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Tumor-Reactive CD4+ T Cell Responses to the Melanoma-Associated Chondroitin Sulphate Proteoglycan in Melanoma Patients and Healthy Individuals in the Absence of Autoimmunity

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To avoid immune escape by down-regulation or loss of Ag by the tumor cells, target Ags are needed, which are important for the malignant phenotype and survival of the tumor. We could identify a CD4+ T cell epitope derived from the human melanoma-associated chondroitin sulfate proteoglycan (MCSP) (also known as high m.w.-melanoma-associated Ag), which is strongly expressed on >90% of human melanoma lesions and is important for the motility and invasion of melanoma cells. However, MCSP is not strictly tumor specific, because it is also expressed in a variety of normal tissues. Therefore, self tolerance should prevent the induction of strong T cell responses against these Ags by vaccination strategies. In contrast, breaking self tolerance to this Ag by effectively manipulating the immune system might mediate antitumor responses, although it would bear the risk of autoimmune disease. Surprisingly, we could readily isolate CD4+ Th cells from the blood of a healthy donor-recognition peptide MCSP_H11545 on HLA-DR11-expressing melanoma cells. Broad T cell reactivity against this Ag could be detected in the peripheral blood of both healthy donors and melanoma patients, without any apparent signs of autoimmune disease. In some patients, a decline of T cell reactivity was observed upon tumor progression. Our data indicate that CD4+ T cells are capable of recognizing a membrane glycoprotein that is important in melanoma cell function, and it may be possible that the sizable reactivity to this Ag in most normal individuals contributes to immune surveillance against cancer. The Journal of Immunology, 2007, 178: 7703–7709.
With respect to the functional role that MCSP seems to play in the oncogenic behavior of melanoma cells, it is not surprising that MCSP is broadly expressed on >90% of melanoma lesions (15). However, MCSP expression has also been described in a variety of normal tissues (e.g., endothelial cells, activated pericytes, chondrocytes, smooth muscle cells, certain basal keratinoyctes within the epidermis, as well as cells within the hair follicles) (16).

MCSP-targeted T cell-based immunotherapy, therefore, faces two problems. First, the induction of T cell responses by vaccination should be hampered by pre-existing tolerance. Second, in the case of induced T cell responses, autoimmunity might be a potential and serious drawback.

However, we could readily isolate CD4+ Th type 1 cells from the blood of a healthy donor that recognizes a peptide located in the core protein of MCSP on melanoma cells. Unequivocal T cell reactivity against this peptide could be detected in the peripheral blood of both healthy donors and melanoma patients in the absence of the clinical signs of autoimmunity.

Materials and Methods

Experiments were performed with cells from healthy donors and melanoma patients after having obtained informed consent and approval by the ethics committee of the Medical Faculty of the University of Erlangen-Nuremberg.

Cell lines, reagents, and Abs

EBV-transformed B (EBV-B) cell lines and tumor cell lines were cultured in RPMI 1640 medium (Cambrex) supplemented with 10% FCS (PAA Laboratories), 20 μg/ml gentamicin (PAA Laboratories), 2 mM l-Glutamine (PAA Laboratories), 10 mM Hepes (PAA Laboratories), and 10 mM sodium pyruvate (PAA Laboratories). Dendritic cells (DC) and CD4+ T cells were cultured in the same medium supplemented with 1% autologous plasma or 10% human serum, hereafter referred to as DC or T cell medium, respectively. Human IL-2 was purchased from Roche Diagnostic Systems; IL-4, IL-1β, IL-6, and GM-CSF from Strathmann; IL-7 from BioMOL; TNF-α from Bender MedSystems; and PGE, from Pfizer. The following mouse anti-human mAbs were used for blocking experiments: anti-HLA-DQ (SPV31) from Immunotech and anti-HLA-DP (B7/21) and anti-HLA-DR (L234) from BD Biosciences. For flow cytometric analysis, the following conjugated Abs were used: anti-CD4 (RPA-T4), anti-IFN-γ (25723.11), and respective mouse and rat isotype controls (all from BD Biosciences).

Peptides

Peptide LAQSGSAMPILANPSYVENTAGDVSYLFRVTGLQFGE LQK = MCSP643–714 was selected by using a database, allowing the prediction of a given HLA class I or II molecule ([17]: SYFPEITHI-Database: www.uni-tuebingen.de/uni/kxii/). The chosen peptide of 42 amino acid length (aa 673–714) covers most of the predicted HLA-DR binding motifs within the sequence of the MCSP core protein. All peptides were synthesized by conventional solid-phase peptide synthesis using 9-fluorenylmethoxycarbonyl (Fmoc) for transient NH-terminal protection and was characterized using mass spectrometry.

The pMFG Retrovirus encoding human invariant chain (II)-MCSP

A cDNA encoding aa 659–735 of the MCSP core protein (provided by Dr. G. Pluschke, Swiss Tropical Institute, Basel, Switzerland), was ligated downstream of the first 80 amino acids of the Ii, followed by an internal ribosomal entry site sequence derived from the encephalomyocarditis virus and by the sequence encoding a truncated form of the human low-affinity nerve growth factor receptor. Cell lines were also transduced with I-MAGE-3 as a control, as described previously (18).

DCs and CD4+ responder cells

DCs were generated from leukapheresis products from donor 4800, as described previously (19). In brief, 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 250 U/ml IL-4 for 6 days to generate immature, monocyte-derived DCs. Maturation was induced by adding a cytokine mixture consisting of 400 U/ml IL-1β, 1000 U/ml IL-6, 10 ng/ml TNF-α, and 1 μg/ml PGE2 (20). Mature DCs were harvested on day 7. CD4+ T lymphocytes were isolated by PBMC by negative selection using anti-CD5−, anti-CD14+, and anti-CD19+ mAbs coupled to magnetic microbeads (Miltenyi Biotec).

Generation of peptide-specific CD4+ T cell clones

Autologous immature DCs were loaded overnight with 10 μg/ml MCSP673–714 peptide and matured after 6 h by adding a cytokine mixture, as mentioned above. CD4+ T cells (1 x 10^5) were cocultured with 1 x 10^6 peptide-loaded DCs in 100 μl T cell medium/round-bottom microwell, supplemented with IL-4 (1000 U/ml), IL-12 (20 ng/ml), and TNF-α (5 ng/ml). The CD4+ T lymphocytes were restimulated on days 7, 14, and 21 with autologous DCs freshly loaded with peptide MCSP673–714 overnight (10 μg/ml) and matured by the cytokine mixture, adding IL-2 (10 U/ml) and IL-7 (5 ng/ml). Aliquots of each microculture (~4,000 cells) were assessed on day 30 for their capacity to produce IFN-γ when stimulated with ~15,000 autologous EBV-B cells that were loaded overnight with 10 μg/ml of peptide MCSP673–714, or with a 40 amino acid peptide derived from MAGE-12 protein as a negative control. After 20 h of coculture in round-bottom microwells in T cell medium supplemented with IL-2 (25 U/ml), IFN-γ released in the supernatant was measured by ELISA using reagents from MedGenix. Microcultures that were tested positive were then cloned by limited dilution, using irradiated, autologous, peptide-loaded EBV-B cells (10^4 cells/round-bottom microwell) and stimulator autologous immature DCs loaded with irradiated allogeneic LG2-EBV-B cells (10^6 cells/well) as feeder cells in the presence of IL-2 (50 U/ml), IL-4 (5 U/ml), IL-7 (5 ng/ml), and PHA (125 ng/ml; Sigma-Aldrich).

Recognition assays with peptides

CD4+ T cells (4 x 10^4/microwell) were cocultured with 1 x 10^5 peptide-loaded EBV-B cells from donors with different HLA class II typing. Supernatants were harvested after 20 h, and IFN-γ production was measured by ELISA. To screen a set of truncated peptides, autologous EBV-B cells were incubated for 1 h in the presence of different peptides in decreasing concentrations (3 μg/ml to 0.001 μg/ml). In addition, cytokine secretion was measured in the supernatant after stimulation of the clone with peptide-loaded EBV-B cells by using the Cytometric Bead Assay from BD Biosciences. 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 5 μg/ml.

Recognition assay with tumor cells

Several HLA-matched (ER-MEL 3 and ER-MEL 4) or mismatched (MEL 397 and LB 1622 MEL) MCSP-expressing melanoma cell lines were seeded at 2 x 10^4/flat-bottom microwell plates and incubated for 48 h to allow the formation of a monolayer. CD4+ T cells were then added (4 x 10^4/microwell plates) and, after 20 h, IFN-γ secreted in the supernatants was measured by ELISA.

MCSP expression profile analysis by quantitative real-time PCR (qPCR)

Most total RNA from normal tissues, melanoma lesions, and cell lines was prepared by GITC/CSCI procedure (21), and measured by an OD value at 260 nm. Total RNA from liver, heart, brain, testis, and thymus were from Ambion. Reverse transcription was performed using 1 μg mRNA with oligo(dT)18 as a primer. Mock cDNA was obtained by omitting reverse transcriptase. Part of the reverse transcription reactions with RNA from normal tissue (l/40) and from tumors (1/400) were used for qPCR (Eurogentec). The primers were as follows: forward, 5’- CTT CCG AAA ACg CAA CAA gA-20nt, 6735-6754, NCBI: X96753; reverse, 5’- Ttg CAg CAA gtc TCG gTg TC-20nt, 6830-6811. Specific 5’-FAM/3’-TAMRA labeled probe’s sequence was as follows: 5’- AgC TCC Agg Tcc TgA Ctg CCA AgC-24nt, 6767-6780, synthesized by Eurogentec. Thermal cycling and fluorescence monitoring were performed in an Applied Biosystems Prism 7700 sequence detector (Applied Biosystems). PCR conditions were 2 min at 55°C, 10 min at 95°C and 1 cycle at 95°C, 1 min and 60°C. The probe corresponds to the last exon of MCSP, near the 3’ end of the reading frame. Mock cDNA was used to test residual genomic DNA. Eight samples contained residual genomic DNA, and the MCSP data were corrected by subtracting the number of copies of MCSP estimated with the mock cDNA from the number of copies of MCSP estimated with cDNA. 18S rRNA copies were estimated with SYBR green dye to detect the 18S rDNA amplification accumulation. cDNA reverse transcribed with oligo(dT)18 was used as a template, because preliminary experiments indicated that equivalent results were achieved with oligo(dT)18 and random primers. A
total of 1/20,000 of the cDNA product was taken for each amplification reaction. The primers used were as follows: forward, 5’ TCg AAC gTC TgC CCT ATC AA and reverse, 5’/H11032 gCT ATT ggA gCA ggA ATT ACC. PCR conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C, followed by a one-cycle reaction for melting curve: 95°C for 20 s; 55°C for 20 s; increase from 55°C to 95°C in 20 min; and kept at 95°C for 20 s. The mRNA expression level of MCSP was expressed as relative unit per 10^5 18S rRNA copies.

Measurement of MCSP T cell reactivity by ELISPOT analysis

PBMC from healthy blood donors and melanoma patients (after having obtained informed consent) were plated in triplicates at 3*10^5/flat-bottom 96-well in medium containing 10% heat-inactivated human serum, and stimulated with 10^g/ml of peptide MCSP693–708. After 20 h, wells were washed and incubated with biotinylated mAb to IFN-γ (7-B6-1; Mabtech) for 2 h. Final staining and computer-assisted analysis was done as described before (22). Responses were considered significant if a minimum of 10 spot-forming cells per well were detected, and additionally, this number was at least twice that in negative control wells. In addition, PBMC (2.5*10^6 per 24-well) were once stimulated with peptide (10^g/ml) in the presence of IL-2 (5 U/ml) and IL-7 (10 ng/ml) and the ELISPOT was performed on day 7.

In some experiments, CD4+ T cells were isolated from PBMC by negative selection using magnetic microbeads (Miltenyi Biotec) and subsequently divided into CD45RA- and CD45RA+ T cells by positive selection. Both populations were stimulated with peptide for 1 week and tested for IFN-γ production following antigenic stimulation by ELISPOT as described above.

Intracellular cytokine staining

PBMC from healthy donors were stimulated with 10^g/ml of peptide MCSP693–708. On day 7, cells were restimulated with peptide or not or PHA (10 μg/ml) as a positive control in the presence of brefeldin-A (10 μg/ml; Sigma-Aldrich). After overnight stimulation, cells were first stained for CD4 and subsequently fixed with formaldehyde and permeabilized with saponine (fix/perm solution; BD Biosciences) to allow for intracellular staining with an anti-IFN-γ Ab or isotype control (BD Biosciences). Analysis was done by flow cytometry (FACScan and CellQuest Software, BD Biosciences).

Results

To identify a CD4+ T cell epitope of MCSP, CD4+ T cells of a healthy donor were stimulated with autologous DCs loaded with a 42 amino acid-long MCSP fragment (aa 673–714). This approach should allow the identification of naturally processed peptides as
the DCs take up the long peptide, process, and present the incorporated T cell epitopes in the groove of their HLA class II molecules.

**Generation of CD4\(^+\) T cell clones**

A total of 96 microcultures were set up, each containing CD4\(^+\) T cells and autologous-stimulator DCs loaded with peptide MCSP\(_{673-714}\) as stimulator cells. Responder cells were restimulated three times with DCs loaded with peptide and tested 10 days after the last restimulation for IFN-γ production after contact with autologous EBV-B cells loaded either with peptide MCSP\(_{673-714}\) or with a control peptide. Three positive microcultures were cloned by limiting dilution. Stably growing T cell clones were obtained from microculture C2. Additional experiments were performed with CD3\(^+\)CD4\(^+\) T cell clone C2.25, hereafter referred to as clone 25. Clone 25 specifically recognized autologous EBV-B cells loaded with the MCSP peptide (Fig. 1a).

**Identification of the 16-mer antigenic peptide**

To determine the core epitope recognized by clone 25 within the 42-mer, a set of 16-mer peptides, overlapping each other by 12 amino acids, was tested for recognition. Clone 25 recognized two overlapping 16-mer peptides, ETNAVGQDVSVLFRVT and VGQDVSVLFRTGALQ = MCSP\(_{693-708}\) (data not shown). In a second step, a set of truncated peptides derived from the sequence of the 16-mer peptide recognized best by the clone was tested to define the fine specificity of clone 25. Truncation of either D at the N terminus or A at the C terminus resulted in loss of recognition by the T cell clone (Fig. 1b). Peptide titration experiments with the truncated peptides revealed that the 16-mer peptide VGQDVSVLFRTGALQ was most efficiently recognized by clone 25. Significant T cell stimulation was only seen when using peptide concentrations >300 nM (Fig. 1c). Peptide recognition was abolished in the presence of an anti-HLA-DR Ab, and testing several EBV-B cell lines with known HLA class II typings revealed that the peptide was presented to clone 25 by HLA-DR11 (data not shown). To analyze the cytokine profile of clone 25, the concentration of different cytokines produced after stimulation with the 16-mer peptide was measured using a flow cytometry-based multiplexed assay. Clone 25 showed a TH1 cytokine profile producing IFN-γ and TNF-α, but no IL-4 and only low level of IL-10 (Fig. 1d).

The antigenic peptide is naturally processed and presented

Importantly, clone 25 did not only recognize EBV-B cells loaded with peptide, but also HLA-DR11\(^+\) EBV-B cells transduced with...
Given the expression of MCSP in normal tissues, peripheral T cell tolerance against this Ag might impede the induction of Ag-specific T cell responses. However, we could readily generate T cells from the blood of a healthy donor who did not show any signs of autoimmune disease. Therefore, we set out to detect the T cell reactivity against the identified epitope in the blood of healthy individuals and melanoma patients by ELISPOT analysis. Although, we could not detect ex vivo responses, after one in vitro stimulation, 12 of 14 tested healthy donors showed significant IFN-γ secretion upon peptide stimulation exceeding >300 spots in six donors (Fig. 5). To confirm the T cell responses detected by ELISPOT, and to characterize them in more detail, we additionally performed intracellular cytokine staining. Fig. 6 shows the IFN-γ production by CD4+ T cells from two healthy individuals upon stimulation with the MCSP693–708 peptide. To further define the origin of MCSP-reactive T cells, CD4+ T cells from healthy donors were divided into CD45RA+ and CD45RA− fractions by magnetic bead sorting and stimulated with peptide MCSP693–708. After 1 week, IFN-γ production upon stimulation with peptide or not was detected by ELISPOT.

Because it has been shown that tumor Ag-specific CD4+ T cells can directly recognize HLA class II-expressing tumor cells, and this would be of particular interest with regard to active-specific immunotherapy, we assayed the recognition of MCSP-expressing melanoma cell lines by clone 25. Melanoma cell lines ER-MEL-3 (HLA-DR7/11) and ER-MEL-4 (HLA-DR3/11) expressing MCSP and HLA-DR11 stimulated clone 25 to produce IFN-γ, whereas MCSP-positive but HLA-mismatched cell lines MEL 397 (HLA-DR4/14) and LB 1622 MEL (HLA-DR13/15) did not (Fig. 3).

**MCSP expression in tumors and normal tissues**

As the expression profile of tumor Ags is of crucial interest with respect to their clinical use in immunotherapy qPCR for MCSP was established to systematically analyze the mRNA expression profile in a set of malignant and normal tissues (Fig. 4). As expected, we found a strong mRNA expression in melanoma cell lines and metastases from cutaneous and uveal melanoma, but there was also a significant, albeit lower expression in normal tissues such as lung, heart, kidney, and thymus. It should be mentioned that these data reflect the expression of mRNA in the tested tissues, which does not necessarily correlate with the protein expression.

**FIGURE 7.** MCSP reactive T cells descend from the CD45RA− memory pool. CD4+ T cells from healthy donors were divided into CD45RA+ and CD45RA− fractions by magnetic bead sorting and stimulated with peptide MCSP693–708. After 1 week, IFN-γ production upon stimulation with peptide or not was detected by ELISPOT.
MCSP T CELL REACTIVITY

Discussion

MCSP is broadly expressed in melanoma and appears to be functionally relevant for adhesion, migration, invasion, and proliferation of melanoma cells, making it an interesting target Ag for immunotherapy of melanoma (16). However, MCSP has been shown to be also expressed in a number of normal tissues, raising concerns of the induction of autoimmunity when immunizing with this Ag. Nevertheless, we could readily generate T cells from the blood of a healthy donor, who did not show any signs of autoimmunity. Moreover, there was a broad T cell reactivity against MCSP in the blood of 12 healthy donors of 14 tested, again without any signs of autoimmunity.

To have a better idea of the expression profile of MCSP, we screened a number of tissues by qPCR and found MCSP to be overexpressed in melanoma as compared with normal tissues, a situation which we know from several other tumor Ags, which are successfully targeted in Ab-based cancer therapy such as Her2, CD20, or VEGF (23–25). In this context, it should be mentioned that targeting MCSP in melanoma patients with unlabelled or radio labelled anti-MCSP Ab 9.2.27 has not been associated with significant normal-organ-related accumulation or toxicity (26–28).

With qPCR, we could detect MCSP transcripts in a variety of normal tissues, including the thymus. Expression of MCSP has, in addition, been demonstrated on the protein level in some normal tissues (16). Despite this expression in normal tissues, CD4+ T cells seem to have escaped deletion in the thymus. These T cells should only show low to intermediate affinity to their Ag and, in fact, significant activation of the MCSP-specific T cells was only seen when using peptide concentrations >300 nM. However, the T cells could recognize MCSP- and HLA-DR11-expressing tumor cells without the need for exogenous peptide loading. These findings suggest that a significant number of MCSP-reactive, potentially tumor Ag-reactive Th cells with low to intermediate avidity circulate in the blood, which might be further activated and expanded by immunization strategies. These helper T cells should be capable to recognize tumor cells overexpressing MCSP, but do probably not recognize normal cells, which have a significant lower Ag expression. In addition, with respect to a vaccination approach with the CD4+ Th cell peptide described in this study, the risk of autoimmune pathology would be even more unlikely, because most somatic cells do not express HLA class II molecules under normal conditions.

Our findings are, to some extent, reminiscent of studies on T cell immunity to the ubiquitously expressed p53, where significant p53-specific Th responses have been detected in mice and patients with colorectal cancer (29–31). In addition, p53-specific CTL responses could be demonstrated in mice and humans, but at least in a mouse model, tolerance could be shown to be operative on the CTL level (32). We do not know whether this is also true for MCSP, but CTL responses in humans directed against wild-type MCSP have not been described so far. However, immunization of HLA-A2/Kb transgenic mice with MCSP cDNA-transfected DCs elicited a CD8+ CTL response specific for HLA-A2-binding MCSP peptides (33).

Another important aspect of our immunomonitoring results is the observation that the T cell responses in melanoma patients were infrequent and weaker as compared with normal healthy individuals, and that, at least in some patients, there was a clear correlation between disease progression and the loss or decline of MCSP T cell reactivity. This phenomenon raises the question of whether the precursor T cells are rendered anergic by a growing tumor load or trapped within the tumor and, thus, are no longer detectable in the peripheral blood. We cannot rule out that MCSP-reactive T cells may localize at the tumor site, but when rapidly growing melanoma metastases are excised, in most cases we do not see any lymphocytic infiltrate, indicating that the tumor often grows much faster than the immune system can counteract. Furthermore, our data indicate that the decline of MCSP-specific T cell reactivity is not due to a general immunosuppression caused by the tumor as we could still detect T cell reactivity to Ags other than MCSP in these patients. Be that as it may, MCSP-specific helper T cell reactivity seems to be associated with a favorable clinical course, suggesting a protective role against melanoma.
Taken together, we feel that MCSP represents a promising target Ag for T cell-based immunotherapy of melanoma.

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Disclosures
Two of the authors (G.S. and E.S.S.) have applied for a patent related to the work that is described in the present study.

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