Functional Analysis of Tumor-Specific Th Cell Responses Detected in Melanoma Patients after Dendritic Cell-Based Immunotherapy

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Recently, we have demonstrated that tumor-specific CD4+ Th cell responses can be rapidly induced in advanced melanoma patients by vaccination with peptide-loaded monocyte-derived dendritic cells. Most patients showed a T cell reactivity against a melanoma Ag 3 (MAGE-3) peptide (MAGE-3 243–258), which has been previously found to be presented by HLA-DP4 molecules. To analyze the functional and specificity profile of this in vivo T cell response in detail, peptide-specific CD4+ T cell clones were established from postvaccination blood samples of two HL-A-DP4 patients. These T cell clones recognized not only peptide-loaded stimulator cells but also dendritic cells loaded with a recombinant MAGE-3 protein, demonstrating that these T cells were directed against a naturally processed MAGE-3 epitope. The isolated CD4+ Th cells showed a typical Th1 cytokine profile upon stimulation. From the first patient several CD4+ T cell clones recognizing the antigenic peptide used for vaccination in the context of HLA-DP4 were obtained, whereas we have isolated from the second patient CD4+ T cell clones which were restricted by HLA-DQB1*0604. Analyzing a panel of truncated peptides revealed that the CD4+ T cell clones recognized different core epitopes within the original peptide used for vaccination. Importantly, a DP4-restricted T cell clone was stimulated by dendritic cells loaded with apoptotic or necrotic tumor cells and even directly recognized HLA class II- and MAGE-3-expressing tumor cells. Moreover, these T cells exhibited cytolytic activity involving Fas-Fas ligand interactions. These findings support that vaccination-induced CD4+ Th cell responses play an important functional role in antitumor immunity. The Journal of Immunology, 2004, 172: 1304–1310.

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Materials and Methods

Cell lines, reagents, and Abs

EBV-transformed B (EBV-B) cell lines and tumor cell lines were cultured in RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 10% FCS (PAA Laboratories, Linz, Austria), 20 μg/ml gentamicin (Sigma-Aldrich, Taufkirchen, Germany), 2 mM L-glutamine (BioWhittaker), 10 mM HEPES (Sigma-Aldrich), and 10 mM sodium pyruvate (Invitrogen, Paisley, U.K.). DCs and CD4+ T cells were cultured in the same medium supplemented with 1% autologous plasma or 10% human serum (BioWhittaker). Cultures were supplemented with 1% BSA and subsequently analyzed. In some cases, cells were incubated with the appropriate concentration of F-moc for transient NH2-terminal protection and were characterized as feeder cells. CD4+ T cell clones were restimulated once per week with irradiated peptide-loaded EBV-B cells in T cell medium supplemented with IL-2 (50 U/ml) and feeder cells. Growing T cell clones were tested for their capacity to produce IFN-γ when stimulated with autologous peptide-loaded EBV-B cells. IFN-γ was measured in the supernatants by ELISA using reagents from Medgenix Diagnostics-Biosource (Fleurus, Belgium).

Peptides

Peptides were synthesized by conventional solid-phase peptide synthesis using F-moc for transient NH2-terminal protection and were characterized using mass spectrometry. All peptides were >80% pure, as indicated by analytical HPLC. Lyophilized peptides were dissolved at 2 mg/ml in DMSO/acetic acid (10 mM) and stored at −20°C.

Recognition assay with protein-loaded DCs

Immature DCs were incubated for 48 h with 20 μg/ml of a recombinant MAGE-3 protein (kindly provided by Gábor Smith-Kline, Rixensart, Belgium) or OVA (Sigma-Aldrich) in the presence of IL-4 and GM-CSF. The MAGE-3 protein is a fusion protein with a lipidated protein D derived from Haemophilus influenzae at its N terminus and a sequence of several histidine residues at the C terminus of the protein (ProtD MAGE-3/His). After 6 h, IL-1β, IL-6, TNF-α, and PGE2 were added to induce their maturation. CD4+ T cells (4 × 10^5/flat-bottom microwell) were stimulated with 15 × 10^3 protein-loaded DCs or peptide-loaded DCs (1 μM, 1 h) as a positive control and, after 20 h, IFN-γ released in the supernatant was measured by ELISA.

Recognition assay with peptides

CD4+ T cells (4 × 10^5/round-bottom microwell) were cocultured with 15 × 10^3 peptide-loaded EBV-B cells or DCs from different donors with known HLA class II typing. After 20 h, IFN-γ released in the supernatant

Flow cytometric analysis

Analysis was performed using the BD Biosciences FACScan flow cytometer. In brief, cells were incubated with the appropriate concentration of Abs for 30 min at 4°C. Cells were then washed twice with PBS supplemented with 1% BSA and subsequently analyzed. In some cases, cells were incubated with a polyclonal FITC-labeled goat anti-mouse IgG/IgM Ab as second Ab. FasL expression analysis was performed on T cells activated for 2 h in the presence of 2.5 μg/ml PHA (PHA-P). For intracellular perforin expression analysis, lymphocytes were first fixed and permeabilized using the BD Cytofix/Cytoperm kit from BD PharMingen and subsequently stained with an FITC-conjugated anti-perforin Ab.

DCs and CD4+ responder T cells

DCs were generated from leukapheresis products from patients R12 and R15 essentially as described previously (14). Briefly, PBMC were obtained by Ficoll density gradient centrifugation and monocytes were isolated by plastic adherence and cultured in the presence of 800 U/ml GM-CSF and 500 U/ml IL-4 for 6 days to generate immature, monocyte-derived DCs. Maturation was induced by adding a cytokine mixture consisting of 10 ng/ml IL-1β, 100 U/ml IL-6, 10 ng/ml TNF-α, and 1 μg/ml PGE2 (15). Mature DCs were harvested on day 7. CD4+ T lymphocytes were isolated from postvaccination PBMC by negative selection using anti-CD8°, anti-CD14°, anti-CD19°, and anti-CD56° Abs coupled to magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany).

Generation of peptide-specific CD4+ T cell clones

CD4+ T cells (1 × 10^5) were cocultured with 3 × 10^5 autologous peptide-loaded (10 μg/ml for 3 h) and irradiated (100 Gy) mature DCs in 24-well plates in 2 ml of T cell medium. On day 3, 50 U/ml IL-2 and 10 ng/ml IL-7 were added. On day 7, peptide-specific T cells were cloned by limiting dilution using irradiated autologous EBV-B cells loaded for 1 h with 10 μg/ml peptide (5 × 10^−10−10 cells/round-bottom microwell) as stimulator cells. Irradiated allogeneic LG2-EBV (5 × 10^5−10^6 cells/well) were used as feeder cells. CD4+ T cell clones were restimulated once per week with irradiated peptide-loaded EBV-B cells in T cell medium supplemented with IL-2 (50 U/ml) and feeder cells. Growing T cell clones were tested for their capacity to produce IFN-γ when stimulated with autologous peptide-loaded EBV-B cells. IFN-γ was measured in the supernatants by ELISA using reagents from Medgenix Diagnostics-Biosource (Fleurus, Belgium).

FIGURE 1. CD4+ T cell clones R12-57 (A) and R14-15 (B) recognize a naturally processed MAGE-3 Ag. Autologous immature DCs were incubated for 48 h with 20 μg/ml MAGE-3 protein or OVA. Maturation of DCs was induced after 6 h by adding IL-1β, IL-6, TNF-γ, and PGE2. CD4+ T cells were cocultured with protein-loaded or peptide-loaded (1 μM) DCs and 20 h later IFN-γ secreted in the supernatant was measured by ELISA. Values shown are the means of triplicate determinations; bars, SD.
was measured by ELISA. In some experiments, IL-4, IL-10, and TNF-α released in the supernatant were measured in addition. To identify the HLA restriction of the T cell clones, blocking of the Ag-induced production of IFN-γ was investigated using mAbs against HLA-DR, HLA-DQ, and HLA-DP. All mAbs were used at a final concentration of 10 μg/ml. To screen the set of truncated peptides, autologous EBV-B cells were incubated for 2 h in the presence of different peptides and different peptide concentrations.

Recognition assay with tumor cells

Several HLA-matched or -mismatched MAGE-3-expressing melanoma cell lines were seeded at 15 × 10^4/flat-bottom microwell and incubated for 48 h to allow the formation of a monolayer. CD4^+ T cells were then added (at 4 × 10^3/microwell) and, after 20 h, IFN-γ secreted in the supernatants was measured by ELISA.

Recognition assay with dead or dying tumor cells

Briefly, 5 × 10^6 immature DCs were loaded with apoptotic or necrotic tumor cells at a ratio of 1:1 and cocultured in 200 μl of T cell medium in flat-bottom microwells. Necrotic tumor cell material was generated by repeated freeze-thaw cycles of 1 × 10^6 cells resuspended in 1 ml of RPMI 1640 medium. Apoptotic material was generated by irradiating tumor cells in 24 wells with 0.5–1.0 J/cm² UVB (UV 3003 K; Waldmann Medizintechnik, Villingen-Schwenningen, Germany) which induced apoptosis within 8 h. Apoptosis was measured using an annexin V kit (BD PharMingen). Six hours later, IL-1β, IL-6, TNF-α, and PGE2 were added to induce maturation of the DCs. Forty-eight hours later, CD4^+ T cells (5 × 10^5/microwell) were added to the microwells and, after 20 h, IFN-γ secreted in the supernatants was measured by ELISA.

Chromium release assay

Tumor cells were labeled with 100 μCi of Na^251CrO₄, loaded with peptide, washed, and preincubated for 30 min with the blocking anti-Fas Ab or isotype control at a concentration of 1 μg/ml before adding the responder cells at the indicated E:T ratios. Blocking Abs and isotype controls were present during the whole incubation step and chromium release was measured after incubation at 37°C for 4 h. For further blocking experiments, effector T cells were preincubated for 1 h in the presence of anti-TRAIL (50 ng/ml) or anti-TNF-α (100 ng/ml) Abs. Abs were also present during the 4-h incubation step.

Results

Generation of MAGE-3-specific CD4^+ T cell clones

To analyze the functional and specificity profile of Th cell responses induced by vaccination with peptide-loaded DCs, CD4^+ T cells were isolated from blood samples collected after the fifth vaccination of two patients who had previously shown strong TH1 reactions against peptide MAGE-3_243-258 (13). CD4^+ T cells from patients R12 and R15 were stimulated once in vitro with autologous peptide-loaded DCs. On day 7, peptide-specific CD4^+ T cells were cloned by limiting dilution using autologous EBV-B cells loaded with peptide as stimulator cells and feeder cells. Growing T cell clones were tested for their ability to produce IFN-γ upon stimulation with autologous peptide-loaded EBV-B cells. Several peptide-specific CD4^+ T cell clones were obtained from both patients. Additional experiments were conducted with CD4^+ T cell clone 57 derived from patient R12 and CD4^+ T cell clone 14 derived from patient R15, hereafter referred to as clone R12-57 and R15-14, respectively. It is noteworthy that peptide-specific Th cell clones could only be generated from postvaccination but not from prevaccination blood samples, underlining the efficacy of the vaccinations.

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**FIGURE 2.** HLA restriction analysis of CD4^+ T cell clones R12-57 (A) and R14-15 (B). CD4^+ T cells were stimulated with autologous peptide-loaded EBV-B cells (1 μM) in the presence or absence of anti-HLA-DR, -DQ, or -DP mAbs. Abs were tested for their capacity to inhibit IFN-γ production by the CD4^+ T cells. Values shown are the mean of triplicate determinations; bars, SD.
Importantly, the CD4+ T cell clones did not only recognize peptide-loaded DCs but also DCs loaded with a recombinant MAGE-3 protein demonstrating that these T cells were directed against a naturally processed MAGE-3 epitope (Fig. 1).

**Determination of the HLA restriction of the CD4+ T cell clones**

Since both patients were HLA-DPB1*0401 and the MAGE-3 peptide has been previously shown to be presented by this HLA molecule, we assumed that the peptide-specific CD4+ T cell clones would be HLA-DP4 restricted. To analyze the HLA restriction of the clones, we tested whether monoclonal anti-DR, anti-DQ or anti-DP Abs would inhibit the recognition of APCs by the CD4+ T cells. As expected, recognition of autologous peptide-loaded EBV-B cells by clone R12-57 was completely abolished in the presence of anti-DP Abs, but not in the presence of anti-DR or anti-DQ Abs (Fig. 2A). However, recognition of autologous peptide-loaded EBV-B cells by clone R15-14 was strongly inhibited in the presence of anti-DQ Abs (Fig. 2B). Similar results were obtained with several other T cell clones derived from both patients (data not shown). To further determine the HLA restriction of the two clones several EBV-B cell lines with known HLA class II typing were tested for their ability to present the antigenic peptide to the different T cell clones. All of the EBV-B cell lines expressing HLA-DPB1*0401 were able to present the antigenic peptide to clone R12-57, whereas clone R15-14 only recognized peptide-loaded EBV-B cells expressing HLA-DQB1*0604 (Table I).

**Analysis of the core epitope recognized by the different CD4+ T cell clones**

To test whether the two clones recognized different core epitopes of the 16-mer peptide KLLTQHFVQENLEY (MAGE-3243–258), we assayed the recognition of a set of truncated peptides. Truncation of either Q at the N terminus or L at the C terminus resulted in dramatic reduction or complete loss of recognition by the HLA-DQB1*0604-restricted clone R15-14. The DP4-restricted clone R12-57, however, only recognized peptides containing KLLTQHFVQENLEY as core sequence (Fig. 3).

The MAGE-3-specific CD4+ T cells show a TH1 cytokine profile

Because TH1 cells are thought to be advantageous in antitumor immunity, we analyzed the cytokine profile of the CD4+ T cell clones. Both clones showed a clear TH1 cytokine profile as they produced IFN-γ and TNF-α but no IL-4 or IL-10 upon stimulation with peptide-loaded DCs (Fig. 4, data are shown for clone R12-57).

**The MAGE-3-specific CD4+ T cells are stimulated by DCs loaded with dead or dying tumor cells.**

DCs loaded with apoptotic or necrotic MAGE-3-expressing melanoma cells (MEL 397, HLA-DP4+) efficiently stimulate CD4+ T cell clone R12-57 to produce IFN-γ, whereas DCs loaded with apoptotic or necrotic MAGE-3 tumor cells (BB 90 MEL) do not. Values shown are the mean of triplicate determinations; bars, SD.
Recognition of DCs loaded with dead or dying tumor cells
To be relevant in vivo, tumor-specific Th cells should recognize Ags that are naturally processed and presented by APCs that have taken up dead or dying tumor cells. Therefore, we tested whether the CD4^+ T cells were stimulated by DCs loaded with different preparations of tumor cells. As shown in Fig. 5, clone R12-57 recognized HLA-DP4^+ DCs loaded with apoptotic or necrotic HLA-mismatched MAGE-3^+ melanoma cells, whereas viable, apoptotic, or necrotic HLA-DP4^+ MEL 397 melanoma cells alone were not able to stimulate the CD4^+ T cell clone. In contrast, clone R12-57 was not stimulated by DCs loaded with apoptotic or necrotic BB 90 MEL cells, an uveal melanoma cell line that does not express MAGE-3. However, clone R15-14 did not show a significant recognition of DCs loaded with dead or dying tumor cells (data not shown). Because this finding could be due to a lower avidity of clone R15-14, we performed peptide titration analysis with both clones but there was no significant difference in peptide recognition (Fig. 6).

Direct recognition of tumor cells
Since it has been shown that the MAGE-3.DP4 peptide is naturally expressed on tumor cells (12), we assayed the direct recognition of melanoma cell lines by the different CD4^+ T cell clones (Fig. 7). Clone R12-57 was stimulated to produce IFN-γ by melanoma cell lines expressing MAGE-3 and HLA-DP4, but did not recognize HLA-mismatched MAGE-3-expressing melanoma cell lines. However, clone R15-14 showed only minimal recognition of HLA-matched MAGE-3-expressing tumor cells using an ELISPOT assay (data not shown).

Clone R12-57 lyses melanoma cells involving Fas-FasL interactions
Finally, we tested whether clone R12-57 showed cytolytic activity against MAGE-3- and HLA-DP4-expressing melanoma cell lines. In the absence of exogenous peptide, only poor lysis was observed (data not shown) but when loaded with low amounts of peptide (1 μM) the tumor cells were efficiently lysed (Fig. 8A). The observed lysis was Ag specific as there was no lysis when loading the tumor cells with an irrelevant HLA-DP4-binding tetanus peptide (data not shown). In addition, lysis was strongly inhibited in the presence of anti-TRAIL or anti-TNF-α Abs only weak inhibition was observed (Fig. 8B). Flow cytometric analysis demonstrated expression of Fas by the melanoma cell lines (Fig. 9A) and expression of FasL but not perforin by the CD4^+ T cell clone (Fig. 9B).

Discussion
We have previously shown that MAGE-3-specific CD4^+ Th cells can be readily induced in advanced melanoma patients by vaccination with peptide-loaded, monocyte-derived DCs matured by a cytokine mixture (IL-1β, IL-6, TNF-α, and PGE_2) which mimics monocyte-conditioned medium (13). In the present study, we analyzed the functional and specificity profile of these in vivo Th cell responses. Several Th cell clones recognizing peptide MAGE-3_243-258 were generated from postvaccination blood samples of two immunized patients. The induced Th cells were shown to be TH1 cells, which are thought to be favorable in antitumor immunity. Recently, a disease-associated bias in TH1/TH2 responses against a defined tumor Ag has been demonstrated in patients with renal cell carcinoma or melanoma (16). In this study, the majority of patients with active disease were skewed toward TH2-type responses against MAGE-6-derived epitopes, whereas normal donors and patients with no current evidence of disease either showed mixed TH1/TH2 or strongly TH1-polarized responses, underlining the importance of antitumor TH1 responses.

Importantly, the CD4^+ T cells recognized not only peptide-loaded DCs but as well DCs loaded with a recombinant MAGE-3 protein or (shown only for clone R12-57) apoptotic or necrotic tumor cell material. This finding implies a potential in vivo relevance of these vaccine-induced, tumor-specific Th cells as they should recognize DCs (and other APCs) at the tumor site that have taken up dead or dying tumor cells and further activate them via engagement of CD40 to allow for efficient CTL priming (9, 17–19). Beside providing help for the induction and maintenance of...
tumor-specific CTLs, CD4$^+$ Th cells may also play a broader role in mediating activation of macrophages and eosinophils (20, 21). Furthermore, IFN-$\gamma$ secreted by tumor-specific CD4$^+$ T cells at the tumor site has been shown to be very important for tumor regression involving inhibition of tumor-induced angiogenesis (22). The MAGE-3-specific CD4$^+$ T cells from patient R12 secreted IFN-$\gamma$ upon stimulation with HLA-matched MAGE-3-expressing melanoma cells, demonstrating that direct recognition of tumor cells might be yet another antitumoral effector mechanism of these Th cells. Tumor-specific CD4$^+$ Th cells have been even found to lyse HLA class II-expressing tumor cells (12, 23–25). In this study, clone R12-57 showed only poor lysis of HLA-matched tumor cells but this lysis was strongly enhanced in the presence of low amounts of exogenous peptide.

Different mechanisms by which CD4$^+$ T cells exert their cytotoxicity have been described, including the perforin/granzyme pathway (26–29), Fas-FasL interactions (30–32) and apoptosis induced by another member of the TNFR ligand family, TRAIL (33, 34). The latter was suggested to play an important role in the killing of melanoma cells by CD4$^+$ T cells, while the tumor cells were resistant to apoptosis induced by FasL (33). In contrast to these findings, our data suggest that the MAGE-3/HLA-DP4-specific CD4$^+$ T cells exerted their cytotoxicity via Fas-FasL interactions as the lysis of peptide-loaded melanoma cells was strongly inhibited in the presence of a blocking Ab against Fas, whereas only weak inhibition was observed in the presence of a blocking Ab against TRAIL or TNF-$\alpha$. We believe that this discrepancy simply reflects the diversity of mechanisms that can be used by cytolytic CD4$^+$ T cells. For instance, tumor-infiltrating CD4$^+$ T cells have been shown to lyse autologous lung carcinoma cells via both perforin/granzyme- and TRAIL-mediated mechanisms (34). In addition, cytolytic CD4$^+$ T cells specific for purified protein derivative of Mycobacterium tuberculosis have been shown to either use the perforin/granzyme or the Fas/FasL pathway depending on the kind of target cell (35). Some authors have argued that the observation of cytolytic activity in CD4$^+$ T cells would be an in vitro artifact (36) but only recently cytolytic CD4$^+$ T cells have been isolated ex vivo (26).

It is well known that class II peptides exist which can bind to different HLA molecules. This has been mainly reported for peptides binding to HLA-DR molecules and peptide-binding motifs largely shared by several common HLA-DR types have been identified (37). Recently, several promiscuous HLA-DR-restricted Th cell epitopes derived from tumor Ags such as HER2/neu, carcinoembryonic Ag, NY-ESO-1, and MAGE-3 have been described (24, 38–41). In the present study, we could demonstrate that promiscuous binding of tumor-specific peptides does not only occur for HLA-DR molecules but also for other class II molecules as peptide MAGE-3243–258 was recognized by CD4$^+$ Th cells in the context of HLA-DPB1*0401 and HLA-DQB1*0604. This makes it an excellent candidate for cancer immunotherapy because it is very immunogenic in vivo at least if delivered on DCs (13), and ~76% of Caucasians express HLA-DPB1*0401, HLA-DPB1*0402, or HLA-DQB1*0604. It is noteworthy that the CD4$^+$ T cell clones obtained from the two vaccinated patients differed not only in their HLA restriction but also recognized different core epitopes within the 16-mer peptide used for vaccination. For instance, T cell clones from patient R15 were stimulated by the original 16-mer peptide and 7 of 12 truncated peptides tested, whereas T cell clones from patient R12 only recognized the 16-mer peptide and 2 other truncated peptides (Fig. 3). Importantly, these T cell clones did not recognize peptide TQHVFQENYLEY (MAGE-3247–258) which has been previously identified as the minimal epitope efficiently recognized by CD4$^+$ T cells (12). These findings suggest that the use of longer peptides for vaccination is advisable because they may induce a broader Th cell response in patients.

The different peptide recognition pattern of the two clones is also interesting from another point of view as it might be one...
possible explanation for the observed differences in Ag recognition. For instance, clone R15-14 did not recognize peptide KQLL-

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