The Signal Peptide of the Tumor-shared Antigen Midkine Hosts CD4+ T Cell Epitopes*

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Background: The CD4+ T cell response to the tumor antigen Midkine was unknown.

Results: Most of the T cell response to Midkine relies on T cell epitopes contained in its signal peptide.

Conclusion: The signal peptide of Midkine is accessible to HLA class II pathway for CD4+ T cell presentation.

Significance: It is a new function for signal peptides to contribute to tumor-specific CD4+ T cell response.

Because of the key role of CD4+ T cell response in immunity to tumors, we investigated the CD4+ T cell response to the recently identified tumor antigen Midkine (MDK). By weekly stimulations of T lymphocytes harvested from seven HLA-DR-typed healthy donors, we derived CD4+ T cell lines specific for eight MDK peptides. Most of the T cell lines reacted with the peptides 9–23 and 14–28, located in and overlapping the MDK signal peptide, respectively. Accordingly, the MDK signal peptide appeared to be rich in good binders to common HLA-DR molecules. The peptide 9–23-specific T cell lines were specifically stimulated by autologous dendritic cells loaded with lysates of MDK-transfected cells or with lysates of tumor cells naturally expressing the MDK protein. One T cell line was stimulated by HLA-compatible MDK-transfected tumor cells. By contrast, the peptide 14–28-specific T cell lines were not stimulated in any of these conditions. Our data demonstrate that CD4+ T cell epitopes present in the signal peptide can be accessible to recognition by CD4+ T cells and may therefore contribute to tumor immunity, whereas a peptide overlapping the junction between the signal peptide and the mature protein is not.

Midkine (MDK) is a heparin-binding growth factor with multiple functions. It plays a major role in neuronal development during embryogenesis (1) and has been implicated in ischemic renal injury (2), inflammatory responses (3), and blood pressure regulation (4). Injection of MDK diminishes myocardial infarct size in animal models (5, 6). MDK also contributes to tumor progression: blockade of MDK leads to tumor regression (7). This protein is overexpressed in various human malignancies, including bladder, prostate, breast, lung, liver, and colon tumors, although its expression is typically low or undetectable in normal adult tissues (8–10). In tumors, MDK exhibits angiogenic (11), mitogenic (12), and antiapoptotic activities (13). Its expression in tumors (8, 14) and its concentration in plasma (15) are associated with a poor disease outcome. Its various functions and expression in many tumors led us to investigate whether MDK can induce cellular cytotoxic responses in humans (16). By in vitro stimulation of CD8+ T cells collected from HLA-A2 healthy donors and immunization of HLA-A2 transgenic mice, we identified two CD8+ T cell epitopes and demonstrated that MDK-specific cytotoxic T lymphocytes can lyse tumor cells (16). One of these CD8+ T cell epitopes resides in the signal peptide, as described previously for other secreted tumor antigens (17–19). These findings suggest that MDK may be novel candidate for the development of a cancer vaccine.

CD4+ T lymphocytes enhance and sustain the tumor-specific CD8+ T cell response by providing cytokines and co-stimulation signals. CD4+ T lymphocytes also contribute to tumor regression by recruiting and activating phagocytes, by producing inflammatory cytokines, or by exhibiting direct cytolytic functions (20). Many vaccine strategies exploit CD4+ T cell functions to eliminate tumors and combine CD4+ and CD8+ T cell epitopes delivered in various forms such as DNA, recombinant viruses, proteins, or long polypeptide fragments. We investigated whether MDK can prime a CD4+ T cell response in multiple HLA-typed donors. As an overexpressed tumor antigen, a residual expression of MDK persists in healthy donors and may promote a tolerance status that would diminish the induction of a MDK-specific CD4+ T cell response. Also, MDK is a relatively small protein, and MDK-specific CD4+ T cell responses may be limited to particular haplotypes and hence may not be effective in all individuals. As we previously identified CD8+ T cell epitopes in the signal peptide, we considered the entire sequence of MDK including the mature form of the protein secreted by tumors and the signal peptide, which remains in the cell (21). Few CD4+ T cell epitopes have been found in signal peptides (22, 23), but here, we demonstrate that the MDK signal peptide contains both subdominant and cryptic CD4+ T cell epitopes.

MATERIALS AND METHODS

Peptides and Proteins—The human MDK sequence was retrieved from Uniprot (P21741) and comprises the signal peptide (amino acid positions 1–22) and the mature protein (23–143). Overlapping 15-amino-acid-long MDK peptides were

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2 The abbreviations used are: MDK, Midkine; DC, dendritic cell; IMDM, Iscove’s modified Dulbecco’s medium; PBMC, peripheral blood mononuclear cell.
optimized for the requirement of aliphatic or aromatic residues in the N-terminal part of the peptide for binding to HLA class II molecules and therefore covered the sequence 1–133. MDK and biotinylated peptides were purchased from Actiotech (Cambridge, UK) or synthesized using standard N-(9-fluorenyl)methoxycarbonyl (Fmoc) chemistry on an Advanced ChemTech Apex synthesizer (Advanced ChemTech Europe, Brussels, Belgium). Biotin was introduced using biotinyl-6-aminocaproic acid (Fluka Chimie, St. Quentin Fallavier, France) on the N terminus. As appropriate, peptides were purified by RP-HPLC on a C18 Vydac column (Interchim, Montluçon, France) to ~90% purity as determined by analytical HPLC.

**HLA Class II-specific Peptide Binding Assays**—HLA-DR and HLA-DP4 molecules were immunopurified from homologous EBV cell lines by affinity chromatography using the monomeric Mabs, L243 and B7/21, respectively (24–26). Binding to HLA-DR and HLA-DP4 molecules was assessed by a competitive ELISA as reported previously (24–26). The competition for the immunopurified HLA class II molecules is performed between a reporter peptide (biotinylated) and the peptide to be tested. The binding specificity for each HLA class II molecule was ensured by the choice of the biotinylated peptides as described previously (25, 26). Unlabeled forms of the reporter peptides were used as reference peptides to assess the validity of each experiment. A binding experiment is validated if the IC50 of the reference does not differ from more than a factor of 3 with its common value. Their sequences and IC50 values were as follows: HA 306–318 (PKYVKQNTLKLAT) for DRB1*01:01 (2 nM), DRB1*04:01 (6 nM), DRB1*11:01 (9 nM), and DRB5*01:01 (5 μM); YKL (AAAYAAKAALAA) for DRB1*07:01 (4 μM); A3 152–166 (EAEQLRAYLDGTGVE) for DRB1*15:01 (19 nM); MT 2–16 (AKTIAYDEEARGLE) for DRB1*03:01 (240 nM); B1 21–36 (TERRVLRTHYINRE) for DRB1*13:01 (40 μM); LOL 191–210 (ESWGAWDRTPKDHLTGGP) for DRB3*01:01 (21 nM); E2/E168 (AGDLAIEDTAKI) for DRB4*01:01 (3 μM); and Oxy 271–287 (EKKYFAATQFEPLAARL) for DPB1*04:01 (11 nM) and DPB1*04:02 (10 μM).

**Cell Lines and Cell Transfection**—Human hepatocellular carcinoma cells (HepG2), cervical cancer cells (HeLa), and melanoma cells (A375) were purchased from the American Type Culture Collection (Manassas, VA). The pcDNA3.1-MDK (pMDK) was developed as described previously (16). Twenty μg of this plasmid was introduced into HeLa cells (3 × 106 cells) by electroporation (0.9 kV, 250 microfarads, 0.4-cm gap) with a Gene Pulser II (Bio-Rad). The treated cells were suspended in 40 ml of prewarmed RPMI 1640 medium containing 10% fetal bovine serum and incubated under 5% CO2 at 37 °C. They were harvested 48 h later, and MDK expression was assessed by intracellular staining with an anti-MDK-phycoerythrin antibody (BD Biosciences) as described previously (16).

**Blood Samples and HLA-DR Genotyping**—Blood cells were collected from anonymous healthy donors at the Etablissement Français du Sang (EFS, Rungis, France) asuffy coat preparations after informed consent and following EFS guidelines. Peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation on Ficoll-Hypaque gradients (Sigma-Aldrich). The Gold SSP DRB1 typing kit (Invitrogen) was used for HLA-DR genotyping. HLA-DRB1 typing of the donors was as follows: 341 (04/01:15/01), 343 (11/01:15/01), 332 (01/01:14/01), 331 (04/01:07/01), 342 (01/01:11/04), 402 (04/01:13/01), 403 (03/13:01/13), 409 (01/02/12/01), 540 (07/01:16/01), 548 (11/01:03/01), 820 (01/01:04/01), and 821 (01/07:07/01).

**Generation of Antigen-specific T Cell Lines from Healthy Donors**—Monocyte-derived dendritic cells (DCs) were generated from plastic-adherent cells among PBMCs after 5–7 days of culture in AIM V medium (Invitrogen) supplemented with 1000 units/ml of rh-IL-4 (R&D Systems) and rh-GM-CSF (Miltenyi Biotech, Paris, France). Immature DCs were collected on day 5 or 6. Mature DCs were generated after the addition of 1 μg/ml LPS (Sigma). CD4+ T lymphocytes were isolated from the nonadherent phase by positive selection using an anti-CD4 monoclonal antibody coupled to magnetic microbeads (Miltenyi Biotech) and magnetic cell sorting, as recommended by the manufacturer (Miltenyi Biotech). Mature DCs (5 × 105) were incubated at 37 °C, under 5% CO2, for 4 h in 1 ml of complete IMDM containing a pool of MDK peptides, each peptide being at a concentration of 10 μg/ml. Complete IMDM was IMDM (Invitrogen) supplemented with 0.24 mM glutamine, 0.55 mM asparagine, 1.5 mM arginine (all amino acids from Sigma), 50 units/ml penicillin and 50 μg/ml streptomycin (Invitrogen), and 10% human serum. Pulsed cells were washed, and 105 was added to each round-bottom microwell already containing 105 autologous CD4+ lymphocytes in 200 μl of complete IMDM supplemented with 10 units/ml of IL-2 (R&D Systems) and 10 ng/ml IL-12 (R&D Systems). The CD4+ T lymphocytes were restimulated on days 7, 14, and 21 with autologous DCs freshly loaded with the MDK peptide pools and were grown in complete IMDM supplemented with 10 units/ml IL-2 (R&D Systems) and 5 ng/ml IL-7 (R&D Systems). The specificity of the T cell lines was investigated by IFN-γ enzyme-linked immunospot (EliSpot) assays on days 28 and 29 as described previously (27). Two to three consecutive EliSpot assays were performed in the same week. Antigen-presenting cells were autologous PBMCs or autologous immature DCs or murine L cells transfected with one HLA class II molecule (28). T cell lines were considered peptide-specific when their production of spots in the presence of antigens was at least two times higher than in their absence with a minimum of 20 spots. Positivity in a statistically unpaired Student t test was also evaluated (significance determined at p < 0.05). Peptide specificity of each T cell line was evaluated in at least two independent experiments.

**RESULTS**

**CD4+ T Cell Response Specific for MDK Peptides in Healthy Donors**—We first investigated the capacity of 18 peptides overlapping the MDK sequence to prime specifically CD4+ T cells from seven HLA-typed healthy donors. These donors were selected to represent all the most frequent HLA-DR molecules in the Caucasian population (Fig. 1 legend). CD4+ T cells were seeded in 96-well plates and stimulated weekly by mature DCs loaded separately with one of the two peptide pools. Peptide specificity of the T cell lines was evaluated by IFN-γ EliSpot using autologous PBMC as antigen-presenting cells (Fig. 1A). Four peptides were common to multiple T cell lines isolated from the seven donors: peptide 9–23 induced four T cell lines;
CD4+ T Cell Epitopes in the Signal Peptide of Midkine

**FIGURE 1.** Peptide specificity of CD4+ T cell lines raised against MDK peptide pools. CD4+ T cell lines were obtained after weekly stimulations for 3 weeks with autologous mature DCs loaded with one of the two MDK peptide pools (pool 1: 1–15, 4–18, 9–23, 14–28, 18–32, 25–39, 38–52, 52–66, and 64–78; and pool 2: 70–84, 74–88, 78–92, 84–98, 89–103, 99–113, 105–119, 110–124, and 119–133). After an initial IFN-γ EliSpot assay to screen the T cell lines for peptide specificity, positive T cell lines (104 cells) were incubated in duplicate with individual peptides (10 μg/ml) and 104 autologous PBMCs and analyzed by IFN-γ spot staining after 24 h of incubation. Statistical differences with the negative control (no peptide): *, p < 0.05; **, p < 0.01. A, examples of T cell lines specific for MDK peptides. Error bars, S.D. B, number of MDK peptide-specific T cell lines derived from the seven healthy donors tested. Sequences of the T cell epitopes are the following: 9–23, LTLLALTSAVAK; 14–28, LLALTSAVAKKKDKV; 18–32, TSATVKKDKVVKKG; 52–66, CGVG-FREGTCGAGQTG; 64–78, QTQRIRCRPVCNWKK; 70–84, CRPVCNWKEFGAGDC; 74–88, CNWKKEFGADCKYKF; 78–92, KEFGADCKYKFENWG; 99–113, GKTWQGTLKRAYN; 105–119, GTLKKARYNAQCGED; 119–133, ETIRVTPC-TPKTA.

The Peptides 9–23 and 14–28 Are Presented Differentially from Tumor Lysates to T Cells—We then investigated whether the identified MDK CD4+ T cell epitopes were appropriately presented by autologous DCs loaded with lysates of tumor cell lines expressing MDK (Fig. 3). T cell lines specific for the peptides 64–78 (403.7), 99–113 (403.5), and 78–92 (403.37) were indeed stimulated by autologous DCs loaded with a lysate of the hepatocellular carcinoma cells HepG2 expressing the MDK protein. They however did not react to DCs loaded with a lysate of the cervical cancer cells HeLa, which did not express MDK. In contrast, the three T cell lines specific for the peptides 14–28 (Fig. 3A) were not stimulated by either HepG2 or HeLa lysates. To confirm these results, we used HeLa cells transiently transfected with MDK in the T cell presentation assays (Fig. 3B). Two T cell lines specific for the peptide 9–23 were activated by the lysates of MDK-transfected HeLa cells but
not by a lysate of nontransfected HeLa cells. By contrast, the two T cell lines specific for the peptide 14–28 reacted with none of the lysates (HeLa, HeLa transfected with MDK, and HepG2). We concluded from these experiments that epitopes 64–78, 78–92, and 99–113 present in the mature form of MDK were processed appropriately by immature DCs loaded with lysates of MDK-expressing tumor cells. This was also the case for

### TABLE 1

| Peptides | DR1 | DR3 | DR4 | DR7 | DR11 | DR13 | DR15 | DRB3 | DRB4 | DRB5 | DP401 | DP402 | Nb HLA II |
|----------|-----|-----|-----|-----|------|------|------|------|------|------|-------|-------|-----------|
| 1–15     | 21  | 226 | 49  | 7   | 21   | 267  | 204  | 161  | 20   | 19   |       |       | 5        |
| 4–18     | 21  | 136 | 20  | 94  | 19   | 37   | 65   | 46   | 6    | 18   |       |       | 9        |
| 9–23     | 0.2 | 1   | 13  | 0.3 | 5    | 45   |      |    |      |      |       |       | 8        |
| 14–28    | 34  | 599 | 48  | 45  | 132  |      |      |      |      |      |       |       | 4        |
| 18–32    |     |     |     |     |      |      |      |      |      |      |       |       |          |
| 25–39    | 1,251 |   |     |     |      |      |      |      |      |      |       |       | 0        |
| 38–52    | 1,305 | 1,859 | 923 |   |      |      |      |      |      |      |       |       | 0        |
| 52–66    | 32  | 701 | 833 |   |      |      |      |      |      |      |       |       | 0        |
| 64–78    | 246 | 558 | 2,066 | 504 |      |      |      |      |      |      |       |       | 1        |
| 70–84    | 53  | 1,562 |   |     |      |      |      |      |      |      |       |       | 1        |
| 74–88    | 333 | 2   | 1,231 |   |      |      |      |      |      |      |       |       | 1        |
| 78–92    | 299 | 1,457 | 800 | 216 | 226  | 114  | 167  | 378  |      |      |       |       | 1        |
| 84–98    | 187 | 362 | 141 | 292 | 52   | 49   |      |      |      |      |       |       | 2        |
| 99–113   | 1,460 |     |     | 74  | 2,333 |   |      |      |      |      |       |       | 0        |
| 105–119  | 97  | 492 | 1,008 | 225 |      |      |      |      |      |      |       |       | 1        |
| 110–124  | 10  | 6   | 158 | 69  |      |      |      |      |      |      |       |       | 1        |
| 119–133  | 2   | 1,289 | 819 | 763 |      |      |      |      |      |      |       | 2        |

![FIGURE 2. HLA restriction of peptide-specific T cell lines](image1)

MDK-specific T cell lines (1–2 × 10⁴ cells) were incubated in duplicate with their appropriate peptide and 3 × 10⁴ L cells transfected with one HLA class II molecule or 3 × 10⁴ homologous EBV cell lines (STEILIN, DRB1*03:01/DRB3*01:01 or 06AD, DRB1*13:01/DRB3*01:01). Their activation was revealed in duplicate by IFN-γ EliSpot. Names and peptide specificities of the T cell lines are indicated in the panels. Controls evaluate the autopresentation of the peptides to the T cells and did not contain presenting cells (L cells or EBV cells). Statistical differences with autopresentation control: *, p < 0.05; **, p < 0.01. Error bars, S.D.

![FIGURE 3. Stimulation of peptide-specific T cell lines by immature DCs loaded with tumor lysates](image2)

A. Cell lysates of HeLa and HepG2 cells were obtained by five rapid freeze-thaw cycles in AIM V medium. Immature DCs (1 equivalent to 2.5 million of cells per ml) were pulsed for 4 h with the tumor lysates (equivalent to 2.5 million of cells per ml). T cell lines (1–3 × 10⁴ cells) were incubated with 5000 pulsed DCs for 24 h and then tested by IFN-γ EliSpot assay. Statistical differences with the negative control (no peptide): *, p < 0.05; **, p < 0.01. Error bars, S.D. Peptide stimulation was evaluated using PBMCs as antigen-presenting cells.

B. The same protocol was used for other T cell lines and included lysates of MDK-transfected HeLa cells (HeLa pMDK).
epitope 9–23, mostly in the signal peptide (position 1–22). By contrast, epitope 14–28, which straddles the junction between the signal peptide and the mature protein, did not appear to be presented by immature DCs loaded with the lysates.

The Peptides 9–23 and 14–28 Are Presented Differentially to T Cells from a MDK-expressing Tumor Cell Line—To investigate the direct presentation of CD4+ T cell epitopes from MDK-expressing tumor cell lines, we looked for tumor cell lines that concomitantly expressed MDK and HLA-DR molecules. Unfortunately, none of the HLA-DR-expressing tumor cells of our cell collection also expressed MDK. We therefore produced a new cell line by transfecting A375 melanoma cells that express HLA-DR (HLA-DRB1*04:01/07:01) with an MDK-encoding plasmid (Fig. 4). The resulting stable clone (A375.MDK) exhibited a homogeneous level of MDK expression by intracellular staining and FACS analysis. 3 × 10⁶ A375, 3 × 10⁶ A375.MDK cells, or 5 × 10⁶ PBMCs were incubated in duplicate with the T cell lines (1–3 × 10⁶ cells) for 24 h. Peptides (10 μg/ml) and antibodies (L243 anti-HLA-DR or SPVL3 anti-HLA-DQ) were added directly to the culture. T cell activation was assessed by IFN-γ ELISPOT assay. Statistical differences with the negative control (no peptide): *, p < 0.05; **, p < 0.01. Statistical differences with the positive control in anti-HLA inhibition experiments are shown. Error bars, S.D.

FIGURE 4. Presentation to peptide-specific T cell lines of MDK peptides by HLA-DR+/melanoma cells transfected with the MDK construct. A375 melanoma cells (HLA-DRB1*04:01/07:01) were transfected with the MDK construct, and a stable clone expressing MDK (A375.MDK) was isolated after assessment of MDK expression by intracellular staining and FACS analysis. 3 × 10⁶ A375, 3 × 10⁶ A375.MDK cells, or 5 × 10⁶ PBMCs were incubated in duplicate with the T cell lines (1–3 × 10⁶ cells) for 24 h. Peptides (10 μg/ml) and antibodies (L243 anti-HLA-DR or SPVL3 anti-HLA-DQ) were added directly to the culture. T cell activation was assessed by IFN-γ ELISPOT assay. Statistical differences with the negative control (no peptide): *, p < 0.05; **, p < 0.01. Statistical differences with the positive control in anti-HLA inhibition experiments are shown. Error bars, S.D.

DISCUSSION
In this paper, we have evaluated the capacity of MDK to prime CD4+ T lymphocytes in humans and localized several CD4+ T cell epitopes of MDK-restricted to different HLA-DR molecules. Two CD4+ T cell epitopes, overlapping the MDK signal peptide but differing in their processing outcome in tumor cells, were responsible for a large proportion of the T cell response.

By in vitro stimulation of CD4+ T lymphocytes collected from healthy donors, we derived peptide-specific T cell lines and identified several CD4+ T cell epitopes in the MDK sequence including in its signal peptide. Antigen-specific T cell line derivation is a very sensitive method to detect antigen-specific T cells in multiple HLA-typed subjects (27, 29). It usually generates a sufficient number of specific T cells to characterize the restriction elements and the antigen processing of the T cell epitopes, as intended in this study. We found that T cell lines induced by the peptides 9–23, 64–78, 78–92, and 99–113 were specifically stimulated by DCs loaded with lysates of tumor cells naturally expressing MDK or transfected with MDK. We also derived a T cell line specific for the peptide 9–23, which was stimulated by a HLA-DR-compatible tumor cell line transfected with MDK.

As proposed recently (30), these epitopes can be qualified as subdominant, at least, as T cells primed with the corresponding peptides were stimulated by the whole antigen presented in native conditions. They may therefore contribute to a tumor-specific response. The T cell epitopes we identified include the peptide 9–23, which is more than half of the MDK signal peptide. The peptide 9–23 was found T cell stimulating in vitro for approximately one third of the 25 naïve donors tested (data not shown), and the magnitude of T cell response varied from 1 to 6 independent 9–23-specific T cell lines per donor. To our experience, this peptide induces a substantial T cell response. To our knowledge, few CD4+ T cell epitopes contained in signal peptides have been reported (22, 23). They have been delineated in signal peptides of the tumor antigen Muc-1 (23) and the Cafl1 antigen of Yersinia pestis (22).

As stated by the authors, BALB/c mice inoculated with metastatic MUC1-transfected murine DA3 mammary tumor cells exhibited significantly prolonged survival following vaccination with MUC1 signal peptide (23). Some HLA-DR1-transgenic mice infected with Y. pestis responded to peptides contained in the signal peptide of Cafl1, suggesting that after cleavage from Cafl1, the signal sequence is available for antigen presentation during infection (22).

Signal peptides contain numerous hydrophobic residues that serve to anchor the nascent polypeptide to the membrane (31). These residues may also serve as anchor residues for HLA-DR molecules. Indeed, this is consistent with our binding experiments that revealed a large specificity of the MDK signal peptide for HLA-DR molecules. Hydrophobic and aromatic residues constitute primary anchor residues for the pocket P1 of all HLA-DR (32) and HLA-DQ molecules. Interestingly, aromatic residues are also present in the MDK signal peptide (31). These residues may also serve as anchor residues for HLA-DR molecules. Indeed, this is consistent with our binding experiments that revealed a large specificity of the MDK signal peptide for HLA-DR molecules. Hydrophobic and aromatic residues constitute primary anchor residues for the pocket P1 of all HLA-DR (32) and HLA-DP4 (26) molecules. These binding data also revealed that some of the MDK peptides were promiscuous, for example peptides...
1–15 and 4–28, and did not elicit any T cell response, as described previously for other peptides (27). We reported previously (16) that the MDK signal peptide also hosted HLA-A2-restricted CD8+ T cell epitopes, as has been found for numerous tumor and viral antigens (17–19). Again, the presence of several hydrophobic residues in signal peptides favors their binding to HLA-A2 (33). Thus, signal peptides appear to harbor both CD4+ and CD8+ T cell epitopes.

CD4+ T lymphocytes are known to contribute in vivo to tumor immunity by sustaining CD8+ T cell responses, by providing cytokines such as IFN-γ and by recruiting phagocytes (20, 34). It has been also proposed that tumor cells expressing HLA class II can be the target of CD4+ T cells by presenting their specific epitopes (35). Experiments performed with DCs loaded with lysates and with the A375 tumor cells transfected with MDK demonstrated the tumor specificity of T cells specific for the MDK T cell epitopes: 64–78, 99–113, 78–92, and the MDK T cell epitope 9–23 contained in the signal peptide. These T cells are therefore expected to be stimulated in vivo by presentation of MDK present in the tumors but captured by the DCs and possibly by the tumors themselves. They could therefore exert helper functions in vivo and contribute to tumor immunity. Because of the 9–23 also encompasses a HLA-A2-restricted T cell epitope, this peptide should be able to elicit in vivo both CD4+ and CD8+ tumor-specific T cells. An interesting issue would be to investigate whether the endogenous expression of MDK in the tumors initiates specific immune responses in cancer patients, including CD4, CD8, and B cell responses. Because many tumor antigens do not generate such a spontaneous response (36), we do not exclude the lack of spontaneous responses to MDK. However, the preexisting T cells we have detected could be either recruited in vivo by multiple injections or amplified by ex vivo stimulations.

Most of the CD4+ T cell lines raised against MDK peptides were specific for peptide 14–28, which straddles the junction between the peptide signal and the mature protein. However, 14–28-specific T cell lines did not react to DCs loaded with lysates of tumor cells expressing MDK or to HLA-compatible tumor cell lines transfected with MDK. Thus, peptide 14–28 behaved as a cryptic epitope: 14–28-specific T cell lines did not recognize the native antigen expressed by a tumor (30). T cells primed by the MDK peptide 14–28 are therefore not tumor-specific. The signal peptide of secreted proteins, such as MDK, is cleaved in the lumen of the endoplasmic reticulum by signal peptidase and signal peptide peptidase (21). The cleavage site is at the 22nd position of the MDK sequence, the 14–28 epitope is presumably cleaved by signal peptidase and therefore not displayed in MDK produced by the tumor to T cell presentation by HLA class II molecules. This cleavage may also contribute to protect the epitope-specific T cell repertoire from negative selection or peripheral tolerance. It is consistent with the superiority of the 14–28 peptide over all of the other peptides for priming specific T cells. Peptide peptidase has been implicated in the processing of the preprocalcitonin tumor antigen (19). Presentation of the preprocalcitonin 16–25 CD8+ T cell epitope is inhibited by the preincubation of tumor cells with the serine protease inhibitor dichloroisocoumarin. However, in our experiments dichloroisocoumarin was toxic to the A375 melanoma cell line, even a low dose, such that they were uninformative (data not shown). Proteases involved in the MHC class II pathway are difficult to identify unambiguously using inhibitors (37, 38). This may be because the peptide protrudes from the HLA class II molecules and does not require a very precise length to be presented to T cells or because other redundant proteases might cleave the peptide (39). Formally, we therefore can only conclude that location of this 14–28 cryptic epitope is consistent with peptide peptidase being involved in its cleavage during the MDK elongation.

We delineated several CD4+ T cell epitopes with a broad specificity for HLA class II molecules, but the capacity of the mature form of MDK to prime a CD4+ T cell response in humans is limited. Most of the T cell lines are specific for the peptides 9–23 and 14–28, and very few are specific for the mature form of MDK. The number of T cell lines specific for the mature form of MDK we obtained was small compared with the results with other antigens such as survivin (40) or viral peptides (27, 29). In our assays, the yield of T cell lines depends on the number of preexisting T cells in the blood of the donors at the initiation of the T cell culture. It was not the goal of this study to evaluate the size of the MDK-specific T cell repertoire, as we have already described for other proteins (41–43), but the low yield of T cell lines suggests that there were few preexisting CD4+ T cells specific for the mature form of MDK. A low level of MDK is detected in normal subjects (44), and this residual expression may contribute to reduce the MDK T cell repertoire. According to others (45, 46), injection of recombinant MDK is expected to induce a CD4+ T cell response in humans beings with a limited amplitude and consequently be poorly immunogenic (42, 43, 47). This could be an advantage for therapeutic applications of the MDK protein to treat cardiac ischemia (48) and neuronal disorders (49).

In conclusion, by investigating the CD4+ T cell response specific for MDK, we mainly demonstrated the relevance of a CD4+ T cell epitope contained in its signal peptide to contribute to the tumor-specific T cell response. By contrast, another epitope, overlapping the junction between the signal peptide and the matured protein, was cryptic.

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