A Mycoplasma Peptide Elicits Heteroclitic CD4$^+$ T Cell Responses against Tumor Antigen MAGE-A6

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Abstract

Purpose: Although T-helper (Th) epitopes have been previously reported for many tumor antigens, including MAGE-A6, the relevant HLA-DR alleles that present these peptides are expressed by only a minority of patients. The identification of tumor antigenic epitopes presented promiscuously by many HLA-DR alleles would extend the clinical utility of these peptides in vaccines and for the immunomonitoring of cancer patients.

Experimental Design: A neural network algorithm and *in vitro* sensitization assays were employed to screen candidate peptides for their immunogenicity.

Results: The MAGE-A6$_{140\text{-}170}$, MAGE-A6$_{172\text{-}187}$, and MAGE-A6$_{280\text{-}302}$ epitopes were recognized by CD4$^+$ T cells isolated from the majority of normal donors and melanoma patients evaluated. Peptide-specific CD4$^+$ T cells also recognized autologous antigen-presenting cell pulsed with recombinant MAGE-A6 (rMAGE) protein, supporting the natural processing and MHC presentation of these epitopes. Given the strong primary *in vitro* sensitization of normal donor CD4$^+$ T cells by the MAGEA6$_{172\text{-}187}$ epitope, suggestive of potential cross-reactivity against an environmental stimulus, we identified a highly homologous peptide within the *Mycoplasma penetrans* HF-2 permease (MPHF2) protein. MPHF2 peptide – primed CD4$^+$ T cells cross-reacted against autologous APC pulsed with the MAGE-A6$_{172\text{-}187}$ peptide or rMAGE protein and recognized HLA-matched MAGE-A6$^+$ melanoma cell lines. These responses seemed heteroclitic in nature because the functional avidity of MPHF2 peptide-primed CD4$^+$ T cells for the MAGE-A6$_{172\text{-}187}$ peptide was ~1,000 times greater than that of CD4$^+$ T cells primed with the corresponding MAGE-A6 peptide.

Conclusions: We believe that these novel "promiscuous" MAGE-A6/MPHF2 Th epitopes may prove clinically useful in the treatment and/or monitoring of a high proportion of cancer patients.

Melanoma antigen gene (MAGE) proteins are a family of closely related molecules that were initially identified as tumor-associated antigens capable of being recognized by CTLs isolated from the peripheral blood of cancer patients (1). MAGE are classified as either type I (*MAGE-A*, *MAGE-B*, and *MAGE-C* genes located on the X chromosome) or type II (those that are located outside of the type I MAGE genomic cluster; refs. 2, 3). Among normal somatic tissues, type I MAGE proteins are selectively expressed in testicular cells (4). However, they can also be expressed in both premalignant and malignant lesions under conditions of DNA hypomethylation (5). The MAGE-A proteins, composed of 12 members (i.e., MAGE-A1 through MAGE-A12), are expressed by more than half of all human cancers (6). For instance, MAGE-A6 is expressed in more than 60% of melanomas (7), 30% of renal cell carcinomas (8), and by many other cancer types, such as breast, esophageal, head and neck, bladder, and lung carcinomas (7, 9 – 12). This wide range of expression among cancer types, as well as the limited of expression by normal tissues, makes the MAGE family members attractive targets in the design of cancer vaccines and immunotherapies.

Previous studies have shown that melanoma is among the most responsive cancers to immunotherapy (13, 14), making it a prototype for the development of antitumor vaccine models. Although most vaccine studies have focused on the effector CD8$^+$ T cell compartment of the anti-melanoma immune response as being most important for objective clinical responses, it is clear that antitumor CD4$^+$ T cell responses regulate the quality, magnitude, and durability of CD8$^+$ CTL immunity *in vivo* (15, 16). CD4$^+$ T cells have been shown to play a crucial role in the induction of effective cellular antitumor immune responses (16, 17), with type-1 CD4$^+$ T cells mediating delayed type hypersensitivity (DTH)-like responses that can facilitate the cross-presentation of tumor antigens by host APCs and consequent epitope spreading in the antitumor T cell repertoire (18). Furthermore, CD4$^+$ T cells may exhibit direct tumoricidal activity and inhibit tumor angiogenesis (19 – 22).

In the current study, we have identified three naturally processed and poly – HLA-DR presented MAGE-A6 – derived

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epitopes that are effective in eliciting Th1-type (i.e., IFN-γ) responses *in vitro* in the majority of normal donors and melanoma patients tested. Notably, the MAGE-A6$_{172-187}$ epitope was highly homologous to, and immunologically cross-reactive with, a peptide derived from the HF-2 permease protein (MPHF2) of the ubiquitous *Mycoplasma penetrans* bacterium. CD4$^+$ T cells stimulated *in vitro* with this microbial homologue recognized MAGE-A6 protein-loaded, autologous monocytes as well as MAGE-A6$^+$, HLA-DR–matched melanoma cell lines. Indeed, MPHF2 peptide-based stimulation yielded CD4$^+$ T cells exhibiting a higher functional avidity for target cells presenting the MAGE-A6$_{172-187}$ peptide than T cells evoked against the MAGE-A6 peptide itself. We believe that these MAGE-A6/MPHF2 Th epitopes may prove useful in the development of novel cancer vaccines or immunomonitoring strategies for patients harboring MAGE-A6$^+$ tumor lesions, without limiting patient accrual based on the required expression of a limited number of HLA haplotypes that are permissive for peptide presentation.

Materials and Methods

Cell lines. Cell lines used included the melanoma cell lines Mel526, SLM2, and UPCI-Mel 591.8, the SLR20 renal carcinoma cell line (23, 24), and T2.DR4, a human B × T cell hybrid cell line expressing HLA-DR4 class II molecules (8). Cell lines were cultured in T75 culture flasks (Costar), in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% HEPES, 1% L-glutamine, and 1% nonessential amino acids (all reagents from Invitrogen) in a humidified 37°C incubator under 5% CO_2 tension.

Isolation of patient and normal donor peripheral blood mononuclear cells. Peripheral blood was obtained from normal donors or melanoma patients by venipuncture with written consent, under an Institutional Review Board (IRB)–approved protocol. Blood was diluted 1:2 with PBS, applied to Ficoll-Hypaque gradients (Cellgro; Mediatech, Inc.), and centrifuged at 550 × *g* for 25 min at room temperature. Peripheral blood mononuclear cells (PBMC) were recovered from the buoyant interface and washed thrice with PBS to remove residual platelets and Ficoll-Hypaque.

HLA-DR typing. Donor HLA-DR alleles were identified by genotyping. DNA was extracted from PBMC using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's protocol, with consequent HLA-DR genotyping done using the Dynal Allset+SSP DR "low resolution" Kit (Dynal Inc.) with extracted DNA samples. The HLA-DR4$^+$ phenotype of PBMC or tumor cell lines was confirmed using flow-cytometric analysis employing the HLA-DR4–specific monoclonal antibody (mAb) 359-F10 (8, 23).

DC1 preparations. Type-1 polarized dendritic cells (DC1) were generated from CD14$^+$ MACs (MACS; Miltenyi Biotech)–isolated human monocyte precursors as previously described (25). Additional CD14$^+$ monocytes were cryopreserved at -80°C and used as antigen presenting cells in ELISPOT assays.

Synthetic peptides. The MAGE-A6 (GenBank accession no. AAA68875), *M. penetrans* HF-2 permease (GenBank accession no. NP_757962) and *Chlamydia muridarum Nigg* TC0097 (GenBank accession no. AAF_38977) proteins were analyzed using the net-based ProPred HLA-DR peptide-binding algorithm.[6] MAGE-A6 peptide sequences were then selected based on their predicted ability to bind the broadest repertoire of HLA-DR alleles. All peptides were synthesized using *N*-(9-fluorenyl)methoxycarbonyl (FMOC) chemistry by the University of Pittsburgh Cancer Institute's (UPCI) Peptide Synthesis Facility (Shared Resource). Peptides were >95% pure based on high-performance liquid chromatography (HPLC) and tandem mass spectrometry analyses done by the UPCI Protein Sequencing Facility (Shared Resource).

CD4$^+$ T cell isolation from PBMC and* in vitro *stimulation. Following monocyte separation, CD4$^+$ T cells were isolated from CD14^{neg} PBMC by magnetic cell sorting (MACS; Miltenyi Biotech), according to manufacturer's protocol and then cryopreserved until needed. To establish DC-T cell cultures, CD4$^+$ T cells were thawed at 37°C and washed in AIM-V medium (Life Technologies-Invitrogen) and then resuspended in T cell media [AIM-V supplemented with 5% human serum (Life Technologies)]. DC1s were incubated for 1 to 3 h in 1 mL of T cell media with or without MAGE-A6 or MPHF2 peptides (10 μg/mL) at 37°C and, after washing, were then cocultured with autologous CD4$^+$ lymphocytes at a 1:10 DC1-to-T cell ratio in T cell media for 11 days.

Generation of anti-MPHF2 and anti-FluM1$_{60-73}$ CD4$^+$ T cell clones. The CD4$^+$ T cell clones were obtained by limiting dilution as previously reported (26, 27) from bulk CD4$^+$ T cells isolated from HLA-DR4$^+$ normal donor 10 (N.D.10) who had been primed using autologous DC1 pulsed with the MPHF2 or FluM1 peptides. T cell clones were maintained *in vitro* by restimulation every 2 weeks.

ELISPOT assay. On day 11 of *in vitro* stimulation, the frequencies of peptide-specific CD4$^+$ T cell responders were measured using anti-human IFN-γ ELISPOT assays as previously described (8, 23). Tumor cells used in ELISPOT assays were pretreated with IFN-γ (1,000 units/mL) for 24 h to up-regulate MHC class II expression and then irradiated (100 Gy) to prevent their proliferation. CD4$^+$ T cells, along with autologous CD14$^+$ cells or HLA-DR–matched tumor cell lines, were added to ELISPOT wells at a 5:1 T cell/APC ratio. In antibody-blocking tests, APC were preincubated with 20 μg/mL of L243 HLA-DR blocking antibody (American Type Culture Collection) for 1 h at 37°C before loading in ELISPOT wells. Peptides or rMAGE-A6 were added at 10 μg/mL, except in titration experiments where peptide concentrations were varied between 0 and 30 μmol/L. ELISPOT plates were incubated at 37°C for 24 h (peptide and tumor recognition) or 48 h (protein responses), developed, and evaluated using an ImmunoSpot automatic plate reader (Cellular Technology Ltd.) as previously reported (8, 23). The number of peptide-specific CD4$^+$ T cell responders was always statistically compared with the background number of IFN-γ spots produced by T cells in response to APC pulsed with the malarial circumsporozooite CS$_{326-345}$ peptide (for peptide-based assays) or with the TOP10 processed bacterial lysate (for protein-based assays). Positive control wells contained T cells and 10 μg/mL phytohemagglutinin (PHA; Sigma-Aldrich).

Cytokine-secretion assay. The recognition of autologous DCs pulsed with peptides was also assessed by MACS IFN-γ secretion assays (Miltenyi Biotech) as previously described (28). Briefly, 10^5 CD4$^+$ T cell clones were incubated for 6 h at 37°C in the presence of an equal number of autologous DC pulsed with titrated doses of peptides (i.e., 50-0.5 μmol/L). The cells were then labeled for 5 min at 4°C with IFN-γ–specific high-affinity capture matrix per the manufacturer's protocol. After 45 min of incubation, the secreted cytokine was stained with IFN-γ detection antibody-FITC and anti–CD3-Per-CP (BD Biosciences). Cells were then washed and analyzed using a BD LSR II flow cytometer (BD Biosciences) and BD FACSDiva Software (BD Biosciences).

Cytotoxicity assay. A total of 20,000 melanoma cells were cocultured with MPHF2- or FluM1$_{60-73}$–specific CD4$^+$ T cell clones at 1:1, 1:5, and 1:10 tumor-to-T cell ratios in complete media containing 10% HuAB serum for 18 h. Cytotoxicity was measured using the Vybrant Apoptosis Assay Kit 13 (Invitrogen). The kit contains PO-PRO-1 nucleic acid stain that selectively passes through the plasma membranes of apoptotic cells and labels them with violet fluorescence; and 7-aminoactinomycin D (7AAD) a red-fluorescent DNA-selective dye that is membrane impermeant, but easily passes through the compromised plasma membranes of necrotic cells. Briefly, after coculture, tumor cells have been harvested by trypsinization and washed once in cold PBS. Cells were resuspended at 10^6/mL in 2.5 μmol/L PO-PRO-1

[6] http://www.imtech.res.in/raghava/propred/index.html

and 1 μg/mL 7AAD in PBS. Samples were kept on ice and analyzed 15 min after staining. Cell analysis was done on a BD LSR II flow cytometer (BD Biosciences) at 488 nm for 7AAD excitation and 405 nm for PO-PRO-1 excitation.

PCR. Cell lines were screened for MAGE-A6 expression by reverse transcription-PCR (RT-PCR), whereas *M. penetrans* HF-2 contamination was tested by PCR. For MAGE-A6 analysis, RNA was isolated from the cell lines using the RNeasy Tissue Kit (Qiagen) and cDNA prepared using the GeneAmpRNA PCR Kit (Applied Biosystems). MAGE-A6 transcripts were analyzed as previously described (8) using the following primer set forward: 5′-TGGAGGACCAGAGGCCCCC-3′; reverse: 5′-CAGGATGATTATCAGGAAGCCTGT-3′. *M. penetrans* HF-2 DNA contamination of cell lines was tested by PCR as previously described (29) using the primers forward: 5′-CATGCAAGTCGGAC-3′; reverse: 5′-AGCATTTCCTCTTC-3′. *M. penetrans* HF-2 bacteria were used as positive DNA control, as was the assessment for β-actin DNA using the primer set forward: 5′-GGCATCGTGATGGACTCCG-3′; reverse: 5′-GCTGGAAGGTGGACAGCGA-3′. The PCR reaction parameters consisted of an initial 3-min denaturation step at 94°C followed by 32 amplification cycles that consisted of denaturation at 94°C for 45 s, annealing at 68°C for 45 s, and extension at 72°C for 1 min. The final cycle was followed by an additional extension step at 72°C for 10 min.

rMAGE-A6 generation and Western blot analysis. Full-length MAGE-A6 cDNA was generated by RT-PCR using the primer set forward: 5′-TGGAGGACCAGAGGCCCCC-3′; reverse: 5′-AGGATGATTATCAGGAAGCCTGTC-3′. cDNA was isolated from the MAGE-A6$^+$ SLR20 renal carcinoma cell line (23) and inserted into the pBAD TOPO TA (Invitrogen) cloning vector and then amplified in TOP10 (Invitrogen) bacteria, according to the manufacturer's protocol. The sequence was confirmed using the sequencing primers provided in the pBAD TOPO TA Cloning Kit. Bacterial extracted poly–His-tagged recombinant MAGE-A6 (rMAGE) was purified using the BD Talon Purification Kit (BD Biosciences) according to the manufacturer's protocol. Non-transformed TOP10 bacteria were grown and processed in an identical manner as for rMAGE purification, with the processed elution fractions (TOP10) used as a negative control in ELISPOT readouts for immune response to rMAGE. Lipopolysaccharide levels for rMAGE and TOP10 control protein were tested using the QCL-1000 Kit (Bio Whittaker) and determined to be <3 ng/mL (data not shown). For Western blotting, lysates were generated from 5×10^6 cells and 40 μg total protein loaded per lane for separation using 10% SDS-PAGE (Bio-Rad Laboratories). Gel-separated proteins were then transferred onto Immobilon-P membranes (polyvinylidene fluoride microporous membrane; Millipore) and stained using the anti-MAGE antibody, 57B (kindly provided by Dr. G.C. Spagnoli, University Hospital of Basel, Basel, Switzerland; ref. 30) or isotype-matched control Ab (Sigma-Aldrich). Goat anti-mouse horseradish peroxidase–conjugated antibody (Sigma-Aldrich) was used as a detection antibody. After extensive washing with PBS supplemented with 0.05% Tween, Western Lightning Chemoluminescence Reagent Plus (Perkin-Elmer Life Sciences) was added, and the blot was developed by ECL chemiluminescence radiography (Kodak).

ELISA for patient serum immunoglobulin G reactive against rMAGE-A6. Recombinant MAGE-A6 protein was diluted in coating buffer (15 mmol/L Na_2CO_3, 30 mmol/L $NaHCO_3$ (pH, 9.6), with 0.02% NaN_3) to the concentration of 1 μg/mL and adsorbed to polypropylene flat-bottom 96-well plates (Corning-Costar) overnight at 4°C. Plates were washed with PBS and saturated overnight at 4°C by the addition of 200 μL per well PBS containing 2% bovine serum albumin (BSA). After the plates were washed, serum was diluted in PBS-2% BSA buffer and added to the plates (50 μL per well). After 2 h of incubation at room temperature, plates were washed, the secondary Ab [goat anti-human immunoglobulin G (IgG)-AP; Caltag Laboratories] was added (50 μL per well), and the plates were further incubated for 1 h at room temperature. Following two additional washes, 50 μL per well substrate solution (phosphatase substrate system; Kirkegaard & Perry Laboratories) were added, and plates were incubated for 25 min at room temperature and read immediately (MRX Microplate Reader; Dynatech). Sera were tested over a range of 4-fold dilutions as previously described (31).

Statistical analysis. Statistical comparisons were made using a two-tailed Student's *t* test, with a *P* value ≤0.05 considered significant.

Results

Selection and analysis of promiscuous MAGE-A6 Th epitopes. The MAGE-A6 protein sequence was subjected to a computer algorithm screen designed to identify peptides most likely to have "promiscuous" HLA-DR–binding tendencies. Four peptides were targeted for further analysis (Table 1). Although three of these peptides represent novel sequences (MAGE-A6$_{172\text{-}187}$, MAGE-A6$_{192\text{-}214}$, and MAGE-A6$_{280\text{-}302}$), we previously reported that the MAGE-A6$_{140\text{-}170}$ peptide contains an HLA-DR4–restricted Th epitope (23).

These four peptides were initially evaluated for their immunogenicity *in vitro* using normal donor and melanoma patient CD4$^+$ T cells as responders and autologous type 1 polarized DCs (i.e., DC1; ref. 25) as stimulator cells. A single round of *in vitro* stimulation was employed to amplify recall Th responses to these epitopes and to limit the priming of naïve CD4$^+$ T cells, with IFN-γ ELISPOT assays done to enumerate the resultant peptide-specific CD4$^+$ T cell responses in an initial assessment of 14 melanoma patients and 7 normal donors. A summary of donor characteristics and their serum antibody/T cell responses to MAGE-A6 protein/peptides are provided in Table 2 and Fig. 1, respectively. Peptide-specific responses were observed in both melanoma patients and, at typically lower frequencies, in normal donors. Melanoma patients displayed variable reactivity against each of the four peptides tested. The MAGE-A6$_{280\text{-}302}$ peptide was associated with the most frequent CD4$^+$ T cell responses among the patients evaluated, with 9/14 patients evaluated reacting against this sequence (mean response [MR] = 50 specific spots/10^5 CD4$^+$ T cell evaluated) in a statistically significant manner. The MAGE-A6$_{140\text{-}170}$ (5/13 patients reactive; MR = 58.4 specific spots per 10^5 CD4$^+$ T cell evaluated) and MAGE-A6$_{172\text{-}187}$ (7/13 patients reactive; MR = 67 specific spots per 10^5 CD4$^+$ T cell evaluated) peptides were also commonly immunostimulatory, whereas the MAGE-A6$_{192\text{-}214}$ (2/14 patients reactive; MR = 22 specific spots

Table 1. Predicted poly-HLA-DR–binding peptides derived from MAGE-A6

Peptide	Sequence	HLA-DR alleles predicted to bind peptide (%)
MAGE-A6$_{140\text{-}170}$	VGN**W**QYFFPVIFSKASDS**L**QLVFGIELMEVD	DRB1*01, *03, *04, *07, *13, *15; DRB5*01; (80)
MAGE-A6$_{172\text{-}187}$	IGH**VY**IFATCLGLSYD	DRB1*01, *04, *07, *08, *11, *13, *15; DRB5*01; (80)
MAGE-A6$_{192\text{-}214}$	DNQ**I**MPKTGF**L**IILAIIAKEGD	DRB1*01, *03, *04, *07, *08, *11, *13, *15; DRB5*01; (84)
MAGE-A6$_{280\text{-}302}$	ETS**Y**VKVLHHM**V**KISGGPRISYP	DRB1*01, *03, *07, *08, *11, *13, *15; DRB5*01; (78)

Table 2. Normal donor and patient characteristics

Donor	Age/Sex	HLA-DR genotype	Serum IgG* anti – MAGE-A6	CD4 T cells † anti – MAGE-A6	Stage	Status	Treatment received
N.D.01	49/M	07, 13	-	+	-	-	-
N.D.02	64/M	07, 16	-	-	-	-	-
N.D.03	35/M	11, 13	-	+	-	-	-
N.D.04	17/F	03, 15	-	-	-	-	-
N.D.05	30/F	13	-	+	-	-	-
N.D.06	44/M	07, 13	-	+	-	-	-
N.D.07	41/F	07, 15	-	+	-	-	-
N.D.08	42/F	15, 16	-	+	-	-	-
N.D.09	48/M	07	-	-	-	-	-
N.D.10	47/M	04, 15	-	+	-	-	-
Mel01	42/M	01, 11	-	-	II	Met	S, I
Mel02	62/M	03, 07	+	+	IV	Met	S, I, C
Mel03	80/M	01, 04	+	+	I	NED	S
Mel04	69/M	03, 13	-	+	III	Met	S, I
Mel05	37/F	01, 07	++	+	III	Met	S, R, I
Mel06	75/M	13, 14	+	-	II	NED	S
Mel07	69/M	03, 07	-	+	II	NED	S
Mel08	75/M	01, 07	+	+	I	NED	S
Mel09	75/F	04, 15	-	+	IV	NED	S, R
Mel10	58/F	07	-	+	III	NED	S
Mel11	30/M	NT	+	+	IV	Met	S, C
Mel12	34/F	13, 15	-	+	IV	Met	S, I, R
Mel13	63/M	07, 13	++	+	IV	Met	S, I, C
Mel14	32/F	01, 11	NT	-	IV	Met	S
Mel15	67/F	03, 09	+	+	IV	Met	S
Mel16	45/M	15, 17	-	+	IV	Met	S
Mel17	54/F	01, 13	-	+	IV	NED	S
Mel18	31/M	01, 04	+	+	IV	Met	S
Mel19	52/F	11, 13	-	-	II	Met	S, C
Mel20	38/F	11, 13	NT	-	IV	Met	S, R
Mel21	45/F	04	NT	+	I	NED	S

Abbreviations: C, chemotherapy; I, immunotherapy; Mel, melanoma patient; Met, metastatic disease; N.D., normal donor; NED, no evidence of disease at time of blood draw; NT, not tested; R, radiotherapy; S, surgery.

*Serum was evaluated for levels of anti – MAGE-A6 IgG using an ELISA-based protocol described in Materials and Methods. In these assays, a negative (-) or positive (+) Ab response was assigned if the ELISA value obtained from a 1/400 dilution of patient sera did not, or did (respectively), exceed the mean + 3 × SD ELISA value obtained for a 1/400 dilution of sera exceeded from the 10 normal donors. If the patients' ELISA value for a 1/1600 dilution also exceeded this normal donor value (i.e., mean + 3 × SD ELISA value obtained for a 1/400 dilution of sera), a double-plus (++) value was assigned.

†Statistically significant $CD4^+$ T cell response (IFN-γ ELISPOT) against one or more MAGE-A6 – derived peptides ($P < 0.05$ versus CS peptide response).

per 10^5 $CD4^+$ T cell evaluated) epitope exhibited the poorest overall immunogenicity (Fig. 1A). Similarly, in normal donors (Fig. 1B), the MAGE-$A6_{280-302}$ peptide yielded the strongest and most frequent responses (5/7 normal donors reactive; MR = 147 specific spots per 10^5 $CD4^+$ T cell evaluated), whereas the MAGE-$A6_{172-187}$ peptide was the second most stimulatory peptide (3/7 normal donors reactive; MR = 108 specific spots per 10^5 $CD4^+$ T cell evaluated) among the donors evaluated. The MAGE-$A6_{140-170}$ (1/7 normal donors reactive; MR = 50 specific spots per 10^5 $CD4^+$ T cell evaluated) and MAGE-$A6_{192-214}$ (1/7 normal donors reactive; MR = 53 specific spots per 10^5 $CD4^+$ T cell evaluated) epitopes were less effective in promoting specific immune responses. Overall, 11/14 melanoma patients (i.e., with the exception of patients Mel01, Mel06, and Mel14) and 5/7 normal donors (except for N.D.02 and N.D.04) evaluated in these early studies were responsive against at least one of these peptides following a single round of *in vitro* stimulation. In most cases, a patient exhibiting anti – MAGE-A6 $CD4^+$ T cell responses also displayed detectable levels of serum anti – MAGE-A6 IgG antibodies (Table 2).

Recognition of naturally processed MAGE-A6 epitopes by peptide-stimulated $CD4^+$ T cells. Although our preliminary data suggest that the selected MAGE-A6 epitopes could stimulate specific $CD4^+$ T cell responses *in vitro* from the majority of (randomly selected) donors, this does not prove that these peptides are naturally processed and HLA presented. Hence, to provide support for the physiological relevance for these epitopes, we first constructed, produced, and purified recombinant MAGE-A6 (rMAGE; Fig. 1C) as outlined in Materials and Methods and then analyzed whether MAGE-A6 peptide-stimulated T cells could recognize autologous $CD14^+$ monocytes loaded *in vitro* with rMAGE using IFN-γ ELISPOT assays as a readout system (Fig. 1D). As controls, T cells were assessed for reactivity against monocytes loaded with either the relevant MAGE-A6 or irrelevant CS peptides or the TOP10 processed bacterial lysate as negative control for rMAGE protein. An evaluation of peptide-primed $CD4^+$ T cells generated from melanoma patients supported the conclusion that each of these peptides contained epitopes tested that were naturally processed and presented by autologous monocytes.

A representative experiment is shown in Fig. 1D, where after one round of *in vitro* stimulation with individual peptides, responder CD4$^+$ T cells isolated from patient Mel13 recognized autologous APC pulsed with either the relevant MAGE-A6 peptide or rMAGE protein.

Recognition of poly-DR presented MAGE-A6 epitopes by normal donors and potential cross-reactivity against environmental pathogens. We noted that 5/7 normal donors (in Fig. 1B) were able to mount significant Th1-type responses against the MAGE-A6$_{172\text{-}187}$ and/or MAGE-A6$_{280\text{-}302}$ peptides after a single round of *in vitro* stimulation. One possible explanation for this finding is that some normal donors harbor premalignant MAGE-A6$^+$ lesions because MAGE antigens can be expressed in such tissues (32–34). However, we believe it highly unlikely that such a large frequency of donors would be impacted in this manner. An alternative possibility is that the MAGE-A6 peptides evaluated bare sufficient sequence or conformational homologies to proteins present in the environment to which many individuals may have become naturally primed against, allowing for functional cross-reactivity to be detected in our assays. This type of phenomenon has been previously suggested for the HLA-A2–presented MART-1$_{27\text{-}35}$ epitope recognized by CD8$^+$ T cells in patients with melanoma (35).

Although conformational epitope mimics are not simply predicted or easily evaluated, we were able to perform sequence homology searches of the GenBank database for potential sources of cross-reactive linear epitopes. These screens suggested that the MAGE-A6$_{172\text{-}187}$ and MAGE-A6$_{280\text{-}302}$ (but perhaps not the MAGE-A6$_{140\text{-}170}$ or MAGE-A6$_{192\text{-}214}$) epitopes exhibited significant homologies to known microbial sequences. Indeed, we noted that the VYIFATCL octamer within the MAGE-A6$_{172\text{-}187}$ sequence was similar to a peptide (i.e., the HF-2$_{219\text{-}226}$; MPHF2; IYIFAACL) derived from the HF-2 permease protein of *M. penetrans* (a common opportunistic human pathogen associated with pneumonia; ref. 36). The MAGE-A6 peptide contains conservative V1I and A5T positional amino acid substitutions when compared with the pathogen homologue. Similarly, the VLHHMVKI octamer with the MAGE-A6$_{280\text{-}302}$ sequence was highly homologous to a peptide (RVLHEMVKI) derived from the *C. muridarum Nigg* (a rodent tropic strain; ref. 37) conserved

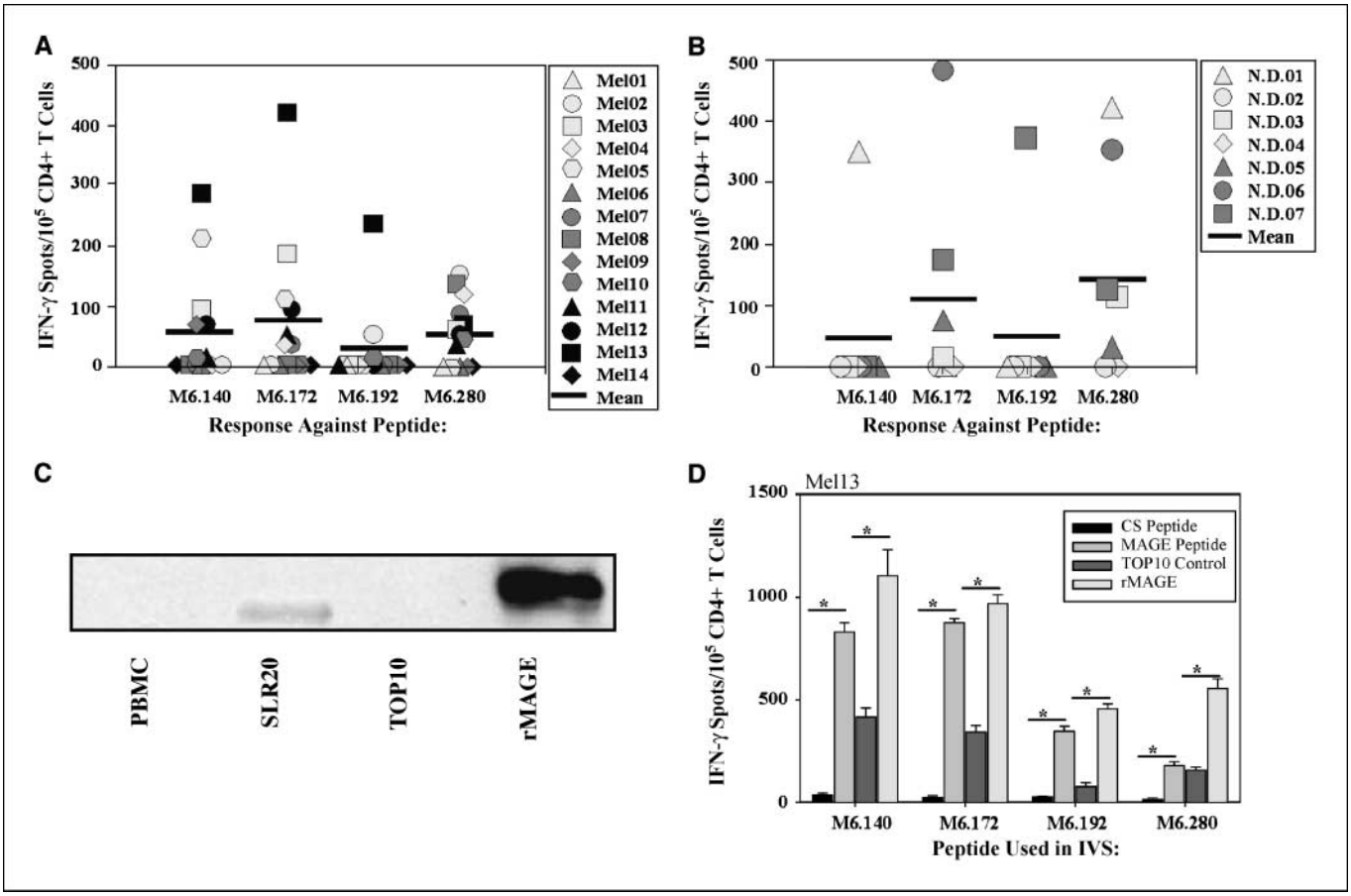

Fig. 1. Type-1 CD4$^+$ T cell responses against MAGE-A6 peptides predicted to be promiscuously presented by HLA-DR alleles and naturally processed rMAGE epitopes. CD4$^+$ T cells were isolated from the indicated (*A*) 14 melanoma patients and (*B*) 7 normal donors and tested for their ability to be stimulated by, and react against, the indicated MAGE-A6 peptides. CD4$^+$ T cells were stimulated once *in vitro* using autologous DC1 pulsed with 10 μg/mL MAGE-A6 peptide for 11 days. Responder T cells were assessed for their functional reactivity in IFN-γ ELISPOT assays against autologous monocytes pulsed with 10 μg/mL of control (CS$_{326\text{-}345}$) or relevant (M6.140, MAGE-A6$_{140\text{-}170}$; M6.172, MAGE-A6$_{172\text{-}187}$; M6.192, MAGE-A6$_{192\text{-}214}$; M6.280, MAGE-A6$_{280\text{-}302}$) peptides. *C,* Western blotting using the anti-MAGE mAb 57B was done against rMAGE or the following controls: normal donor PBMC (MAGE-A6$^-$) lysate; SLR20 renal carcinoma cell line (MAGE-A6$^+$) lysate and TOP10 (MAGE-A6$^-$) bacterial lysate. *D,* IFN-γ ELISPOT assays were done on *in vitro* stimulation (peptide)-primed CD4$^+$ T cells isolated from seven melanoma patients to assess their functional reactivity against rMAGE-pulsed autologous monocytes (a representative experiment for patient Mel13 is shown). Controls included monocytes pulsed with the indicated relevant (MAGE-A6) or irrelevant (CS) peptides, and the TOP10 protein (negative control for rMAGE). *, significant responses ($P < 0.05$ for rMAGE versus TOP10 or MAGE-A6 peptide versus CS peptide, data not shown). Abbreviations used: N.D., normal donor; Mel, melanoma patient.

hypothetical protein TC0097 (CHP-TC0097). When these microbial core peptides were expanded to include three native (from the protein of origin) amino acids on each flank and then analyzed using the ProPred HLA-DR peptide-binding algorithm, they were predicted to bind a wide range of HLA-DR alleles, similar to their MAGE-A6 homologues (data not shown). Subsequently, both microbial peptides were synthesized. Due to its high hydrophobicity index, the MPHF2 peptide-flanking regions were additionally modified to include diaminopropionic acid (Z) groups (i.e., similar to lysine but less bulky) to improve peptide solubility.

CD4$^+$ T cell responses to the MAGE-A6$_{172-187}$ and the MPHF2 homologue peptide are immunologically related. We next evaluated the ability of these two microbial peptides to be recognized by MAGE-A6 peptide–primed CD4$^+$ T cells, as well as to serve as immunogens capable of priming CD4$^+$ T cells capable of cross-reacting against the homologous MAGE-A6 epitopes *in vitro* (Fig. 2). The resulting *in vitro* stimulation responder T cells were assessed for their reactivity against autologous monocytes pulsed with the priming peptide, its homologue peptide or rMAGE in IFN-γ ELISPOT assays. MAGE-A6$_{172-187}$ peptide-primed CD4$^+$ T cells derived from 2/6 patients recognized the stimulating peptide and also cross-reacted against the MPHF2 homologue peptide in a statistically significant manner (Fig. 2A). Similarly, CD4$^+$ T cells generated from 4/6 patients after stimulation with the MPHF2 peptide recognized the stimulating peptide, with half of these responders also cross-reacting against the MAGE-A6$_{172-187}$ homologue peptide. Interestingly, whereas CD4$^+$ T cells generated from patients stimulated with the MAGE-A6$_{172-187}$ epitope only modestly recognized naturally processed rMAGE protein, MPFH2-primed CD4$^+$ T cells from all six patients recognized autologous monocytes pulsed with rMAGE (Fig. 2A). In marked contrast, in the MAGE-A6$_{280-302}$/CHP peptide analyses that were done in parallel, we observed no evidence for peptide cross-recognition by CD4$^+$ T cells after peptide-based *in vitro* stimulation (Fig. 2B).

We extended our analysis of the comparative *in vitro* immunogenicity of the MAGE-A6$_{172-187}$ versus MPHF2 peptides by evaluating CD4$^+$ T cell responses in normal donors. We observed that in normal donor 08 (N.D.08), that MAGE-A6$_{172-187}$ peptide priming did not promote a specific CD4$^+$ T cell response *in vitro*, whereas MPHF2 peptide-stimulated CD4$^+$ T cells cross-reacted against both target peptides and the naturally processed rMAGE-derived epitope(s) presented by autologous monocytes in IFN-γ ELISPOT readout assays (Fig. 2C). By first isolating CD4$^+$ T cells bearing a naïve (i.e., CD45RA$^+$, CCR7$^+$) versus a memory (CD45RA$^-$, CCR7$^-$) from normal donors, we noted that the vast majority of MAGE-A6$_{172-187}$– and MPHF2-specific response after *in vitro* stimulation derives from the memory pool of circulating CD4$^+$ T cells (Fig. 2D), suggesting that these T cells had previously experienced antigenic challenge *in vivo*.

***MPHF2-stimulated CD4$^+$ T cells recognize HLA-DR–matched, MAGE-A6$^+$ melanoma cell lines* in vitro.** To further evaluate the potential physiological relevance of CD4$^+$ T cell priming against the MPHF2 epitope, we compared MAGE-A6$_{172-187}$ and MPHF2-stimulated CD4$^+$ T cells for their ability to recognize HLA-DR–matched, MAGE-A6$^+$ melanoma cell lines *in vitro*. In these experiments, peripheral blood CD4$^+$ T cells isolated from HLA-DR4$^+$ melanoma patient Mel21 were stimulated twice at weekly intervals with autologous DC1 pulsed with either the MAGE-A6$_{172-187}$ or MPHF2 peptides. Two HLA-DR4$^+$, MAGE-A6$^+$ (Fig. 3B) melanoma cell lines, SLM2 and Mel526.DR4 (Fig. 3A and B), were then used as targets for responder T cells in IFN-γ ELISPOT assays (Fig. 3C). Pan-DR mAb (L243) was also added to replicate wells as indicated to show the MHC class II–restricted nature of T cell responses. We observed that both populations of peptide-primed CD4$^+$ T cells recognized the two tumor cell lines in a manner that was partially blocked by the addition of mAb L243, with the MPHF2-stimulated CD4$^+$ T cells exhibiting a greater magnitude of response to tumor cell lines than T cells primed with the MAGE-A6$_{172-187}$ epitope (Fig. 3C). In contrast, the addition of anti–class I mAb W6/32 did not affect peptide-induced CD4$^+$ T cell recognition of either Mel526 or SLM2 target cells (Fig. 3C). To rule out the possibility that T cell recognition was due to specific *Mycoplasma* infection of the target cell lines, both melanoma cell lines were shown to be negative for *M. penetrans* contamination using a sensitive PCR method (Fig. 3D).

MPHF2-stimulated CD4$^+$ T cells exhibit a higher functional avidity for MAGE-A6$_{172-187}$ loaded target cells than T cells primed against the MAGE-A6 peptide itself. To determine whether the superior capacity of MPHF2 (versus MAGE-A6$_{172-187}$) peptide-primed CD4$^+$ T cells to recognize autologous APC pulsed with rMAGE-A6 protein (as well as HLA-matched, MAGE-A6$^+$ tumor cells) could be attributed to differences in T cell functional avidity, we compared the abilities of peptide-primed CD4$^+$ T cells generated from HLA-DR4$^+$ melanoma patient Mel21 to recognize titrated doses of the MAGE-A6 peptide pulsed onto T2.DR4 presenting cells in IFN-γ ELISPOT assays. CD4$^+$ T cells generated using the MAGE-A6 peptide recognized T2.DR4 cells only when pulsed with a relatively high concentration of peptide (with a half-maximal response associated with a peptide dose of 3-10 μmol/L), whereas MPHF2-stimulated cells recognized target cells pulsed with far (~1,000-fold) lower concentrations of peptide (Fig. 3E).

Anti-MPHF2 CD4$^+$ T cell clones were also established by limiting dilution from reactive bulk cultures generated from normal donor N.D.10. As depicted in Fig. 4A, HLA-DR4–restricted clone 9/9 recognized both the priming MPHF2 peptide and the MAGE-A6$_{172-187}$ peptide in a dose-dependent manner (but not the irrelevant MAGE-A6$_{192-214}$ epitope) based on IFN-γ secretion assays. Additionally, clone 9/9 T cells recognized the wild-type (non–Z-amino acid modified) MPHF2 peptide presented by T2.DR4 cells and MP bacteria-pulsed, autologous monocytes in an MHC class II–restricted manner (data not shown). Furthermore, the anti-MPHF2 CD4$^+$ T cell clone 9/9 (Fig. 4B), but not the anti-FluM1 CD4$^+$ T cell Clone 17/2 (Fig. 4C) specifically lysed the HLA-DR4–matched, MAGE-A6$^+$ melanoma cell line SLM2, but not HLA-DR4–negative melanoma cell line UPCI-Mel.591.8, at effector-to-target ratios as low as 1:1.

Discussion

Although a proportion of solid cancers, including (up to 50-70% of) melanoma, have been reported to constitutively express MHC class II molecules *in situ* (38), even those that do not can frequently be induced to express class II complexes *in vitro* and *in vivo* after treatment with IFN-γ (39, 40). Hence, periodic inflammatory responses (accompanied by IFN-γ

production or by provision of IFN-γ as a therapy) within the tumor microenvironment or tumor-draining lymph nodes have the potential to enforce tumor cells as relevant APCs for the priming or boosting of tumor antigen (such as MAGE-A6)–specific $CD4^+$ and $CD8^+$ T cells. Alternatively, or even more likely, migratory host professional APCs (such as class I^+, class II^+ DCs) may activate tumor-reactive $CD4^+$ and $CD8^+$ T cells in secondary lymphoid tissues after first acquiring antigens within the tumor microenvironment (41). In any case, the coordinate activation of type-1 $CD4^+$ T cell responses are viewed as a key variable to sustained levels of protective antitumor immunity mediated by $CD4^+$ T cells directly or via $CD8^+$ T cells that are supported by $CD4^+$ T cell help (16–18, 42, 43). Hence, the ability to survey and modulate type 1 antitumor $CD4^+$ T cells has become an important goal for optimizing cancer immunotherapeutic approaches.

Previous studies of peptide-specific, $CD4^+$ T cell responses against tumor antigens, including MAGE-A6 have been traditionally skewed toward an analysis of a single (i.e., HLA-DR4) or very limited set of HLA-DR restriction elements (23, 44). Given the extreme polymorphism among HLA-DR alleles, the translational utility of the epitopes defined to date would be limited to a modest cohort of 20% to 30% of patients (23). In the current study, we have attempted to circumvent this limitation by identifying peptides that are likely to bind to, and be presented by, as broad a range of HLA-DR alleles (i.e., poly-DR) as possible, thereby expanding the range of patients to which MAGE-A6–based therapies might be applied clinically.

Peripheral blood $CD4^+$ T cells were harvested from normal donors and patients with melanoma, stimulated with a single round of peptide-based *in vitro* stimulation, and evaluated for their ability to recognize MAGE-A6 peptides (selected to be promiscuously presented by HLA class II) in IFN-γ ELISPOT assays. Our data support the identification of three novel MAGE-A6 epitopes recognized by type 1 Th cells and

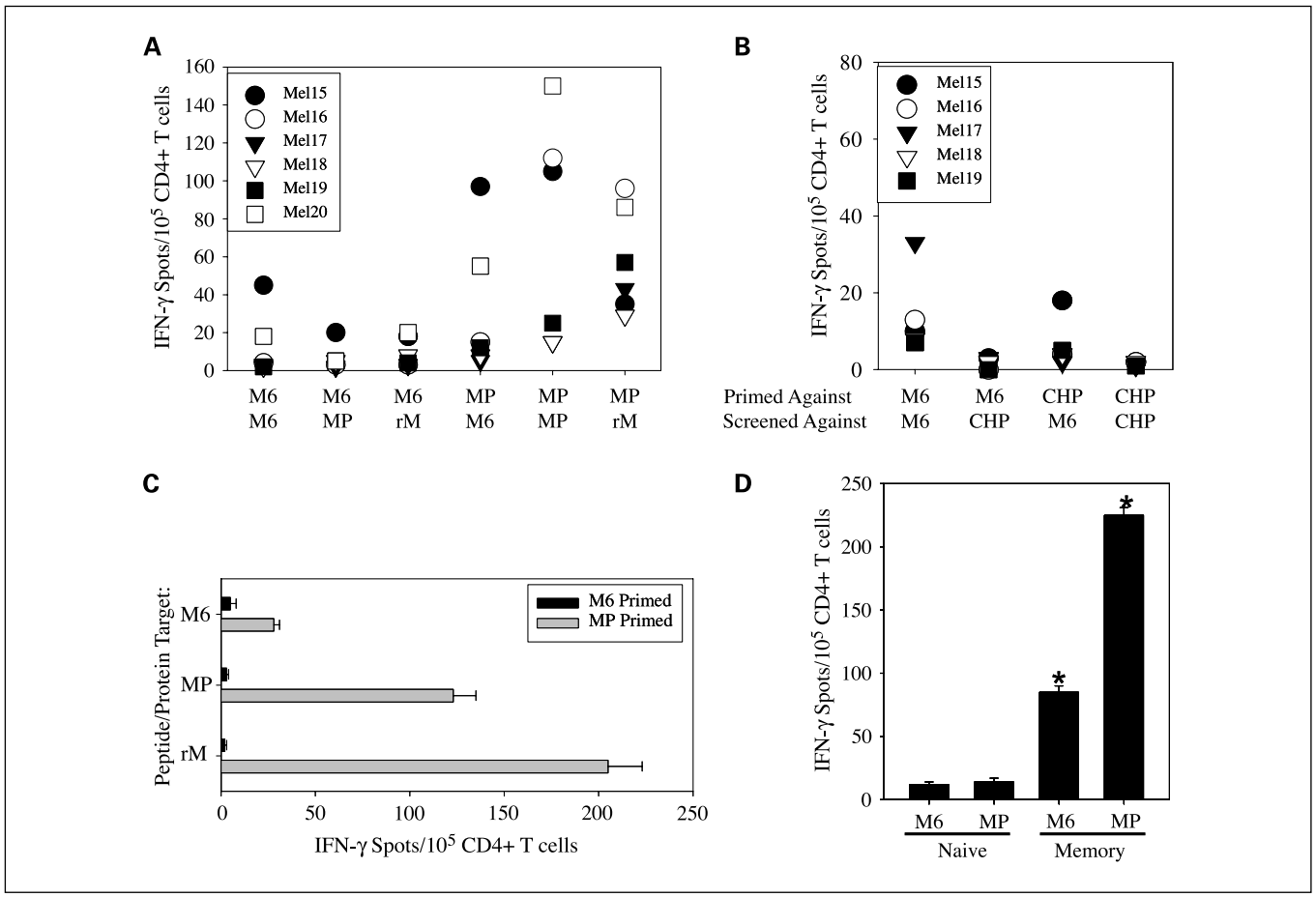

Fig. 2. Normal donor and melanoma patient $CD4^+$ T cell responses against MPHF2 and CHP peptides: evidence for cross-reactivity against MAGE-A6 epitopes. *A,* $CD4^+$ T cells isolated from the indicated melanoma patients were stimulated with autologous DC1 pulsed with either the MAGE-$A6_{172\text{-}187}$ (*M6*) or its homologue MPHF2 (*MP*) peptide, as outlined in the Fig. 1 caption. Responder $CD4^+$ T cells were then analyzed in IFN-γ ELISPOT assays for reactivity against autologous monocytes pulsed with 10 μg/mL of peptide (M6 or MP) or rMAGE protein (*rM*). Negative control values for the $CS_{326\text{-}345}$ peptide or TOP10 bacterial protein have been subtracted in each case. *B,* $CD4^+$ T cells isolated from the indicated melanoma patients were stimulated with autologous DC1 pulsed with either the MAGE-$A6_{280\text{-}302}$ (*M6*) or its homologue CHP peptide, as outlined in the Fig. 1 caption. Responder $CD4^+$ T cells were then analyzed in IFN-γ ELISPOT assays for reactivity against autologous monocytes pulsed with 10 μg/mL of peptide (*M6* or *CHP*). Negative control values for the $CS_{326\text{-}345}$ peptide or TOP10 bacterial protein have been subtracted in each case. *C,* $CD4^+$ T cells isolated from normal donors were stimulated as above using the individual MAGE-$A6_{172\text{-}187}$ (*M6*) or MPHF2 (*MP*) peptides and then analyzed for their reactivity against autologous monocytes pulsed with the indicated peptides or rMAGE protein (rM) in IFN-γ ELISPOT assays. The data displayed in (*B*) for normal donor 08 (N.D.08) are representative of three normal, unrelated donors analyzed. *D,* $CD4^+$ naïve ($CD45RA^+CCR7^+$) versus memory ($CD45RA^-CCR7^-$) T cells were isolated from normal donor 10 (N.D.10) PBMC and stimulated as in (*B*) and then analyzed in IFN-γ ELISPOT assays against the indicated target peptides presented by autologous monocytes. *, $P < 0.05$ versus CS controls (all <25 spots per 10^5 $CD4^+$ T cell responders). Results are reflective of data obtained using two independent normal donors.

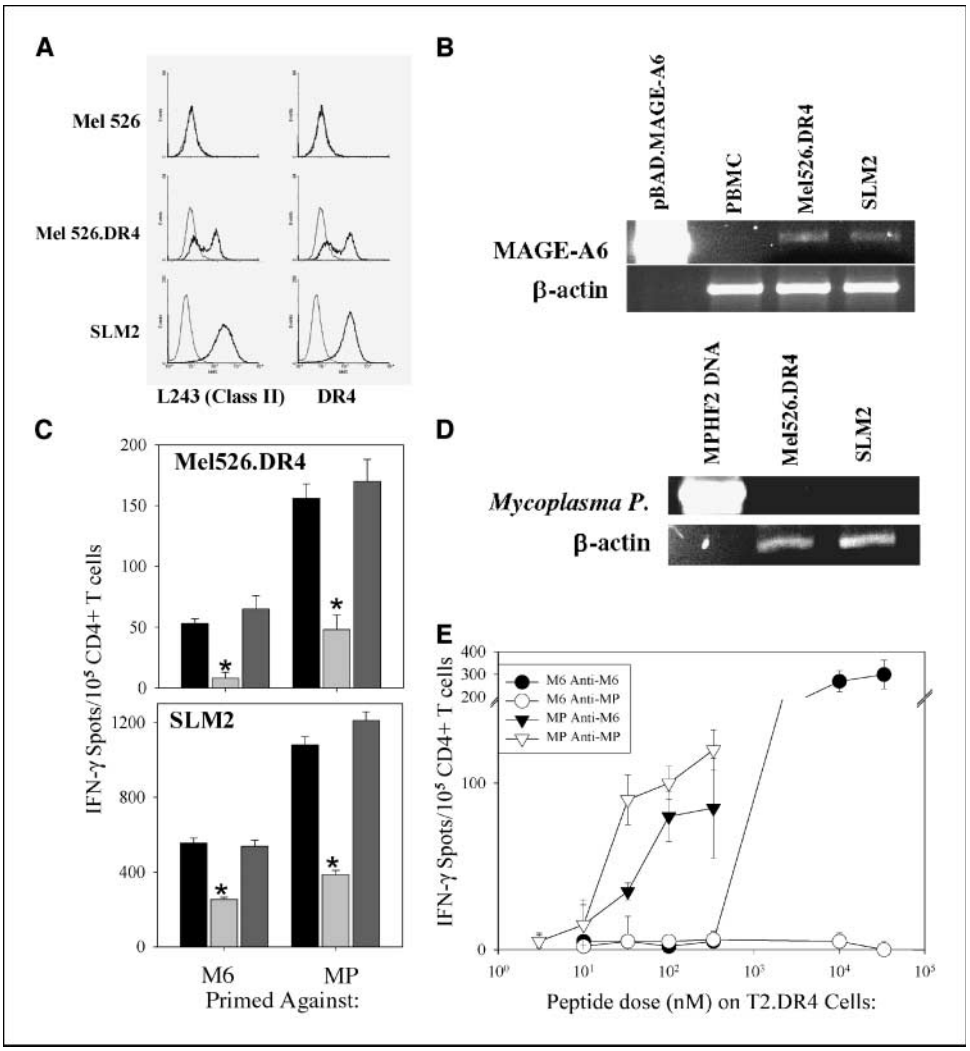

Fig. 3. MPHF2-primed $CD4^+$ T cells more effectively recognize HLA-matched, MAGE-$A6^+$ tumor cells than T cells primed *in vitro* against the homologous MAGE-$A6_{172\text{-}187}$ peptide. In (*A*), flow cytometry was used to assess the target cell lines used in these experiments (Mel526.DR4, SLM2), as well as the MHC class II – negative Mel526 cells for expression of pan-MHC class II (L243) and HLA-DR4 molecules. Thin line, IgG control; thick line, MHC class II or HLA-DR4 antibody. Although SLM2 cells spontaneously expressed HLA-DR4, the Mel526.DR4 cell line is HLA-$DR4^+$ as a consequence of infection with a retrovirus encoding the HLA-DRα/DRβ1*0401 cDNAs (37). Tumor cell lines were also evaluated by (*B*) RT-PCR for expression of MAGE-A6 mRNA expression and (*D*) by PCR for contamination by *M. penetrans*. In (*C*), $CD4^+$ T cells were isolated from an HLA-$DR4^+$ melanoma patient (Mel21) and stimulated with autologous DC1 pulsed with the MAGE-$A6_{172\text{-}187}$ (*M6*) or MPHF2 (*MP*) peptides as outlined in Fig. 1 caption, with responder T cells evaluated for functional reactivity against the Mel526.DR4 (*C, top*) and SLM2 (*C, bottom*) tumor cell lines in IFN-γ ELISPOT assays. L243 (anti – pan-class II mAb; 20 μg/mL; light-gray filled histograms), W6/32 (anti – pan-class I mAb; 20 μg/mL; dark-gray filled histograms), or no mAb (black filled histograms) were added to wells to confirm the class II – restricted nature of IFN-γ secretion by responder $CD4^+$ T cells. *, $P < 0.05$, all data for L243 versus no mAb; W6/32 versus no mAb, no significant difference. In (*E*), to test the functional avidity of MAGE-$A6_{172\text{-}187}$ (*M6*) and MPHF2 (*MP*) peptide – primed $CD4^+$ T cells, lymphocytes were evaluated for their ability to recognize titrated doses of the M6 or MP peptides pulsed onto the HLA-$DR4^+$ T2.DR4 – presenting cell line in IFN-γ ELISPOT assays (as described in Materials and Methods). MAGE-$A6_{172\text{-}187}$, MW = 1,728 g/mol (e.g., 10 μmol/L = 17.3 μg/mL); MPHF2, MW = 2,115 g/mol (e.g., 10 μmol/L = 21.2 μg/mL). All data are reflective of three independent assays done.

substantiate the ability of these peptides, along with the previously defined MAGE-$A6_{140\text{-}170}$ peptide, to be recognized by a large proportion of individuals (who overall exhibited a diverse array of HLA-DR haplotypes). Of the four peptides analyzed, the MAGE-$A6_{172\text{-}187}$ and MAGE-$A6_{280\text{-}302}$ peptides were recognized at the highest frequencies in peptide ELISPOT readouts by $CD4^+$ T cells. In contrast, the MAGE-$A6_{192\text{-}214}$ seemed comparatively non-immunogenic, and the MAGE-$A6_{140\text{-}170}$ peptide was moderately effective in this capacity. Based on the ability of peptide-primed $CD4^+$ T cells to recognize autologous monocytes pulsed with rMAGE protein, each of the peptides evaluated seem to contain naturally processed and presented Th epitopes.

These MAGE-A6 poly-DR epitopes displayed a high degree of homology with sequences contained in other MAGE-A family members, especially MAGE-A3. MAGE-$A6_{140\text{-}170}$, MAGEA$6_{172\text{-}187}$, and MAGE-$A6_{280\text{-}302}$ differed from their MAGE-A3 counterparts by only a single amino acid in each case. For MAGE-$A6_{140\text{-}170}$, this difference is at position 156, where a D → S substitution occurs in the MAGE-A3 protein. The MAGE-$A6_{172\text{-}187}$ peptide differs from its MAGE-A3 counterpart based on a conservative V175L substitution, and the MAGE-$A6_{280\text{-}302}$ to MAGE-A3 difference reflects an R298H substitution. Hence, whereas it remains to be formally evaluated, we believe that in many cases, the selected MAGE-A6 epitopes will likely elicit $CD4^+$ T cell responses in a high

frequency of patients that are capable of cross-reacting against their MAGE-A3 homologues when presented by autologous APCs. As a result, these peptides could represent promising candidates for inclusion in peptide-based vaccines designed to treat the majority of patients harboring tumors that exhibit MAGE-A6$^+$ and/or MAGE-A3$^+$ phenotypes *in situ*.

It was also noted in this study that CD4$^+$ T cell lines isolated from several normal donors were able to effectively recognize the MAGE-A6$_{172\text{-}187}$ and MAGE-A6$_{280\text{-}302}$ epitopes after a single round of *in vitro* stimulation. This observation could be attributed to epitope mimicry (45), a process believed to underlie the induction of autoimmune diseases due to pathologic T cells primed against infectious microorganisms that cross-react against host proteins in susceptible individuals. Diseases such as viral myocarditis, lyme disease, rheumatoid arthritis (45), multiple sclerosis (46), and virus-induced autoimmune diabetes (47, 48) have long been considered to be initiated or exacerbated by microbial pathogens. As was previously noted for the HLA-A2–presented, melanoma-associated MART-1$_{27\text{-}35}$ epitope (35), we hypothesized that the high degree of normal donor response against the MAGE-A6 Th peptides might be due to the cross-reactivity of T cells initially primed *in vivo* against highly homologous peptides within environmentally encountered proteins. After performing a homology search of the GenBank database, we selected the MPHF2$_{216\text{-}229}$ and CHP$_{42\text{-}55}$ peptides as two likely candidate homologues of the MAGE-A6$_{172\text{-}187}$ and MAGE-A6$_{280\text{-}302}$ peptides, respectively. The MPHF2 peptide derives from *M. penetrans* HF-2, a ubiquitous species of *Mycoplasmataceae*, which infects the urogenital and respiratory tracts of

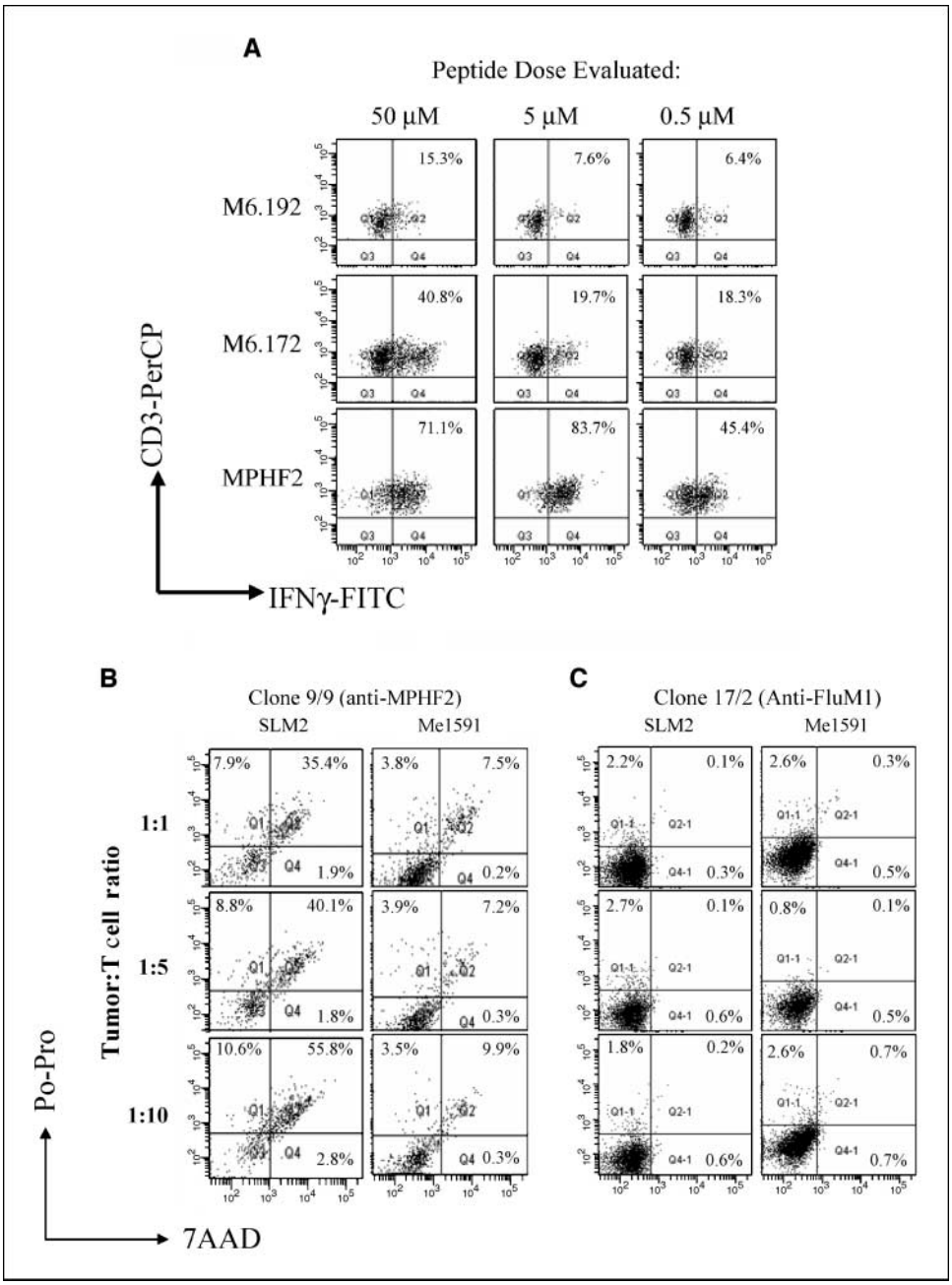

Fig. 4. The type-1 CD4$^+$ T cell clone 9/9 primed against the MPHF2 epitope recognizes the MAGE-A6$_{172\text{-}187}$ peptide in a dose-dependent manner and promotes the death of HLA-DR4$^+$ melanoma cell line SLM2 that constitutively expresses MAGE-A6 protein. The HLA-DR4–restricted, CD4$^+$ T cell clone 9/9 was obtained by limiting the dilution from the bulk culture of MPHF2 peptide-primed CD4$^+$ T cells isolated from HLA-DR4$^+$ normal donor 10 (N.D.10; Table 2) as described in Materials and Methods. In (*A*), clone 9/9 cells (10^5) were incubated with an equal number of autologous DC pre-pulsed with 50, 5, or 0.5 μg/mL of relevant (MPHF2 or MAGE-A6$_{172\text{-}187}$) or irrelevant MAGE-A6$_{192\text{-}214}$ peptide. IFN-γ production was then analyzed in 6-h cytokine secretion assays monitored by flow cytometry as described in Materials and Methods. In (*B*), the cytotoxic capacity and specificity of clone 9/9 was analyzed against the relevant HLA-DR4$^+$, MAGE-A6$^+$ melanoma cell line SLM2 versus the irrelevant HLA-DR4$^-$ UPCI-Mel 591.8 melanoma cell line. T cell–mediated cytotoxicity at the indicated tumor-to-T cell ratios was measured using the Vybrant Apoptosis Assay Kit as described in Materials and Methods, with results monitored by flow cytometry. In this assay, apoptotic tumor cell events are reflected in the top left quadrant, necrotic tumor cell events are reflected in the top right quadrant, and viable tumor cells are reflected in the bottom left quadrant. Results are representative of two independent assays done in each instance. As shown in (*C*), tumor cell lines were not generically sensitivity to CD4$^+$ T cell–mediated killing because the cytotoxic anti-FluM1$_{60\text{-}73}$ (restricted by HLA-DR4) CD4$^+$ T cell clone (clone 17/2) failed to mediate the death of either SLM2 or UPCI-Mel591. Data are representative of three independent assays.

humans. A typical feature of this microorganism is penetration into human cells and long-term intracellular replication and persistence. In human disease, *M. penetrans* is clinically observed in cases of HIV-1 infection, but has also been suggested to represent a primary cause of non-HIV–related urethritis and respiratory disease (36). Given the link between clinical *Mycoplasma* infections and pulmonary disease, including pneumonia (36) and anectodal reports of spontaneous tumor regression in cancer patients recovering from pneumonia (49–52), it is tempting to consider the possible involvement of cross-reactive immunity in these complex biological processes.

Our results suggest that the MPHF2 peptide is immunogenic and capable of promoting type 1 effector $CD4^+$ T cells in a majority of melanoma patients and normal donors. Responder $CD4^+$ T cells typically derived from the $CD45RA^-CCR7^-$ population of memory T cells (rather than the $CD45RA^+$ $CCR7^+$ naïve T cell cohort) and were able to cross-react against the MPHF2 and MAGE-$A6_{172\text{-}187}$ peptides and to recognize the naturally processed rMAGE epitope. Notably, anti-MPHF2 $CD4^+$ T cell lines and clones also seemed to preferentially recognize HLA-DR–matched melanoma cell lines that constitutively express the MAGE-A6 gene product, resulting in specific T cell production of IFN-γ and tumor cell apoptosis/necrosis. Importantly, whereas many strains of *Mycoplasma* can commonly infect laboratory cultures, we did not detect *M. penetrans* in any of cell lines and conclude that MPH2-specific $CD4^+$ T cell line/clone recognition of HLA-DR^+ target cells is likely due to the cross-reactivity against the MAGE-A6 homologue epitope presented in MHC complexes on the cell surface of tumor cells endogenously or on autologous DCs after the processing of exogenous rMAGE-A6 protein.

The CHP peptide derives from *C. muridarum* strain *Nigg*, a mouse-tropic strain capable of causing respiratory disorders in mice. This strain of *Chlamydia* diverges significantly from human-tropic strains (37). Given the low degree of likelihood that humans would encounter this microbe, it was perhaps not surprising that we did not observe any evidence for the cross-reactivity of these peptides by T cells in our studies. This does not rule out a pathogenic homologue for the MAGE-$A6_{280\text{-}302}$ peptide being responsible for our common observation of *in vitro* stimulation responses against this MAGE-A6 peptide among normal donors, but suggests that additional studies will need to be done to illuminate its identity.

Finally, whereas the (memory) response rates of normal donor and cancer patient $CD4^+$ T cells against MP and MAGE-A6 were noted to be high in this study, this may not be that surprising. MP infections are clinically observed under conditions of chronic immune suppression (36), suggesting that many healthy, normal donors may have experienced this environmental pathogen and have acquired ($CD4^+$ and/or $CD8^+$ T cell–mediated) protective immunity against it. Because most melanoma patients are not immunosuppressed with regard to T cell responsiveness to infectious pathogens (23), they would presumably retain protective anti-MP immunity. The highly homologous MAGE-A family (in particular MAGE-A3/-A6) members are expressed at a high frequency by a broad range of cancer types (1-13). Hence, it would be expected that (at least cross-reactive) memory $CD4^+$ T cell responses (manifest as Th-dependent humoral responses or direct T cell recognition of class II^+ tumor cells, as reported in Table 2, etc.) against MAGE-A3/-A6 epitopes would be observed in patients with cancer, such as melanoma. The ability of the promiscuous $MPHF2_{216\text{-}229}$ peptide to promote heteroclitic immunity against the MAGE-A6 (and presumably MAGE-A3) protein(s) may make this epitope extremely attractive as a vaccine candidate in patients bearing tumor types in which MAGE-A3/MAGE-A6 expression is commonly observed.

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