used T2 cells as APCs, which leads to variable background reactivity (8, 9). This makes the evaluation of a low level of reactivity difficult and has prompted exploration of alternate APCs (30). We therefore used autologous APCs (PBMCs or DCs), which leads to minimal background. It is notable that another recent study using a similar ELISPOT assay with autologous PBMCs as APCs in uncultured cells also failed to find significant reactivity to one of the antigens (MelanA/MART-1), as seen here (7). Similar paucity of cytokine production in preimmunization specimens was noted recently with another technique, real-time reverse transcription-PCR, when the peptide was directly added to PBMCs in a short-term assay (31).

An important distinguishing feature of our study is the use of mature DCs as APCs, which may be critical to assay T-cell function in vitro. We have also examined recently immune...
responses to other viruses (e.g., EBV) using similar DC-based assays (26). Use of mature DCs as APCs led to heightened detection of immune responses, as seen here with influenza but not melanoma antigens. This is likely attributable to an increase in cytokine production/cell and elicitation of “subthreshold” responses (32, 33) because of better antigen presentation and costimulation with DCs (26). The absence of this effect using DCs pulsed with melanoma antigens, either peptides or viral vectors, suggests a paucity of memory cells to MAs, or that these T cells are dysfunctional (34).

Influenza matrix (Flu-MP)-specific CD8+ T cells readily recognize endogenously processed antigen on influenza-infected monocytes in the 16-h ELISPOT assay (14). In contrast, freshly isolated MA-specific T cells did not recognize endogenously processed antigen on tumor cells in this short-term assay. In prior studies, recognition of endogenously processed antigen by melanoma-reactive T cells and their cytolytic function have been examined mostly after repetitive in vitro stimulation in the presence of cytokines (4, 8, 35). However, even with in vitro stimulation, little tumor cell-reactive cytokine production was observed in preimmunization samples, which is consistent with our observations (8, 35). We have shown previously that virus-specific memory T cells are efficiently activated by mature DCs (12). Thus, Flu-MP-specific CD8+ T cells readily proliferate and differentiate into cytolytic effectors in 1-week DC:T cocultures. However, under these conditions, MA-specific cytolytic effectors could not be elicited, even from samples wherein the prestimulation reactivity to Flu-MP and melanoma antigens was similar. This may be critical for protective immunity and rapid responses after re-exposure to antigen and again points to the paucity of functional T-cell memory to MAs in vivo.

Modifications in the MelanA/MART-1 peptide to increase its binding to the HLA-A2 molecule may lead to enhanced stability and immunogenicity of the peptide in vitro (36, 37). We used a MelanA/MART-1-specific CD8+ T-cell clone as a positive control in our assays to confirm that the naturally processed MelanA/MART-1 peptide that we used was stable in solution. It is notable, however, that a recent study using this heteroclitic peptide in a similar ELISPOT assay also found low functional reactivity to this antigen in healthy humans, consistent with our results (7).

Recently, MHC tetramers have been used to quantify MA-specific T cells in the circulation of melanoma patients and healthy subjects (7, 34, 38). In two studies, high frequencies of MelanA/MART-1 tetramer binding T cells were observed in both cohorts (7, 38). Phenotypic studies on MA tetramer binding cells have yielded variable results, with predominantly memory or a naïve phenotype (7, 34, 38). However, in some of these studies (7, 38), tetramer staining was only performed at 4°C, which may have led to nonspecific staining (39). We did not use MHC tetramers to concurrently quantify antigen-specific T cells in this study. However, the detection of a high frequency of tetramer binding cells does not necessarily imply functional immunity, as has been demonstrated recently (34). Here again, it has been difficult to directly examine the function of circulating tetramer binding cells without prior ex vivo culture (7). Indeed, in one of these studies (7), only a low level of MelanA/MART-1-specific functional reactivity was observed with a similar ELISPOT assay as in this study (7), and this was despite high levels of tetramer binding cells.

In conclusion, these data demonstrate that even with the use of mature DCs as APCs, only low levels of circulating MA-reactive functional T cells are detected in melanoma patients and healthy donors. Moreover, these cells are less functional under similar assay conditions, when compared with control virus-specific memory T cells from the same individual. These data therefore support current strategies aimed at enhancing immunity to these antigens in melanoma patients. The qualitative differences between melanoma- and virus-specific T cells illustrated here underscore the need to monitor not only quantitative (e.g., by MHC tetramer analysis) but also qualitative aspects of elicited T cells in vaccination protocols. Paucity of functional T-cell memory also emphasizes the need to target professional APCs (like mature DCs) to prime naïve T cells and elicit high avidity effectors in patients (40). Protective tumor immunity in patients may require an antitumor immune response that functionally and quantitatively resembles antiviral immunity.

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