

# Disease-associated Bias in T Helper Type 1 (Th1)/Th2 CD4<sup>+</sup> T Cell Responses Against MAGE-6 in HLA-DRB1\*0401<sup>+</sup> Patients With Renal Cell Carcinoma or Melanoma

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## Abstract

T helper type 1 (Th1)-type CD4<sup>+</sup> antitumor T cell help appears critical to the induction and maintenance of antitumor cytotoxic T lymphocyte (CTL) responses in vivo. In contrast, Th2- or Th3/Tr-type CD4<sup>+</sup> T cell responses may subvert Th1-type cell-mediated immunity, providing a microenvironment conducive to disease progression. We have recently identified helper T cell epitopes derived from the MAGE-6 gene product; a tumor-associated antigen expressed by most melanomas and renal cell carcinomas. In this study, we have assessed whether peripheral blood CD4<sup>+</sup> T cells from human histocompatibility leukocyte antigens (HLA)-DRB1\*0401<sup>+</sup> patients are Th1- or Th2-biased to MAGE-6 epitopes using interferon (IFN)- $\gamma$  and interleukin (IL)-5 enzyme-linked immunospot assays, respectively. Strikingly, the vast majority of patients with active disease were highly-skewed toward Th2-type responses against MAGE-6-derived epitopes, regardless of their stage (stage I versus IV) of disease, but retained Th1-type responses against Epstein-Barr virus- or influenza-derived epitopes. In marked contrast, normal donors and cancer patients with no current evidence of disease tended to exhibit either mixed Th1/Th2 or strongly Th1-polarized responses to MAGE-6 peptides, respectively. CD4<sup>+</sup> T cell secretion of IL-10 and transforming growth factor (TGF)- $\beta$ 1 against MAGE-6 peptides was not observed, suggesting that specific Th3/Tr-type CD4<sup>+</sup> subsets were not common events in these patients. Our data suggest that immunotherapeutic approaches will likely have to overcome or complement systemic Th2-dominated, tumor-reactive CD4<sup>+</sup> T cell responses to provide optimal clinical benefit.

Key words: melanoma • renal cell carcinoma • helper T lymphocyte • MAGE-6 • epitope

## Introduction

Although renal cell carcinoma (RCC)\* and melanoma are considered among the most responsive cancers to immunotherapy, the vast majority of recent immunotherapeutic

approaches have focused solely on the induction of CD8<sup>+</sup> antitumor T cells in vivo as a surrogate of clinical benefit (1–3). It appears clear that the ability to promote effector CD8<sup>+</sup> T cells reactive against tumor antigens is a necessary, but not sufficient, event in objective clinical responses (4). CD4<sup>+</sup> T cells can also recognize tumor antigen-derived peptides, either directly (as some RCCs and melanomas express MHC class II molecules in situ; references 5 and 6) or via cross-presentation mechanisms by host antigen-presenting cells, such as dendritic cells (DCs; references 7 and

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\*Abbreviations used in this paper: DC, dendritic cell; IVS, in vitro stimulation(s); RCC, renal cell carcinoma.

8). Th1-type CD4<sup>+</sup> T cells secreting IFN- $\gamma$  appear crucial to the optimal generation and durability of specific CTL in vivo and may also serve to recruit these effector cells into the tumor microenvironment via delayed-type hypersensitivity responses (8). Hence, the lack of effectively promoting specific Th1-type CD4<sup>+</sup> T cell generation, maintenance, and direction for antitumor CD8<sup>+</sup> T cells may represent a significant limitation in current vaccine trials.

Tumor-induced deviation of CD4<sup>+</sup> T cell responses in progressive disease and the role of Th1- and Th2-type CD4<sup>+</sup> effector cells have been evaluated in a limited number of murine models (9–19). Studies using the B16 melanoma model have documented a gradual shift of initial Th0-, mixed Th1-/Th2-type CD4<sup>+</sup> T cell response to Th2/Tr-type dominated responses by 14–20 d of progressive tumor growth (13, 17–19). Injection of neutralizing anti-IL-4, -IL-10, or -TGF- $\beta$ 1 antibodies can prevent this tumor-induced functional transition, resulting in enhanced CD8<sup>+</sup> CTL generation and protection against tumor growth (17). Depletion of CD4<sup>+</sup> T cells in late-stage progressive B16 models, where Th2/Tr-type response dominate, restores CTL effector function and can result in tumor regression and vitiligo, particularly upon administration of rIL-12 (13). Analyses of the anti-tumor efficacy of Th1- and Th2-type CD4<sup>+</sup> T cells has also been evaluated in prophylactic and adoptive transfer tumor models (9, 12, 13, 15). In these latter cases, Th1- and Th2-type can mediate complementary antitumor effector functions, via contrasting mechanisms (9). Although Th2-type CD4<sup>+</sup> T cells can promote the recruitment of tumoricidal eosinophils and macrophages into the tumor microenvironment and promote acute tumor rejection (9), on a cell-per-cell basis Th1-type T cells appear to provide a greater therapeutic index (12, 14, 15) and only Th1-type CD4<sup>+</sup> T cells appear to promote durable anti-tumor CTL responses (15).

Interestingly, tumor infiltrating lymphocytes in patients with spontaneous and therapeutically induced regressing lesions appear to be characterized by dominant Th1-type responses to mitogens, whereas tumor infiltrating lymphocytes from patients with progressor lesions have been reported to exhibit functionally dominant Th2-type (IL-4, IL-5) and/or Th3-/Tr-type (IL-10, TGF- $\beta$ 1) CD4<sup>+</sup> T cell responses (20–22). However, prior analyses of patient bulk CD4<sup>+</sup> T cell responses to mitogenic stimuli have yielded equivocal results, with generally no clear-cut Th1- or Th2-type bias observed (23–25). As this difference in results may reflect the stringency with which tumor-specific CD4<sup>+</sup> T cell responses were evaluated, significant recent emphasis has been placed on the identification of tumor antigen-derived helper T cell epitopes that may be used to quantitate and assess tumor-specific CD4<sup>+</sup> T cell numbers and function.

Based on IFN- $\gamma$  as a read-out cytokine or proliferation as an endpoint, Th1-type epitopes have been identified for the MART-1/Melan-A, gp100/pmel17, tyrosinase, MAGE-3, and MAGE-6 (unpublished data) melanoma-associated antigens, among others (26–28). We have recently

defined a set of helper epitopes derived from the MAGE-6 protein and have focused on these targets in the current study, since expression of MAGE-6 (unpublished data) has been observed in premalignant lesions in situ and at high frequencies in primary and metastatic tumors (29–31). Hence, CD4<sup>+</sup> T cell responses to MAGE-6 epitopes may represent early etiologic events. The bias of MAGE-6 (tumor)-specific CD4<sup>+</sup> T cell responses (i.e., Th1, Th2, Th3/Tr) may impact cancer incidence in susceptible individuals, the progression status of established MAGE-6<sup>+</sup> tumors or time to recurrence of MAGE-6<sup>+</sup> disease in the adjuvant setting. Indeed, melanoma expression of HLA-DR has been reported to be a marker of poor prognosis (32, 33), suggesting that the nature of CD4<sup>+</sup> T cell recognition of MHC class II-presented tumor epitopes may play a decisive immunoregulatory role in situ.

This study is the first to demonstrate tumor antigen-specific Th2-type polarization of CD4<sup>+</sup> T cell responses in the peripheral blood of patients with RCC or melanoma. We have implemented IFN- $\gamma$  and IL-5 ELISPOT assays to assess the magnitude of Th1- and Th2-type CD4<sup>+</sup> T cell responses to MAGE-6 epitopes, respectively. We report that HLA-DR $\beta$ 1\*0401<sup>+</sup> patients with active melanoma or RCC displayed strongly polarized Th2-type reactivity to these peptides, whereas normal donors and patients that were disease-free following therapeutic intervention exhibited either weak mixed Th1-/Th2-type or strongly-polarized Th1-type responses to these same epitopes.

## Materials and Methods

**Cell Lines and Media.** The T2.DR4 (DRB1\*0401<sup>+</sup>) cell line (provided by Dr. Janice Blum, Indiana University School of Medicine, Indianapolis, IN) was used as the peptide-presenting cell in these studies. This cell line uniformly expresses HLA-DR\*0401 molecules that contain moderate-to-low affinity binding peptides derived mainly from intracellular invariant chain (class II-associated invariant chain peptide [CLIP]) due to a genetic deficiency in HLA-DM (34). T2.DR4 cells were maintained in RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10 mM L-glutamine (all reagents GIBCO BRL).

**Peptide Selection and Synthesis.** The MAGE-6 (unpublished data), influenza A matrix<sub>60–73</sub> (35), malarial circumsporozoite<sub>326–345</sub> (26) and EBV EBNA-1<sub>519–533</sub> (36) HLA-DR4-presented epitopes were synthesized by FMOC chemistry by the University of Pittsburgh Cancer Institute's (UPCI) Peptide Synthesis Facility (Shared Resource). Peptides were >90% pure based on HPLC profile and MS/MS mass spectrometric analysis performed by the UPCI Protein Sequencing Facility (Shared Resource).

**Isolation of Patient and Normal Donor PBMC-Derived T Cells.** 40–100 ml of patient or normal donor heparinized blood was obtained with informed consent under IRB-approved protocols and diluted 1:2 with HBSS, applied to ficoll-hypaque gradients (LSM; Organon-Teknika) per the manufacturer's instructions, and centrifuged at 550 g for 25 min at room temperature. Patient and normal donor information is provided in Table I. PBMCs at the buoyant interface were recovered and washed twice with HBSS to remove residual platelets and ficoll-hypaque. HLA-DR4<sup>+</sup> status was confirmed by flow cytometry using the anti-HLA-DR4

**Table I.** HLA-DRB1\*0401-positive Patients Evaluated in this Study

Patient	Age	Sex	Stage	Treatment	Disease status at time of evaluation (y)	RT-PCR MAGE-6 (+/-)	α-MAGE response		
							121	140	246
Melanoma ( <i>n</i> = 18)									
SLM1	71	F	IV	S, DC/peptide	NED (1.5)	+	Th1	Th1	Th1
SLM2	64	F	IV	S, IFN-α	NED (5.0)	+	Th1	Th1	Th1
SLM5	34	M	IV	S, IFN-α	NED (4.1)	NA	Th1	Th1	–
SLM6	37	F	I	S, IFN-α	NED (1.9)	NA	Th1	Th1	Th1
SLM9	35	F	III	S, C, R	NED (0.3)	+	Th1	–	–
SLM10	74	M	IV	S	NED (0.4)	NA	Th1	–	–
SLM12 <sup>a</sup>	39	F	IV	S, C, IFN-α	Stable	+	Th2	Th2	Th2
SLM14	52	M	IV	C	Mets, brain	+	Th2	Th2	Th2
SLM16	64	M	IV	None	Mets, liver/lung	+	–	–	Th2
SLM17	74	M	IV	S	Mets	+	Th2	Th2	Th2
SLM18	31	F	IV	S, IFN-α	Mets, brain	+	Th2	Th2	Th2
SLM19	56	M	IV	S, IFN-α, C	Mets	+	–	Th2	Th2
SLM21	45	M	IV	S, IFN-α	Mets	–	–	–	–
SLM22	57	F	IV	S	Mets	–	Th2	Th2	Th2
SLM23	63	M	I	S, IFN-α	NED (1.2)	+	Th2	Th1/2	–
SLM24	36	F	IV	S	Mets	+	Th2	Th2	Th2
SLM25	42	M	IV	S	Mets	+	–	Th2	–
SLM26	41	F	IV	S	Mets	–	–	–	–
RCC ( <i>n</i> = 18)									
SLR2	51	F	IV	S	NED (0.3)	+	Th1	Th1	Th1
SLR3	45	M	IV	S	Mets	NA	–	Th2	–
SLR4	49	F	IV	S, IL-2	Mets	NA	Th2	Th2	Th2
SLR5	79	M	IV	S, IFN-α	Mets	NA	Th2	Th1/2	Th2
SLR6	64	M	I	S	NED (0.3)	NA	Th1	Th1	Th1
SLR7	52	F	I	S	Local Dis.	NA	–	–	Th2
SLR8	49	M	IV	C, R	Mets	NA	–	Th2	–
SLR9	53	F	I	S	NED (0.1)	NA	Th1	–	–
SLR10	41	M	IV	S, C, R	Mets	NA	Th2	–	–
SLR11	58	M	IV	S, IFN-α, R	Mets	–	–	–	–
SLR12	58	M	I	S	Local Dis.	NA	–	–	Th2
SLR13	71	M	I	S	Local Dis.	NA	–	–	–
SLR14	75	F	I	S	Local Dis.	NA	–	Th2	–
SLR15	58	M	I	S	NED (0.1)	NA	Th1	–	–
SLR16	57	M	IV	S, R	Mets	–	–	–	Th2
SLR17	53	M	II	S	Local Dis.	NA	–	–	Th2
SLR18	62	F	II	S	Local Dis.	NA	Th2	Th2	Th2
SLR19	67	M	IV	S, IFN-α, R	Mets	NA	Th2	Th2	Th2

AIT, adoptive immunotherapy (VDLN cells); C, chemotherapy; Mets, metastatic disease; R, radiotherapy; S, surgery; DC/peptide, dendritic cell plus synthetic melanoma peptide vaccine; IFN-α, IFN-α therapy; NED, no evidence of disease at time of blood draw; NA, not available for evaluation. <sup>a</sup>Patient with ocular melanoma. Th1 or Th2 assignment for peptide reactivity reflects donor responses of ≥10 spots/50,000 CD41 T cells as determined in IFN-γ or IL-5 ELISPOT assays, respectively.

reactive mAb clone 359–13F10 (IgG; provided by Dr. Janice Blum, Indiana University School of Medicine, Indianapolis, IN) in indirect immunofluorescence assays. PBMCs were diluted to  $10^7$ /ml in AIM-V medium (GIBCO BRL) and incubated for 60 min at  $37^\circ\text{C}$  in T75 vented flasks (COSTAR), with subsequently harvested adherent cells used to generate DCs (see below) and nonadherent cells frozen in 90% FCS containing 10% DMSO (Sigma-Aldrich) at  $10^7$  lymphocytes/vial using controlled-rate freezing technique. On the day of establishing DC–T cell cultures, nonadherent cells were thawed and washed twice with HBSS.  $\text{CD4}^+$  T cells were then isolated using MACS™ (Miltenyi Biotec) anti-human CD4 beads and MiniMACS™ columns per the manufacturer's protocol.  $\text{CD4}^+$  T cell yields were typically 25–35% of starting PBMC numbers loaded, with purity exceeding 97% as assessed by flow cytometry.

**Induction of Antitumor T Effector Lymphocytes.** Autologous DCs were prepared as described previously in 7-d cultures of plastic-adherent PBMCs in AIM-V media supplemented with rhGM-CSF and rhIL-4 (26). Harvested, nonadherent DC ( $2 \times 10^5$ ) were then cocultured with  $2 \times 10^6$  autologous  $\text{CD4}^+$  T cells in the presence of 10  $\mu\text{M}$  synthetic peptides for 7 d in RPMI-1640 containing 10% FBS and no exogenously added cytokines. Responder T cells were then harvested and analyzed for MAGE-6 peptide specificity in ELISPOT assays.

**IFN- $\gamma$  and IL-5 ELISPOT Assays for Peptide-Reactive  $\text{CD4}^+$  T Cell Responses.** To evaluate the frequencies of peripheral blood  $\text{CD4}^+$  T cells recognizing peptide epitopes, ELISPOT assays for IFN- $\gamma$  and IL-5 were performed as described previously (26, 27, 37). Briefly,  $\text{CD4}^+$  T cell responses were evaluated by both IFN- $\gamma$  (Th1) and IL-5 (Th2) ELISPOT assays. For ELISPOT assays, 96-well multiscreen hemagglutinin antigen plates (Millipore) were coated with 10  $\mu\text{g}/\text{ml}$  of anti-human IFN- $\gamma$  mAb (1-D1K; Mabtech) or 5  $\mu\text{g}/\text{ml}$  of anti-human IL-5 (BD Biosciences) in PBS (GIBCO BRL/Life Technologies) overnight at  $4^\circ\text{C}$ . Unbound antibody was removed by four successive washing with PBS. After blocking the plates with RPMI1640/10% human serum (1 h at  $37^\circ\text{C}$ ),  $10^5$   $\text{CD4}^+$  T cells and T2.DR4 cells ( $2 \times 10^4$  cells) were seeded in multiscreen hemagglutinin antigen plates. Synthetic peptides (stocks at 1 mg/ml PBS) were then added to appropriate wells at a final concentration of 10  $\mu\text{g}/\text{ml}$ . Negative peptide control wells contained  $\text{CD4}^+$  T cells with T2.DR4 cells pulsed with Malaria-CS<sub>326–345</sub> peptide, with T2.DR4 cells alone serving as the APC control. Positive controls were T cells plated in the presence of 5  $\mu\text{g}/\text{ml}$  PHA (Sigma-Aldrich). Culture medium was AIM-V (GIBCO BRL/Life Technologies) at a final volume of 200  $\mu\text{l}/\text{well}$ . Plates were incubated at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  for 24 h in the case of IFN- $\gamma$  ELISPOT assays and for 40 h in IL-5 ELISPOT assays. After incubation, supernatants of culture wells were harvested for ELISA analysis, and plates washed with PBS/0.05% Tween 20 (PBS/T) to remove cells. Captured cytokine was detected at sites of its secretion by incubation for 2 h with biotinylated mAb anti-human IFN- $\gamma$  (7-B6–1; Mabtech) at 2  $\mu\text{g}/\text{ml}$  in PBS/0.5% BSA or biotinylated mAb anti-human IL-5 (BD Biosciences) at 2  $\mu\text{g}/\text{ml}$  in PBS/0.5% BSA. Plates were then washed six times with PBS/T, and avidin-peroxidase complex (diluted 1:100; Vectastain Elite Kit; Vector Laboratories) was added for 1 h. Unbound complex was removed by three successive washings with PBS/T and three rinses with PBS alone. AEC substrate (Sigma-Aldrich) was added and incubated for 5 min for the IFN- $\gamma$  ELISPOT assay and the TMB substrate for peroxidase (Vector Laboratories) was added and incubated for 10 min for the IL-5 ELISPOT assay. All determinations were performed in triplicates, with spots imaged using the Zeiss AutoImager (and statis-

tical comparisons determined using a Student two-tailed *t* test analysis). The data are represented as mean IFN- $\gamma$  or IL-5 spots per 100,000  $\text{CD4}^+$  T cells analyzed.

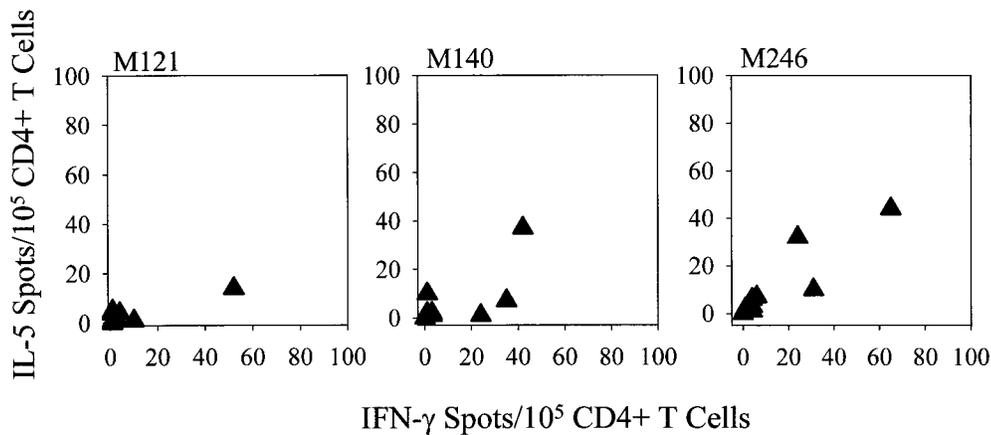
**TGF- $\beta$ 1 and IL-10 ELISAs.** Supernatants were harvested from ELISPOT plates at the endpoint of the culture period and pooled for a single stimulus (i.e., a given peptide, etc.) and frozen at  $-20^\circ\text{C}$  until analysis by cytokine-specific ELISA. Cytokine capture and detection antibodies and recombinant cytokine were purchased from BD Biosciences and used in ELISA assays per the manufacturer's instructions. The lower limit of detection for the TGF- $\beta$  assay was 60 pg/ml, while that of the IL-10 ELISA was 7 pg/ml. In the case of IL-10, 1–5 responder  $\text{CD4}^+$  T cells spots imaged in an IL-10 ELISPOT equated with  $\sim 12$ –17 pg/ml IL-10 as determined in the IL-10 ELISA assay (unpublished data).

**PCR Analysis.** PCR analyses were performed to determine patient HLA-DR4 genotype using a commercial PCR panel according to the manufacturer's instructions (Dynal) and PBL. RT-PCR analysis was also used to determine tumor expression of MAGE-6 mRNA. The following primer set was used: MAGE-6 (forward: TGGAGGACCAGAGCCCCC, reverse: CAGGATGATTATCAGGAAGCCTGT, product size 728 bp with cycles: melting  $94^\circ\text{C}$  for 1 min, annealing  $68^\circ\text{C}$  for 1 min, extension  $72^\circ\text{C}$  for 1 min).

## Results

**Normal HLA-DR $\beta$ 1\*0401<sup>+</sup> Donors Fail to React or Display a Mixed Th1/Th2  $\text{CD4}^+$  Response to MAGE-6 Epitopes after Primary In Vitro Stimulation.** MAGE-6 peptides were not recognized by freshly-isolated  $\text{CD4}^+$  T cells harvested from normal HLA-DR $\beta$ 1\*0401<sup>+</sup> donors (unpublished data). To determine if  $\text{CD4}^+$  precursors were present in normal donors and to identify the balance of Th1-type versus Th2-type responses, we implemented IFN- $\gamma$  and IL-5 ELISPOT assays, respectively. Although both IL-4 and IL-5 have been previously reported as signature cytokines for Th2-type T cell responses (38, 39), we chose the IL-5 assay over the IL-4 ELISPOT assay to screen for Th2-type  $\text{CD4}^+$  T cell reactivity for technical reasons, mainly the much lower backgrounds and higher signal-to-noise ratio observed for the IL-5 ELISPOT system (37, 40).

To evaluate whether normal donors could be prompted to recognize any of these sequences, isolated  $\text{CD4}^+$  T cells derived from the PBMCs of HLA-DR4<sup>+</sup> normal donors were stimulated with autologous immature DCs in the presence of the individual MAGE-6 peptides (i.e., MAGE-6<sub>121–144</sub>, MAGE-6<sub>140–170</sub>, or MAGE-6<sub>246–263</sub>). We chose this in vitro stimulation (IVS) protocol since the DCs generated were poor IL-12 producers upon CD40 ligation and these antigen presenting cells do not appear to skew  $\text{CD4}^+$  T cell responses in either a Th1-type or Th2-type manner (unpublished data). Hence, we consider these as “neutral” DC for  $\text{CD4}^+$  T cell stimulations. 1 wk after stimulation,  $\text{CD4}^+$  T cells were used as responders against T2.DR4 target cells pulsed with the candidate DR4-binding peptides. As shown in Fig. 1, an analysis of 10 normal HLA-DR4<sup>+</sup> donors revealed either very low responsiveness or responses that were equivocal with regard to their balance of IFN- $\gamma$  versus IL-5 spot production. Although

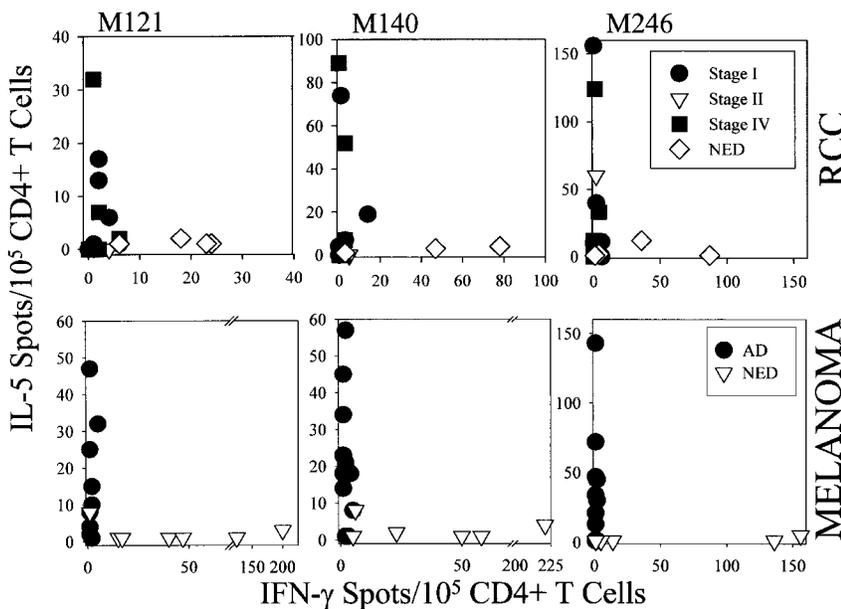


**Figure 1.** Analysis of the Th1-type vs. Th2-type CD4<sup>+</sup> T cell response to MAGE-6 peptides in HLA-DRβ1\*0401<sup>+</sup> normal donors. Peripheral blood CD4<sup>+</sup> T cells were isolated from normal donors and stimulated with autologous, “immature” DCs in the presence of the MAGE-6<sub>121-144</sub> (M121), MAGE-6<sub>140-170</sub> (M140), or MAGE-6<sub>246-263</sub> (M246) peptides for 7 d, in the absence of exogenous cytokines. Responder CD4<sup>+</sup> T cells were then analyzed for their reactivity to T2.DR4 cells pulsed with individual MAGE-6 peptides in both IFN-γ and IL-5 ELISPOT assays. Each symbol within a panel represents the combined IFN-γ/IL-5 ELISPOT data for an individual normal donor.

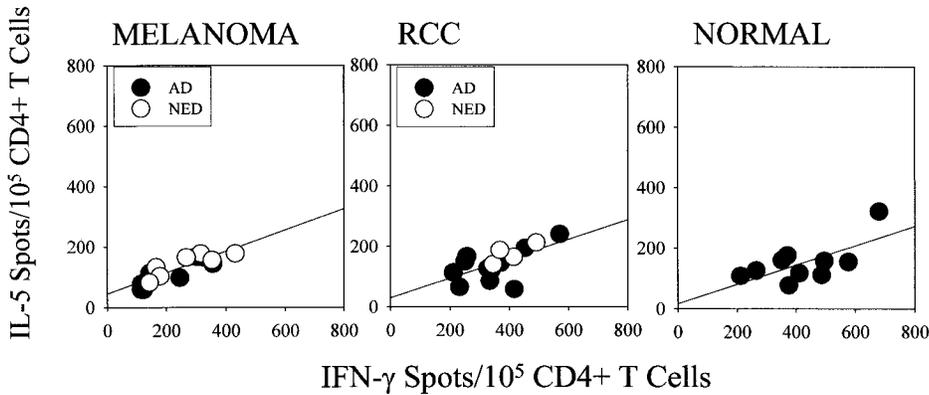
we did not perform these analyses in a manner that allowed for an assessment of coordinate cytokine production from a given CD4<sup>+</sup> T cell, we considered these results from normal donors to reflect Th0-type or mixed Th1-type/Th2-type responses.

*Th1-type versus Th2-type Immunoreactivity of CD4<sup>+</sup> T Cells Against HLA-DR4-Presented MAGE-6 Epitopes in HLA-DR4<sup>+</sup> RCC or Melanoma Patients.* Recent reports have suggested that CD4<sup>+</sup> T cells infiltrating progressor RCC and melanoma may display a predominant Th2-bias in response to TCR ligation (41, 42). We used the IFN-γ (Th1-type) and IL-5 (Th2-type) ELISPOT assays to discern whether such a bias existed in the peripheral blood CD4<sup>+</sup> T cell repertoire of HLA-DRβ1\*0401<sup>+</sup> patients with RCC or melanoma using the identical IVS system outlined above for normal donors.

Overall, the peripheral blood CD4<sup>+</sup> T cell responses were evaluated from 18 RCC and 18 melanoma patients (Table I). Among these patients, 4/18 RCC patients and 7/18 melanoma patients were disease-free (i.e., no-evidence of disease [NED]) at the time of analysis, with all other patients presenting with active disease. As shown in Fig. 2, patients with active disease (either RCC or melanoma) displayed strongly Th2-polarized CD4<sup>+</sup> T cell responses, whereas patients that were disease-free at the time of analysis were strongly Th1-polarized in their reactivity to the three MAGE-6 epitopes evaluated. Not every patient reacted against each of the peptides tested, but if they did respond to a given epitope, this response was strongly polarized in accordance with the disease status of the individual (i.e., Th2 if active disease, etc., Table I). Whereas the melanoma patients were all essentially diagnosed with



**Figure 2.** Analysis of the Th1-type vs. Th2-type CD4<sup>+</sup> T cell response to MAGE-6 peptides in HLA-DRβ1\*0401<sup>+</sup> patients with RCC or melanoma. Peripheral blood CD4<sup>+</sup> T cells were isolated from patients with RCC or melanoma and stimulated for 7 d with autologous “immature” plus MAGE-6 peptides as described in the legend to Fig. 1. Responder CD4<sup>+</sup> T cells were then analyzed for their reactivity to T2.DR4 cells pulsed with individual MAGE-6 peptides in both IFN-γ and IL-5 ELISPOT assays. Each symbol within a panel represents the combined IFN-γ/IL-5 ELISPOT data for an individual patient, with filled circles indicating patients with active disease and open inverted triangles reflecting patients that were free of disease at the time of evaluation, as described in Table I.



**Figure 3.** Patient CD4<sup>+</sup> T cells from IVS cultures exhibit normal, mixed Th1/Th2 responses to PHA mitogenic stimulation regardless of disease status. CD4<sup>+</sup> T cell cultures obtained from IVS outlined in Figs. 1 and 2 were analyzed for their response to PHA mitogen (5 μg/ml). Each symbol represents the combined IFN-γ/IL-5 ELISPOT data for an individual, with melanoma or RCC patients with active disease denoted by filled circles, and patients with no evidence of disease indicated by open circles.

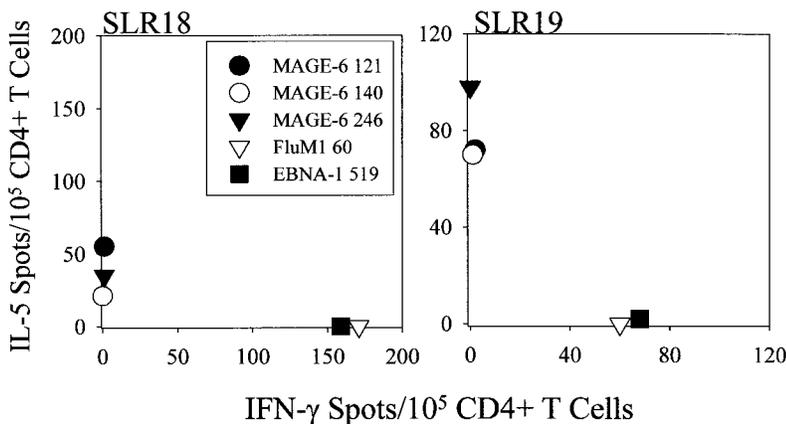
stage IV disease (with the exception of patients SLM6, SLM9, and SLM23), the RCC patients were approximately equally represented by individuals with stage I or stage IV disease (with patient SLR17 exhibiting stage II disease). A comparison of whether the observed Th2-polarization in CD4<sup>+</sup> T cell response to MAGE-6 peptides (Fig. 2, top series) was correlated with disease stage in RCC patients, provided no statistically significant associations (stage I versus stage IV for MAGE-6<sub>121-144</sub> [P = 0.89]; for MAGE-6<sub>140-170</sub> [P = 0.27]; for MAGE-6<sub>246-263</sub> [P = 0.50]).

Patients with active disease were not predisposed to a general Th2-type polarization in their CD4<sup>+</sup> T cell responses. An evaluation of CD4<sup>+</sup> T cells from the patients with melanoma or RCC, or normal donors revealed mixed Th1-/Th2-type responses to PHA mitogenic stimulation in IFN-γ and IL-5 ELISPOT assays (Fig. 3). As groups, patients with active disease, patients with no evidence of disease and normal donors proved indistinguishable in their bulk CD4<sup>+</sup> T cell responses to mitogen. Using a series of newly defined HLA-DR4-presented helper epitopes defined from the influenza virus matrix protein (FluM1<sub>60-73</sub>; reference 35) and EBV EBNA-1 (EBNA-1<sub>519-533</sub>; reference 36), we were able to evaluate CD4<sup>+</sup> T cell responses in patients SLR18 and SLR19 who each presented with active disease at the time of analysis (Fig. 4). In each case, strongly Th2-type biased CD4<sup>+</sup> T cell reactivity was noted against

the three MAGE-6 epitopes, with concurrent Th1-type polarized CD4<sup>+</sup> T cell antiviral responses.

*Patients with RCC or Melanoma Do Not Exhibit Th3-/Tr-type CD4<sup>+</sup> T Cell Responses to MAGE-6 Epitopes.* Although no ELISPOT is currently available to evaluate TGF-β production, an index for the bioactivity of the Tr-type CD4<sup>+</sup> T cells, we analyzed the supernatant from the peptide-stimulated ELISPOT wells for TGF-β levels using a cytokine specific ELISA assay. All supernatants were below the level of detection for secreted TGF-β (i.e., <60 pg/ml, unpublished data). We also evaluated these supernatants for the presence of IL-10 production and were unable to demonstrate peptide-specific secretion of this cytokine (i.e., <7 pg/ml, unpublished data). Based on direct comparison to a newly developed IL-10 ELISPOT assay, as few as 1–5 IL-10 secreting CD4<sup>+</sup> T cells (per 50,000) would have registered as 12–17 pg IL-10/ml in our ELISA assay (unpublished data). Hence we believe that these patients have, at best, very few (frequencies ≤1/50,000 CD4<sup>+</sup> T cells) in their peripheral blood.

Successful therapy associated with NED status is linked to a conversion of peripheral Th2-type to Th1-type CD4<sup>+</sup> T cell response to MAGE-6 epitopes. RCC patient SLR12 with stage I disease was surgically managed, resulting in disease-free status. Peripheral blood CD4<sup>+</sup> T cell responses were evaluated pre- and postsurgery in IFN-γ and IL-5 ELISPOT assays against the MAGE-6<sub>121-144</sub>, MAGE-



**Figure 4.** Peripheral blood CD4<sup>+</sup> T cells from patients SLM18 and SLM19 display Th2-type reactivity to MAGE-6 epitopes, but Th1-type reactivity to viral epitopes. CD4<sup>+</sup> T cells were stimulated with immature autologous DCs pulsed with the indicated peptides for 1 wk, as outlined in Figs. 1 and 2. Responder CD4<sup>+</sup> T cells were then analyzed for their reactivity to T2.DR4 cells pulsed with individual MAGE-6, influenza matrix, or EBV peptides in both IFN-γ and IL-5 ELISPOT assays.

6<sub>140–170</sub>, and MAGE-6<sub>246–263</sub> epitopes, as outlined above. Weak Th2-biased responses were noted against the MAGE-6<sub>121–144</sub> and MAGE-6<sub>246–263</sub> peptides before surgery, while weak, but Th1-biased responses against these epitopes were noted 1 mo postsurgery (Fig. 5 A).

Patient SLM1 with stage IV melanoma was treated with an autologous DC-based vaccine (UPCI 95–060) and achieved a complete response in March 1997. Peripheral blood CD4<sup>+</sup> T cells were isolated both pre- (8/96 and 3/97) and post- (4/97, 11/98) regression of disease. Patient SLM1 was disease-free at the time of both postregression time points. As shown in Fig. 5 B, this patient reacted to all three MAGE-6 epitopes in a strongly Th2-biased manner before regression, but displayed only Th1-type reactivity after the tumor burden was clinically eradicated.

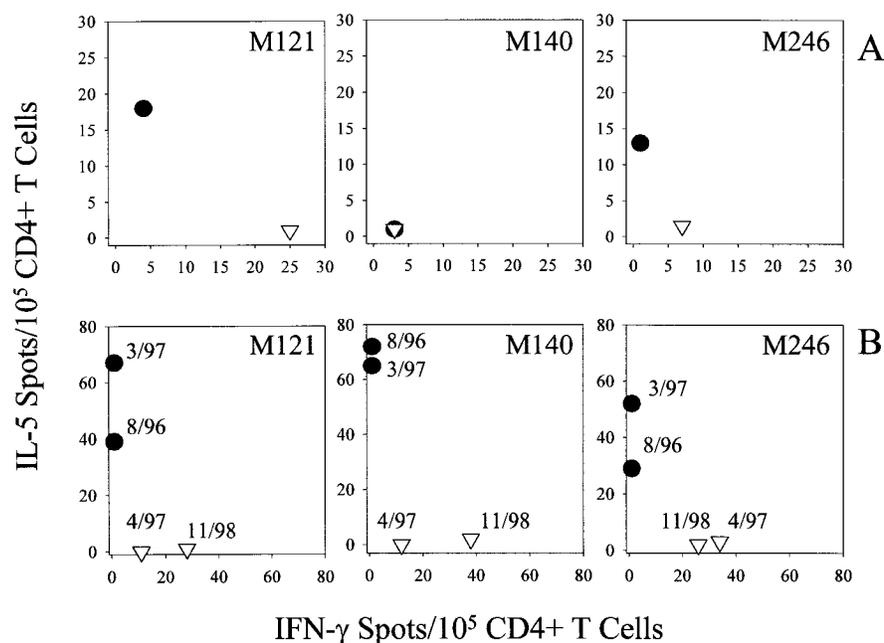
## Discussion

We analyzed peripheral blood T cells harvested from HLA-DRβ1\*0401<sup>+</sup> normal donors and patients with RCC or melanoma for the magnitude and nature of CD4<sup>+</sup> T cell responses to 3 MAGE-6 epitopes that we have recently identified as HLA-DRβ1\*0401-presented epitopes (unpublished data). We observed a dominance of Th2-type (and a frequent lack of any Th1-type) CD4<sup>+</sup> T cell responses to these MAGE-6 epitopes in RCC and melanoma patients with active disease (Fig. 2). In marked contrast, normal donors and patients that had been successfully treated and were disease free at the time of analysis, displayed either mixed Th1-/Th2-type or strongly Th1-polarized immunity to these same peptides. It should be stressed that these polarized CD4<sup>+</sup> T cell responses are specific for the tumor peptides tested and do not reflect the general tendency of the donor to respond in a generically Th2- (or Th1-) biased fashion, as the mitogen (PHA) con-

trol spot frequencies obtained for both the IL-5 and IFN-γ ELISPOT assays were indiscriminant between patients with cancer, patients that were free of disease, and normal donors (Fig. 3). In addition, for two RCC patients (SLR18 and SLR19) evaluated, Th2-type immunity to MAGE-6 peptides coexisted with strong Th1-type immunity to influenza- and EBV-derived helper epitopes (Fig. 4). Overall, although these data derive from relatively few patients, they suggest that Th2-type dominated CD4<sup>+</sup> T cell responses against MAGE-6 epitopes may correlate with active disease status in the patient.

However, when the results of RCC patients with stage I disease were compared with those of RCC patients with stage IV disease, we were unable to determine any significant linkage between the degree of Th2-polarization to MAGE-6 epitopes and disease-stage in patients with active disease. As MAGE-6 appears to represent an early tumor-associated antigen, observed in even premalignant lesions (29–31), our results may suggest that skewing of a normally mixed Th1-/Th2-type MAGE-6-specific CD4<sup>+</sup> T cell responses toward Th2-dominated immunity may also be an early event in disease progression. Such polarization could result from chronic antigenic restimulation *in situ* throughout disease ontogeny, and could be initiated even in individuals with premalignant MAGE-6<sup>+</sup> lesions, potentially serving as a facilitator of disease progression.

Clearly, far more extensive longitudinal studies will be required to determine the prognostic significance of differential Th1- versus Th2-type in the progression, clearance, and/or recurrence of disease. Although we are now in the process of initiating these types of comprehensive studies at the UPMC and UPCI, our preliminary data provided in Fig. 5, suggests that in an RCC patient that was surgically managed and a melanoma patient that was treated with an autologous DC-based vaccine, that Th2-biased responses



**Figure 5.** Analysis of the Th1-type vs. Th2-type CD4<sup>+</sup> T cell response to MAGE-6 peptides in HLA-DRβ1\*0401<sup>+</sup> RCC and melanoma patients pre/posttherapy. Peripheral blood T cells were isolated from an RCC patient SLR12 (A) and melanoma patient SLM1 (B) pre- or posttherapy. In both cases, therapy resulted in disease-free status. In the case of patient SLR12, blood was drawn at the time of surgery (filled circle) and 2 mo postsurgery (open inverted triangle). Patient SLM1 was treated with a DC-based vaccine, resulting in a complete response in 3/97. Blood was drawn pretherapy and during therapy, but before regression (filled circles) and after complete regression (open inverted triangles). 7-d IVS were performed as outlined in Fig. 1, with responder CD4<sup>+</sup> T cells analyzed for their reactivity to T2.DR4 cells pulsed with individual MAGE-6 peptides in both IFN-γ and IL-5 ELISPOT assays.

to MAGE-6 epitopes shifted to Th1-biased response after the patient achieved disease-free status. Furthermore, based on preliminary MHC-peptide tetramer analysis, MAGE-6 reactive CD4<sup>+</sup> T cells were observed to increase in the peripheral blood, at least transiently, as soon as 1–2 mo after successful therapy (unpublished data), which may support the register of these helper T cell responses with clinical benefit.

An additional important consideration in our prospective analyses will be a careful comparison of systemic versus tumor-associated CD4<sup>+</sup> T cell response polarization to MAGE-6 epitopes. It would be hypothesized that the most dramatic polarizations and highest frequencies of non-Th1 polarized tumor antigen-specific CD4<sup>+</sup> T cells would be identified in the tumor microenvironment and tumor-draining lymph nodes. Although we have reported an essentially qualitative Th2-type bias in response to MAGE-6 peptides in the peripheral blood of patients with active disease, we have been thus far, unable to demonstrate systemic antigen-specific Th3/Tr-type CD4<sup>+</sup> T cell responses to these epitopes. This may suggest that these latter responses are rare-events in the patient, or alternatively, that they may be concentrated and best observed within the tumor-involved tissues of the patient.

An analysis of the data presented in Table I indicates that 12/15 (i.e., 80%) evaluable melanoma biopsies expressed the MAGE-6 antigen as deduced by RT-PCR analysis. In all 12 of these MAGE-6<sup>+</sup> patients, Th1-type or Th2-type CD4<sup>+</sup> T cell responses were detected by ELISPOT analysis against at least one of the three MAGE-6 epitopes analyzed in this study. In 2/3 cases where the patient's tumor failed to express the MAGE-6 gene product, the patient's CD4<sup>+</sup> T cells did not react to MAGE-6 epitopes. The resected tumor in patient SLM22, however, failed to express the MAGE-6 mRNA, yet Th2-type CD4<sup>+</sup> T cell responses were observed against all three MAGE-6-derived helper epitopes. This result may be due to MAGE-6 expression by nonresected metastatic lesions in this stage IV patient with active disease. Alternatively, the anti-MAGE-6 CD4<sup>+</sup> T cells may be reacting or cross-reacting against homologous epitopes derived from other MAGE-A family member proteins. An analysis of all MAGE-A family members suggests that this possibility would be most likely for the MAGE-6<sub>121–144</sub> peptide, which is identical to the homologous MAGE-3 sequence, but which differs in sequence at 3 or more key positions with all other MAGE-A members (unpublished data). This possibility is less likely for the MAGE-6<sub>140–170</sub> and MAGE-6<sub>246–263</sub> epitopes that differ from the homologous MAGE-3 sequence by two nonconservative D156S and Y249H substitutions within the putative core binding epitope (unpublished data). Additional nonconservative changes in these two epitope sequences are noted when comparing MAGE-6 to all other MAGE-A members. As corollary analyses, we will prospectively determine whether patient CD4<sup>+</sup> T cells isolated using specific HLA-DR4/MAGE-6 peptide tetramers recognize the homologous MAGE-A family sequences.

In these studies, we chose an *in vitro* induction assay us-

ing autologous immature monocyte-derived (i.e., myeloid) DCs that appeared not to skew the nature of the isolated CD4<sup>+</sup> T cell response to a given epitope. In preliminary studies, we analyzed freshly-isolated CD4<sup>+</sup> T cells from melanoma patients as the responders (arguably recall responses) to peptide-pulsed T2.DR4 targets in our ELISPOT assays and discerned the same cytokine (IFN versus IL-5) secretion bias, as we report after IVS. We systematically implemented the 7-d IVS (using immature myeloid DCs) protocol as this amplified the technically-detectable (ZEISS AutoImager) spot numbers in each of the assays, without changing the bias of cytokines produced. We feel that this is a reasonable amplification tool in the outlined work. As this report was designed to reflect, as closely as possible, the *in situ* peripheral repertoire, we have not attempted to delineate how other DC subsets or conditioning regimens alters the bias of the responder repertoire in the current manuscript. Our data, however, may suggest that immature DCs, such as have been implemented in a number of vaccine trials (43, 44), may be clinically-inferior to strong DC1-type cells (producing high quantities of IL-12; references 45 and 46) due to their comparative inability to promote strong Th1-biased immunity in the face of existing Th2-type responses.

So, why does the tumor-reactive CD4<sup>+</sup> T cell repertoire shift to a Th2-dominated phenotype *in situ* in cancer bearing patients? A number of nonmutually exclusive hypotheses have been proffered. These include: immune deviation via chronic antigenic stimulation and selective sensitivity of Th1-type CD4<sup>+</sup> T cells to activation-induced apoptosis (47), the promotion of DC2-type (i.e., Th2-type promoting) antigen-presenting cell function conditioned by tumor-secreted cytokines and chemokines (i.e., IL-10, TGF- $\beta$ , SDF-1; references 48 and 49), and the enforced repolarization of Th1 type-responses into Th2-type responses *in situ* (50), among others. Using MHC/MAGE-6 peptide tetramers, we anticipate the ability to assess the proapoptotic phenotype of specific CD4<sup>+</sup> T cells in patients with active disease versus those that have achieved NED status in future studies. Our current studies investigating CD4<sup>+</sup> T cell response to viral epitopes suggest that patient DCs do not exert a dominant DC2 functional phenotype *in vitro*. Clearly these important issues demand *in-tense* prospective evaluation.

Although we are currently analyzing DC-based vaccines that are capable of repolarizing Th2-type tumor-reactive CD4<sup>+</sup> T cells toward Th1-type immunity, this may not necessarily represent the clinically preferred modality for the treatment of existing disease. As previously mentioned, Th2-type tumor-specific CD4<sup>+</sup> T cells may well prove productive collaborators to Th1-type CD4<sup>+</sup> T cells in mediating tumor regression via different, but complementary mechanisms (9–12, 14, 20). This may be particularly important in the case of MHC class I-loss variant tumors that will be impervious to Th1-type CD4<sup>+</sup> T cell-sponsored cytotoxicity mediated by CD8<sup>+</sup> antitumor T cells (10, 11). Regardless of which underlying mechanism leads to tumor regression, it appears clear that strong Th1-type tumor-spe-

cific T cell responses will be important in the maintenance of durable cellular immunity (12) and extended disease-free intervals in those patients at high-risk for recurrence.

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