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CD4⁺ Th2 Cell Recognition of HLA-DR-Restricted Epitopes Derived from CAMEL: A Tumor Antigen Translated in an Alternative Open Reading Frame

Elisabeth H. Slager,* Martina Borghi,* Carolien E. van der Minne,* Corlien A. Aarnoudse,* Menzo J. E. Havenga,† Peter I. Schrier,* Susanne Osanto,* and Marieke Griffioen†*†

Tumor Ag NY-ESO-1 is an attractive target for immunotherapy of cancer, since both CD8⁺ CTL and CD4⁺ Th cells against NY-ESO-1 have been described. Moreover, NY-ESO-1 as well as the highly homologous tumor Ag LAGE-1 are broadly expressed in various tumor types. Interestingly, the NY-ESO-1 and LAGE-1 genes also encode for proteins translated in an alternative open reading frame. These alternatively translated NY-ESO-ORF2 and CAMEL proteins, derived from the NY-ESO-1 and LAGE-1 genes, respectively, have been demonstrated to be immunogenic, since CTL specific for these proteins have been isolated from melanoma patients. In this study a panel of advanced melanoma patients was screened for the presence of Th cells specific for the alternatively translated tumor Ags NY-ESO-ORF2 and CAMEL. PBMC of melanoma patients were stimulated for 4 days with mixes of overlapping peptides covering the entire NY-ESO-ORF2 and CAMEL protein sequences and were tested for the release of type 1 (IFN-γ) and type 2 (IL-13) cytokines in ELISPOT assays. In three of 15 patients, T cells specific for two CAMEL peptides (CAMEL₈₁₋₁₀₂ and CAMEL₈₁₋₁₀₂) could be detected. From one of these patients, CD4⁺ T cell clones specific for CAMEL₈₁₋₁₀₂ could be generated. These clones recognized a naturally processed epitope presented in both HLA-DR11 and HLA-DR12 and produced high levels of IL-4, IL-5, and IL-13. In conclusion, this study shows the presence of Th cells specific for the alternatively translated tumor Ag CAMEL in melanoma patients and is the first report that describes the isolation of tumor Ag-specific CD4⁺ Th2 clones. The Journal of Immunology, 2003, 170: 1490–1497.

Dendritic cells (DC)² are APC that have the unique capacity to prime naive T cells (reviewed in Ref. 1). Immature DC reside in peripheral tissues and are efficient in Ag uptake. Several signals have been described to trigger the maturation of these Ag-capturing cells into efficient APC, expressing high levels of MHC class I and II and costimulatory molecules. Upon maturation, DC migrate to the draining lymphoid organs where they can prime naive CD4⁺ and CD8⁺ T cells. The importance of Ag-specific CD4⁺ T cells in the induction and maintenance of an optimal antitumor immune response has been demonstrated by Ossendorp et al. (2). In a murine model, CTL-mediated protective immunity against a MHC class II-negative tumor could be obtained by vaccination with a tumor-specific Th epitope, whereas vaccination with an unrelated Th epitope did not lead to tumor protection. Ag-specific CD4⁺ Th cells have been shown to stimulate the induction of CD8⁺ cytotoxic T cell responses via the APC. Upon interaction with CD40 ligand on CD4⁺ T cells, CD40 triggers maturation of DC, resulting in up-regulation of costimulatory molecules and induction of CD8⁺ CTL (3–5). The importance of Th cells and CD40-CD40 ligand interactions has been further supported by Diehl et al. (6), who have shown that an activating Ab against CD40 can convert CD8⁺ T cell tolerance into immunity. However, Schnell et al. (7) have shown that the requirement of Ag-specific Th cells in the induction of an adequate anti-tumor response cannot be fully bypassed by CD40-CD40 ligand interactions, illustrating the diversity of interactions between CD4⁺ T cells and APC. Hence, the induction of tumor-specific CD4⁺ T cells should be addressed in the development of vaccination strategies for treatment of cancer.

CD4⁺ Th cells can be divided into Th1 and Th2 cells, producing type 1 (IFN-γ, TNF-α, and IL-2) and type 2 (IL-4, IL-5, and IL-13) cytokines, respectively. Th1 cells are involved in the induction of cellular immunity, whereas Th2 cells stimulate humoral immunity (8–10). The type of the Th response has been shown to be dependent on the origin of DC and cytokine environment during the priming of CD4⁺ T cells. Mature myeloid-derived DC produce IL-12 and promote differentiation of Th cells into Th1 cells, whereas mature plasmacytoid-derived DC do not produce IL-12 and polarize T cells to a Th2 profile (11).

Several tumor Ags have been identified that can be recognized by CTL. This led to the development of immunotherapies aimed at stimulating T cell responses against these identified Ags. Attractive targets for immunotherapy are the so-called cancer/testis Ags, such as MAGE-1 (12) and MAGE-3 (12), and NY-ESO-1 (13), which are expressed in tumors of various histological types, but are silent in normal tissues, except for testis. In addition to HLA class I-presented epitopes, several MHC class II-restricted epitopes encoded by NY-ESO-1, MAGE-1, and MAGE-3 have now been identified (14–19).

LAGE-1 is a cancer-testis Ag that shows 94% identity to NY-ESO-1 (20). The LAGE-1 and NY-ESO-1 genes are both located on chromosome Xq28 and are frequently coexpressed (13, 20, 21). The LAGE-1 and NY-ESO-1 genes have been shown to be expressed

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2 Abbreviations used in this paper: DC, dendritic cell; CBA, cytometric bead array; ORF, open reading frame.
in 25–50% of various tumor types, such as melanoma, breast carcinoma, prostate, and bladder cancers (20). Interestingly, both genes have been reported to be translated from an alternative open reading frame (ORF). The ATG start site of the alternative ORF (ORF2) is located 40 bp downstream of the ATG of ORF1. Translation from the alternative start site leads to a 109-aa LAGE-ORF2 protein, also called CAMEL (22) and a 58-aa NY-ESO-ORF2 protein (23). The N-terminal 54 aa of NY-ESO-ORF2 and CAMEL are completely identical. Wang et al. (23) isolated an HLA-A*31-restricted CTL specific for NY-ESO-ORF2 from tumor-infiltrating lymphocytes of a melanoma patient, whereas in our laboratory, Aarnoudse et al. (22) isolated an HLA-A*0201-restricted CTL specific for CAMEL from peripheral blood of a melanoma patient. The CTL specific for NY-ESO-ORF2 has been shown to recognize target cells transfected with either the LAGE-1 or NY-ESO-1 cDNA, demonstrating that the epitope is encoded in the ORF2 of both genes. Although several HLA class II-restricted epitopes have been described for the NY-ESO-1 protein translated in ORF1, it is unknown whether HLA class II-binding peptides derived from the alternatively translated CAMEL and NY-ESO-ORF2 proteins can be recognized by CD4+ Th cells.

The aim of this study was to investigate whether CD4+ T cells specific for CAMEL or NY-ESO-ORF2 could be detected in PBMC of melanoma patients. PBMC were stimulated for 4 days with mixes of overlapping 22-mer peptides covering the CAMEL and NY-ESO-ORF2 protein sequences and were tested for the release of type 1 (IFN-γ) and type 2 (IL-13) cytokines in ELISPOT assays. In three of 15 melanoma patients, T cells specific for CAMEL specifically showed cytokines IL-4, IL-5, and IL-13 and minute amounts of IL-10, but no IFN-γ, TNF-α, or IL-2. Furthermore, we showed that CAMEL is a naturally processed epitope presented by HLA-DR1 and HLA-DR12.

**Materials and Methods**

**Peptides and recombinant protein**

Peptides were synthesized by solid phase methods, using an automated multiple peptide synthesizer (AMS 422; Abimed Analys-Technik, Langenfeld, Germany) and F-moc chemistry. After reverse phase HPLC analysis, peptides were dissolved in DMEM at 50 mg/ml and stored at −70°C. From this stock solution, peptide was diluted in PBS to a final concentration of 1 mg/ml and stored at −20°C. The peptides were overlapping 22-mer covering the entire NY-ESO-ORF2 (aa 1–20, 14–33, 21–42, and 31–52) and CAMEL (aa 1–20, 14–33, 21–42, 31–52, 41–62, 46–65, 51–72, 61–82, 71–92, 81–102, and 88–109) proteins.

Recombinant CAMEL protein (rCAMEL) was obtained by cloning the coding cDNA sequence in frame with an N-terminal histamine tag into pET19b vector (Novagen, Madison, WI). Expression of pET19b-CAMEL was induced in Escherichia coli strain BL21(DE3) (Stratagene, Amsterdam, The Netherlands) by the addition of 1 mM isopropyl-β-D-thiogalactoside (Sigma-Aldrich, Zwijndrecht, The Netherlands). After 4 h, rCAMEL was purified by nickel-chelate affinity chromatography using nickel-nitrilotriacetic acid agarose according to manufacturer’s instructions (Westburg, Leusden, The Netherlands). Recombinant CAMEL was eluted in 8 M urea, 100 mM NaH2PO4, and 10 mM Tris, pH 4.5, and dialyzed in distilled water. After lyophilization, rCAMEL was dissolved in distilled water and stored at −70°C.

**Adenoviral constructs and transduction**

Ad5F35-CAMEL is a recombinant adenoviral type 5 construct modified to express the fiber shaft and knob of serotype 35, which has been demonstrated to infect PBMC very efficiently (24). The CAMEL cDNA was cloned into the Ad5.Fib35 vector and produced in PER.C6 cells (25). The virus was purified using cesium chloride banding, and the titer of the virus batch was determined by HPLC as described previously (26). Target cells were seeded in 48-well plates at 2.5 × 105 cells/well in serum-free medium and incubated with Ad5F35-CAMEL (4 × 105 virus particles/cell) for 2 h at 37°C. Infection was blocked by addition of serum-containing medium. After 48 h cells were used as targets in ELISPOT assays.

**In vitro stimulation of PBMC**

PBMC were isolated by Ficoll gradient centrifugation from heparinized blood from advanced melanoma patients (American Joint Cancer Committee stages III and IV) and stored in liquid nitrogen. All patients were HLA-typed using standard serological techniques. PBMC were thawed and seeded in 24-well plates at 2 × 105 cells/well in RPMI (Invitrogen, Breda, The Netherlands) containing 5% heat-inactivated FCS, 4 mM l-glutamine, 50 μg/ml of penicillin, and 50 μg/ml streptomycin. PBMC were stimulated with mixes of three or four peptides (mix 1: aa 1–20, 14–33, and 46–65; mix 2: aa 21–42, 31–52, 41–62, and 51–72; mix 3: aa 61–82, 71–92, 81–102, and 88–109). Each peptide was added at 5 μg/ml. As a control, 2 × 105 PBMC were stimulated with 50 ng/ml PMA and 1 μM ionomycin (PMI-ioniomycin). After 4 days of stimulation, PBMC were collected and seeded at 1 × 105 cells/well in three to five wells of IFN-γ and IL-13 ELISPOT plates. The number of spots produced by stimulated PBMC was considered to differ significantly from the number of spots produced by nonstimulated PBMC when p < 0.05 using Student’s t test for unpaired samples and a mean number of more than five spots.

**Generation of CAMEL-specific CD4+ T cell clones**

To generate a CAMEL1–110-specific T cell line, 15 × 106 patient of cancer WKH were stimulated with 5 μg/ml of CAMEL1–110 in IMDM (Invitrogen) supplemented with t-arginine (116 μg/ml), t-asparagine (36 μg/ml), l-glutamine (215 μg/ml), 10% human serum, and antibiotics as mentioned above. Bulk T cells were weekly restimulated with an equal number of irradiated, autologous PBMC pulsed with 5 μg/ml CAMEL1–110 and 10 U/ml IL-2. On day 28, the remaining bulk T cells were seeded at 1 cell/well in 96-well U-bottom plates, with each well containing 103 irradiated, allogeneic PBMC, 5 × 103 irradiated, allogeneic EBV-transformed B cell lines, and 5 × 104 autologous PBMC pulsed with 10 μg/ml CAMEL1–110, 1 μg/ml leukaegoglobin (Sigma-Aldrich), and 120 U/ml IL-2. Growing clones were weekly restimulated as described above.

**IFN-γ ELISPOT assay**

Nonstimulated PBMC, PBMC stimulated with peptide mixes, and PBMC stimulated with PMA + ionomycin were seeded at 105 cells/well in triplicate and tested for the release of IFN-γ by ELISPOT assay. The IFN-γ ELISPOT assay was performed as described previously (27), except that 96-well nylons Silent Screen plates (Nalge Nunc International, Rochester, NY) were used.

**IL-13 ELISPOT assay**

The IL-13 ELISPOT assay was performed as described previously for the Th2 response, CAMEL1–110–ELISPOT assay by Allen et al. (28). Briefly, 96-well ELISA plates (Greiner, Alphen aan den Rijn, The Netherlands) were coated with 100 μl of an Ab against human IL-13 (QS-13; U-CyTech, Utrecht, The Netherlands) diluted to 10 μg/ml in PBS overnight at 4°C. Wells were washed several times with PBS/0.05% Tween 20 and blocked with 100 μl of PBS/1% BSA for 1 h at 37°C. Nonstimulated PBMC, PBMC stimulated with peptide mixes, and PBMC stimulated with PMA + ionomycin were seeded at 105 cells/well in triplicate. CAMEL1–110-specific CD4+ T cells were seeded at 5 × 105 cells/well together with 1–25 × 105 target cells/well in duplicate. For blocking studies, target cells were preincubated with Abs against HLA class I (w6/32), HLA class II (IC-2), HLA-DR (B8.11.2), HLA-DQ (SPV-L3), or HLA-DP (B7.21) (29). After 5 h at 37°C, wells were incubated with 250 μl of precooled, distilled water on melting ice for 10 min, washed several times, and subsequently incubated with 100 μl of a biotinylated polyclonal Ab against human IL-13 diluted in PBS/1% BSA overnight at 4°C. After several washes, wells were incubated with 50 μl of a gold-labeled, anti-biotin Ab for 1 h at 37°C. Wells were washed several times and incubated with 30 μl of activator mix (U-CyTech) until silver salt precipitates had been formed at the site of gold clusters. Plates were washed, and spots were counted using light microscopy.

**Human Th1/Th2 cytokine cytometric bead array (CBA) assay**

HLA-DR12-positive EBV-transformed B cells were pulsed with CAMEL1–110 (10 μg/ml) for 2 h at 37°C. Cells were washed and seeded at 3 × 105 cells/well together with clone 12-WKH (3 × 105) in a 48-well plate overnight at 37°C. The release of IFN-γ, TNF-α, IL-2, IL-4, IL-5, and IL-10 by CAMEL1–110-specific T cells was analyzed using the human Th1/Th2 cytokine CBA assay according to the manufacturer’s instructions (BD Biosciences, Franklin Lakes, NJ).
Granzyme A and B ELISA

Release of granzymes A and B by CAMEL-specific CD4+ T cells in the culture supernatant was measured by ELISA according to the manufacturer’s instructions (CLB, Amsterdam, The Netherlands). Briefly, 96-well ELISA plates were coated overnight at room temperature with mAbs directed against human granzyme A or B. Wells were washed several times with PBS/0.02% Tween 20 and subsequently blocked with kit buffer for 1 h at room temperature. Wells were incubated with serial dilutions of culture supernatants for 1 h at room temperature. In each assay serial dilutions of purified human granzyme A (0–1200 pg/ml) and granzyme B (0–960 pg/ml) were included. After several washes, 100 μl of biotinylated Ab against human granzyme A or B was added for 1 h at room temperature. Wells were washed several times and incubated with streptavidin-poly-HRP conjugate for 30 min at room temperature. After washing, wells were incubated with 100 μl of substrate solution. The reaction was stopped by the addition of 100 μl of 0.18 M sulfuric acid solution, and OD492 was measured using a microplate reader (Wallac, Turku, Finland). Concentrations of granzymes A and B were determined by interpolation on the standard curves.

Results

Screening of melanoma patients for the presence of NY-ESO-ORF2- and CAMEL-specific T cells by IFN-γ and IL-13 ELISPOT assays

To determine the presence of T cells reactive against the alternatively translated NY-ESO-ORF2 and CAMEL proteins, PBMC derived from 15 advanced melanoma patients were tested for the release of type 1 (IFN-γ) and type 2 (IL-13) cytokines in ELISPOT assays after 4 days of stimulation with mixes of overlapping peptides. CAMEL (109 aa) and NY-ESO-ORF2 (58 aa) are proteins translated from the LAGE-1 and NY-ESO-1 genes in an alternative ORF. The N-terminal 54 aa of CAMEL and NY-ESO-ORF2 are completely identical. Three mixes of overlapping peptides covering the entire CAMEL and NY-ESO-ORF2 protein sequences were used to screen total PBMC for the presence of CAMEL-specific T cells. Mix 1 (aa 1–20, aa 14–33, and aa 46–65) contains three peptides with the predicted binding motifs for various HLA-DR alleles (data not shown). Mix 2 contains four overlapping 22-mer peptides covering the aa 22–72 sequence, whereas mix 3 consists of four overlapping 22-mer peptides covering the C-terminal part of CAMEL (aa 62–109). In three of the 15 melanoma patients significant numbers of spots were produced by total PBMC after stimulation with peptide mix 3 (Table I). In two patients (no. 5 and 9), CAMEL-specific T cells were detectable in both the IFN-γ and IL-13 ELISPOT assays, whereas a third patient (no. 3) showed only peptide-specific IL-13 spots. All patients produced significant numbers of IFN-γ and IL-13 ELISPOTs after stimulation with PMA and ionomycin (data not shown), demonstrating that all PBMC were able to produce cytokines upon stimulation. As a control, total PBMC from 18 healthy controls were screened for the presence of CAMEL-specific T cells. None of these PBMC produced significant numbers of IFN-γ or IL-13 spots upon stimulation with peptide mix 3 (data not shown).

To determine against which CAMEL peptide the T cells were directed, PBMC from the three patients who showed significant numbers of spots upon stimulation with CAMEL peptide mix 3 (patients 3, 5, and 9) were tested for IFN-γ and IL-13 release after stimulation with single peptides (Table II). CAMEL171–92-specific
release of IFN-γ as well as IL-13 was observed for PBMC from patient 3, whereas PBMC from patient 9 released only IFN-γ after stimulation with CAMEL81-102. The failure to detect CAMEL81-102-specific IL-13 spots in patient 9 might be due to the high background number of spots produced by nonstimulated PBMC. Remarkably, a high number of CAMEL81-102-specific IL-13 spots was produced by PBMC from patient 5, whereas no IL-13 production was detectable in nonstimulated PBMC.

Isolation and characterization of CAMEL81-102-specific IL-13-producing CD4+ T cells

Since melanoma patient 5 demonstrated high numbers of CAMEL81-102-specific T cells in IL-13 ELISPOT assays, we studied these T cells in more detail. PBMC from patient 5 were weekly stimulated in vitro with autologous, irradiated PBMC loaded with CAMEL81-102. On day 28 the number of cells in the bulk culture decreased dramatically, and cells were seeded at 1 cell/well by limiting dilution to generate T cell clones. Growing clones were tested for peptide-specific cytokine release in IL-13 ELISPOT assays. Of the 41 clones tested, eight clones produced IL-13 in response to CAMEL81-102. All CAMEL81-102-specific T cell clones were CD4+ (data not shown). To determine whether CAMEL81-102 encodes a naturally processed epitope, several CAMEL81-102-specific CD4+ T cell clones were tested for recognition of autologous PBMC pulsed with rCAMEL protein. All CD4+ T cell clones tested specifically released IL-13 upon stimulation with PBMC loaded with CAMEL81-102 as well as rCAMEL, as illustrated for clone 12-WKH in Fig. 1A. Furthermore, the response against CAMEL81-102 is mediated by HLA-DR, since Abs against HLA class II as well as HLA-DR blocked recognition of CAMEL81-102, whereas Abs against HLA class I, HLA-DQ, or HLA-DR had no effect (Fig. 1B).

To determine which HLA-DR allele presented the epitope, a panel of PBMC expressing one or more HLA-DR alleles of patient 5 (DR4, DR12, DR52, DR53) was pulsed with CAMEL81-102, rCAMEL protein, or Ad5F35-CAMEL and tested for recognition by clone 12-WKH. All PBMC pulsed with 10 μg/ml of CAMEL81-102 were recognized by clone 12-WKH, whereas recognition of PBMC

![Figure 1](http://www.jimmunol.org/content/journals/10.4049/jimmunol.149.7.1493/DC1/492fda.jpg)

FIGURE 1. CD4+ T cell clones isolated from PBMC of melanoma patient 5 recognize a naturally processed CAMEL81-102 epitope presented in HLA-DR. A, Autologous PBMC were pulsed with CAMEL81-102 (10 μg/ml) or rCAMEL (10 μg/ml) for 2 h at 37°C, seeded at 2.5 × 10⁴ cells/well in duplicate, and tested for recognition by clone 12-WKH (5 × 10⁴ cells/well) in an IL-13 ELISPOT assay. B, Autologous PBMC were incubated with CAMEL81-102 (10 μg/ml) for 2 h at 37°C and subsequently preincubated with Abs against HLA class I (w6/32), HLA class II (IC-2), HLA-DR (B8.11.2), HLA-DQ (SPV-L3), or HLA-DP (B7.21) for 30 min at 37°C. Target cells were seeded at 2.5 × 10⁴ cells/well together with clone 12-WKH (5 × 10⁴ cells/well) in duplicate in an IL-13 ELISPOT assay.

![Figure 2](http://www.jimmunol.org/content/journals/10.4049/jimmunol.149.7.1493/DC1/0201fda.jpg)

FIGURE 2. The CAMEL81-102 epitope is presented to clone 12-WKH by HLA-DR12. A, PBMC of healthy donors expressing one or more HLA-DR alleles of patient 5 (DR4, DR12, DR52, DR53) were pulsed with 1 and 10 μg/ml of CAMEL81-102 or rCAMEL (10 μg/ml) for 2 h at 37°C or were infected with the adenoviral vector Ad5F35-CAMEL (4 × 10⁵ virus particles/cell). Target cells were seeded at 2 × 10⁴ cells/well and tested for recognition by clone 12-WKH (2 × 10⁴ cells/well) in an IL-13 ELISPOT assay. PBMC pulsed with CAMEL81-102, rCAMEL, or Ad5F35-CAMEL alone do not produce IL-13. HLA-DR alleles matching with those of patient 5 (DR4, DR12, DR52, DR53) are depicted in bold. B, PBMC of patient 5 (HLA-A*0201) were pulsed with CAMEL81-11 (10 μg/ml) or rCAMEL (10 μg/ml) for 2 h at 37°C or were infected with Ad5F35-CAMEL (4 × 10⁵ virus particles/cell). Target cells were seeded at 1 × 10⁴ cells/well together with 1 × 10⁶ cells/well of the CAMEL81-11-specific, HLA-A2-restricted CTL clone 1/37 in duplicate in an IFN-γ ELISPOT assay. PBMC pulsed with CAMEL81-11, rCAMEL, or Ad5F35-CAMEL alone do not produce IFN-γ.
in contrast to the HLA-A*0201-restricted, CAMEL1 protein is processed and presented in the MHC class II pathway. This

CAMEL-specific CD4-Th cell clone 12-WKH recognizes CAMEL when exogenous protein is presented and presented in the MHC class II pathway. This in contrast to the HLA-A*0201-restricted, CAMEL-specific CD8-Th cell clone that recognizes intracellular CAMEL processed and presented in the MHC class I pathway.

Peptide-bindings motifs of HLA-DR molecules are less strict than motifs of HLA class I molecules, and antigenic peptides can be presented to the same TCR by several HLA-DR alleles (30, 31). To test the specificity of clone 12-WKH, a large panel of PBMC expressing various HLA-DR alleles was pulsed with rCAMEL protein and tested for recognition by clone 12-WKH. Fig. 3 demonstrates that not only HLA-DR12-positive PBMC but also HLA-DR11-expressing PBMC pulsed with rCAMEL were capable of stimulating the production of IL-13 by clone 12-WKH. To exclude that HLA-DR1- and HLA-DR12-positive targets were recognized by a mixture, instead of a monoclonal, CD4-Th cell culture, clone 12-WKH was stained with a panel of Abs specific for 24 different TCRVβ (TCRVβ repertoire kit; Immunotech, Westbrook, ME). All cells were stained with an Ab specific for Vβ2 (data not shown), strongly suggesting that clone 12-WKH recognizes CAMEL presented in HLA-DR11 and -DR12 by the same TCR.

To identify the anchor residues of the epitope encoded by CAMEL81–102, a panel of overlapping 15-mer peptides was synthesized and tested for their ability to activate clone 12-WKH. As shown in Fig. 4, recognition of CAMEL81–102 was completely abolished when the proline residue at position 86 was deleted. At the C-terminal end of the peptide, removal of the alanine residue at position 93 resulted in loss of recognition by clone 12-WKH, demonstrating that the minimal epitope recognized by clone 12-WKH is PWKRSWSA (aa 86–93).

To determine whether the CD4-Th clone 12-WKH produces cytokines other than IL-13, the release of type 1 (IFN-γ, TNF-α, IL-2) and type 2 (IL-4, IL-5, IL-10) cytokines upon stimulation with CAMEL81–102-pulsed target cells was examined in the human Th1/Th2 cytokine CBA assay. Fig. 5A shows that clone 12-WKH also produced high levels of IL-4 and IL-5, but no or very low levels of IFN-γ, TNF-α, IL-2, and IL-10. In addition, the release of granzymes A and B could be demonstrated in the culture supernatant of clone 12-WKH stimulated with CAMEL81–102 (Fig. 5B). In conclusion, these data show the isolation of CAMEL81–102-specific CD4-Th cells from PBMC of a melanoma patient. These CD4-Th cells release type 2 cytokines (IL-4, IL-5, IL-13) and recognize a naturally processed epitope presented in HLA-DR11 and -DR12.

**Discussion**

NY-ESO-1 and LAGE-1 are highly homologous tumor Ags expressed in a variety of tumors (20, 21). The genes encoding NY-ESO-1 and LAGE-1 have been shown to be translated in different ORFs, generating the NY-ESO-ORF2 and CAMEL proteins (22, 23). Both NY-ESO-ORF2 and CAMEL have been demonstrated to be immunogenic in vivo, since CTL directed against these alternatively translated proteins have been isolated from melanoma patients (22, 23). In this study we screened peripheral blood of 15 advanced melanoma patients for the presence of NY-ESO-ORF2 and CAMEL-specific Th cells by IFN-γ and IL-13 ELISpot assays. In two patients T cells specific for CAMEL81–102 were found, whereas CAMEL81–92-specific T cells could be detected in a third patient. The short in vitro peptide stimulation step (4 days) as well as the absence of CAMEL-specific T cells in healthy donors strongly suggest that CAMEL-specific T cells in melanoma patients have been primed in vivo. From one of the patients, CD4-Th2 clones recognizing a naturally processed epitope were isolated.

Target cells that were exogenously pulsed with high concentrations of CAMEL81–102 were recognized by clone 12-WKH, regardless of the HLA-DR alleles expressed. A motif that has been described for peptides binding to multiple HLA-DR alleles has been found twice in CAMEL81–102, i.e., at positions 87–95 (WKRSWSAGS) and 91–99 (WSAGSCPGM) (30, 31). The presence of two general HLA-DR binding motifs may account for the
promiscuous binding of \( \text{CAMEL}_{1-102} \) when pulsed at high peptide concentrations. However, recognition of target cells loaded with \( r\text{CAMEL} \) protein or low concentrations of \( \text{CAMEL}_{1-102} \) by clone 12-WKH has been shown to be restricted by HLA-DR11 and -DR12.

Clone 12-WKH clearly recognizes PBMC pulsed with \( r\text{CAMEL} \) protein, but not PBMC infected with Ad5F35-CAMEL. This lack of recognition cannot be attributed to low levels of CAMEL expression, since Ad5F35-CAMEL-infected PBMC, in contrast to PBMC pulsed with \( r\text{CAMEL} \) protein, are clearly recognized by a \( \text{CAMEL}_{1-11} \)-specific, CD8\(^+\) CTL clone. These data suggest that exogenously delivered CAMEL is preferentially loaded in MHC class II molecules, whereas intracellularly expressed CAMEL predominantly enters the MHC class I pathway. In addition to Ad5F35-CAMEL-infected PBMC, we failed to demonstrate recognition of HLA class II-positive, CAMEL-expressing tumor cells by clone 12-WKH (data not shown). Similar results have been obtained by Chaux et al. (32), who isolated an anti-MAGE-A3, HLA-DR13-restricted CD4\(^+\) T cell clone recognizing APC loaded with MAGE-A3 protein, but not MAGE-A3-expressing tumor cells. However, other HLA-DP4- and HLA-DR11-restricted CD4\(^+\) T cell clones directed against different MAGE-A3 epitopes have been described that directly recognize MAGE-A3-expressing, MHC class II-positive tumor cells (18, 19), indicating that some intracellular proteins can be presented in the MHC class II pathway of the tumor cell dependent on the epitope processed. We speculate that the in vivo function of \( \text{CAMEL}_{1-102} \) is not to lyse tumor cells, but to regulate the tumor-specific immune response.

The \( \text{CAMEL}_{81-102} \)-specific CD4\(^+\) T cell clones obtained from patient 5 belong to the Th2 subtype, producing high levels of IL-4, IL-5, and IL-13. IL-13, like IL-4, enhances the expression of MHC class II on monocytes and stimulates the proliferation and differentiation of B cells, leading to the production of IgG and IgE Abs. Ab production has been shown to require costimulatory signals delivered by CD4\(^+\) T cells (33, 34). Interestingly, Abs against LAGE-1 and NY-ESO-1 have been detected in sera of melanoma patients (13, 35, 36), and the presence of NY-ESO-1-specific Abs in melanoma patients has been shown to be associated with NY-ESO-1-specific, HLA-DP4-restricted CD4\(^+\) T cells (15). It remains to be shown whether the sera of melanoma patients contain Abs against CAMEL and NY-ESO-ORF2 and whether these Abs are associated with the presence of specific CD4\(^+\) Th cells.

Several studies have reported that DC in tumor tissues have a diminished stimulatory capacity (37–40). Priming of T cells by malfunctioning DC in tumors has been suggested to result in the induction of anergic or regulatory T cells (41). Furthermore, tumor cells of several different histological origins, such as melanoma, breast, and renal cell carcinoma, have been shown to produce immunomodulatory factors that inhibit DC maturation (38, 42–44). Immature DC have been shown to skew the Th1/Th2 balance in favor of Th2, whereas mature DC are required for a Th1 response (45). Therefore, by producing factors that inhibit DC maturation, tumor cells may promote the development of Th2 responses. This is in agreement with the detection of IL-13-producing, CAMEL-specific T cells in melanoma patients, as described in this study, and a disturbed Th1/Th2 balance in cancer patients, as reported by others (46, 47). Recently, Tatsumi et al. (48) have found a Th2 polarization of CD4\(^+\) T cell responses against MAGE-6 epitopes in renal cell carcinoma and melanoma patients with active disease, whereas normal donors and disease-free patients displayed either Th1-type or mixed Th1/Th2-type immunity. Also in animal models, a shift toward Th2-biased responses has been demonstrated during tumor progression (49, 50). Neutralizing Abs against Th2 cytokines as well as depletion of CD4\(^+\) Th2 cells have been shown to enhance the generation of antitumor CD8\(^+\) CTL (50–52). Although CD4\(^+\) Th2 cells can promote the recruitment of eosinophils and macrophages into the tumor, only Th1 cells seem to be required for durable antitumor CTL responses (53–55). Therefore, future immunotherapies for cancer patients should address the generation of an optimal tumor-specific CD4\(^+\) Th1 response.

By screening a panel of melanoma patients we provide strong evidence for the occurrence of Th2 responses against CAMEL, a tumor Ag translated in an alternative ORF. Furthermore, this is the first report that describes the isolation and characterization of CD4\(^+\) Th2 clones specific for an identified tumor Ag. Vaccination with MHC class I- and II-binding peptides has been shown to lead to optimal tumor protection in mice (2). We propose that presentation of the newly identified MHC class II-binding CAMEL epitope in combination with the previously described MHC class I-binding peptides by well-functioning, mature DC might be an effective antitumor vaccine.

**References**


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