Identification of Novel Subdominant Epitopes on the Carcinoembryonic Antigen Recognized by CD4$^+$ T Cells of Lung Cancer Patients[1]

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The carcinoembryonic Ag (CEA) is an attractive target for immunotherapy because of its expression profile and role in tumor progression. To verify the existence of spontaneous anti-CEA CD4$^+$ T cells in lung cancer patients, we first identified CEA sequences forming naturally processed epitopes, and then used the identified epitopes to test their recognition by CD4$^+$ T cells from the patients. We had previously identified CEA$_{177–189/355–367}$ as an immunodominant epitope recognized by CD4$^+$ T cells in association with several HLA-DR alleles. In this study, we identified four additional subdominant CEA sequences (CEA$_{99–111}$, CEA$_{425–437}$, CEA$_{568–582}$, and CEA$_{666–678}$), recognized in association with one or more HLA-DR alleles. Peptide-specific CD4$^+$ T cells produced proinflammatory cytokines when challenged with the native protein and CEA-expressing tumor cells, thus demonstrating that the identified CEA sequences contain naturally processed epitopes. However, CEA is expressed in the thymus and belongs to the CD66 family that comprises highly homologous molecules expressed on hemopoietic cells, raising concerns about tolerance interfering with the in vivo development of anti-CEA immunity. We thus tested the spontaneous reactivity to the identified epitopes of peripheral blood CD4$^+$ T lymphocytes from eight early-stage lung cancer patients bearing CEA-positive tumors. We found GM-CSF- and IFN-γ- producing CD4$^+$ T cells in two patients. Our data indicate that CD4$^+$ immune responses against CEA develop in neoplastic patients, suggesting that tolerance toward CEA or cross-reactive CD66 homologous molecules might be either not absolute or be overcome in the neoplastic disease. *The Journal of Immunology,* **2006, 176: 5093–5099.**

The carcinoembryonic Ag (CEA)[3] is a 180-kDa glycoprotein expressed at high levels during embryonic development but at very low levels in adult gastrointestinal epithelium (reviewed in Ref. 1). It is instead overexpressed in almost all colorectal cancers and in a large percentage of non-small cell lung (70%) and breast (50%) cancers (1), making it an attractive target for immunotherapy (reviewed in Refs. 2 and 3).

It has been recently reported (4), however, that human medullary thymic epithelial cells express CEA. Moreover, CEA, or CD66e, belongs to the CD66 Ig supergene family that also comprises molecules (5), which in addition to epithelial cells, are expressed in normal cells of hemopoietic origin (i.e., neutrophils) and share with CEA regions of high homology (5). These considerations raise concerns both on the possible existence of central or peripheral tolerance interfering with the in vivo development of anti-CEA immunity and the induction of autoimmunity in therapeutic settings.

Evidences exist that CEA is immunogenic: 1) anti-CEA Abs were found in the sera of colon and breast cancer patients (6, 7), 2) cancer vaccines expressing CEA have induced T cell responses in vaccinated patients (8–12), and 3) CEA epitopes recognized by CD8$^+$ (13–17) and CD4$^+$ (18–21) T cells have been identified after in vitro priming with repeated peptide stimulation.

Little is known so far, however, about spontaneous anti-CEA T cell immunity in neoplastic patients. Naturally elicited anti-CEA CD8$^+$ T cell responses have been shown in colon cancer, but not in breast cancer patients (22), while evidence of CD4$^+$ T cell immunity against CEA is still lacking.

Among CEA-expressing tumors, lung cancer is the leading cancer killer in both men and women. Therefore, we decided to address the existence of spontaneous anti-CEA CD4$^+$ T cell responses in the blood of lung cancer patients. To this aim, we first identified CEA sequences forming naturally processed epitopes, and then used the identified epitopes to test their recognition by CD4$^+$ T cells from the patients. Collectively, our data demonstrate that CEA induces in vivo spontaneous CD4$^+$ T cell immunity.

Materials and Methods

Subjects and cell

PBMC were obtained from seven healthy subjects and eight lung cancer patients. The Institutional Ethics Committee had approved the study protocol and informed consent was obtained from all donors before blood sampling. Lung adenocarcinoma Calu-1 (DR*07, DR*14) and colon carcinoma Lovo (DR*13) cell lines were purchased from the American Type Culture Collection (ATCC). HLA-DR homozygous LCL used were: Com (DR*03), established in our laboratory; TEM (DR*14), provided by K. Fleischhauer (H. San Raffaele, Milan, Italy); and Pitout (DR*07), purchased from the European Collection of Cell Culture (Salisbury, U.K.).

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[3] Abbreviations used in this paper: CEA, carcinoembryonic Ag; LCL, lymphoblastoid cell line; Pt, patient.

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Selection and synthesis of CEA peptides

Selection of CEA sequences, based on the TEPITOPE algorithm (23), has been previously described (19). The CEA sequences used here are reported in Table I. Sequences corresponding to analogs on homologous proteins CD66a, b, c, and d were also synthesized: CD66a/c$_{99-111}$ (ETIYPNASL LIQN), CD66b$_{99-111}$ (ETIYPNASLLMRN), CD66d$_{99-111}$ (ETIYTNASL LIQN), CD66a$_{247-259}$ (TYYRPGANLSLSC), CD66b$_{247-259}$ (TYYHAG VNLNLSC), and CD66c$_{247-259}$ (ANYRPGENLNLSC). CEA and analog sequences were synthesized by the stepwise solid-phase method as previously described (24), and synthetic peptides were purified by semipreparative reverse-phase HPLC. The purity of the peptides was confirmed by analytical reverse-phase HPLC, and the mass was determined by MALDI-TOF analysis with a Voyager-RP Biospectrometry Workstation (PE Biosystem). Observed experimental values were in agreement with the theoretical calculated ones. The peptides were lyophilized, reconstituted in DMSO at 10 mg/ml, and diluted in RPMI 1640 (Invitrogen Life Technologies) as needed.

In vitro propagation of CD4$^+$ T cell lines and clones

Synthetic peptides were pooled (CEA subdominant pool) and used to stimulate the PBMC from the donors as described previously (19). Briefly, 20×10^6 PBMC were cultured for 7 days in RPMI 1640 (Invitrogen Life Technologies) supplemented with heat-inactivated human AB serum (10%; BioWhittaker), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (50 mg/ml; BioWhittaker) (tissue culture medium) in the presence of the CEA subdominant pool (1 μg/ml of each peptide). The reactive lymphoblasts were isolated on a Percoll gradient (25), expanded in IL-2 (10 U/ml) containing medium (TCGF, Lymphocult; Biotest Diagnostic), and restimulated at weekly intervals with the same amount of peptides plus irradiated (4000 rad) autologous PBMC as APC. CD4$^+$ T cell clones were obtained by limiting dilution from polyclonal lines as described elsewhere (26).

Flow cytometry

Cytofluorimetric analyses were performed on a FACStar^{Plus} (BD Biosciences). We used the following mAbs: anti-CD4-PE and anti-CD8-FITC (BD Biosciences), anti-DR (D1.12 hybridoma; ATCC), and anti-HLA class I (W6/32 hybridoma; ATCC). FITC-conjugated rabbit anti-mouse Ig Ab (DakoCytomation) was used as second step reagent in indirect immunofluorescence stainings.

CD4$^+$ T cell proliferation and stimulation assays

CD4$^+$ T cells (1×10^4) were seeded in 96-well plates in the presence of CEA-expressing tumors (3×10^4), or irradiated autologous PBMC (1×10^5), or autologous or homozygous HLA-DR-matched LCL ($0{,}5 \times 10^5$) as APC plus each single peptide forming the pool (10 μg/ml), or the purified CEA protein (5–20 μg/ml; BiosPacific) or normal human Ig (20 μg/ml, (Venimmun N; Aventis Behring), or the heat-inactivated cathepsin L protein (5 μg/ml; Calbiochem). In peptide titration experiments, the following concentrations of peptides were added: 10–5-1–0.5–0.1–0.05 and 0.001 μg/ml. Triplicate wells with CD4$^+$ T cells alone and tumor cells or APC alone were used as controls. Three wells with CD4$^+$ T cells plus APC did not receive any stimulus to determine the basal growth rate. In inhibition experiments, mAb D1.12 or an isotype-matched irrelevant mAb (W6/32; ATCC) was added at 25–50 μg/ml. After 48 h, one-half of the medium was removed for cytokine secretion assays, and the cultures were pulsed for 16 h with [^{3}H]TdR (1 μCi/well, 6.7 Ci/mol; Amersham). The cells were collected with a FilterMate Universal Harvester (Packard Instrument) in specific plates (Unifilter GF/C; Packard Instrument), and the thymidine incorporated was measured in a liquid scintillation counter (TopCount NXT; Packard Instrument). GM-CSF and IFN-γ secretion was measured using standard ELISAs (BioSource Europe) according to the manufacturers' instructions. In competition assays, increasing amounts of competitor peptides (1, 5, 10, and 50 μg/ml) were preincubated with the APC for 2 h and then CD4$^+$ T cells were added in the presence of a suboptimal concentration (5 μg/ml) of the stimulating peptides.

Cytotoxicity assay

CD4$^+$ T cells were tested for specific lytic activity in a standard 4-h ^{51}Cr release assay as described previously (27). The following targets were used: Calu-1 cells, unpulsed and peptide-pulsed LCL. To allow the expression of MHC class II molecules, tumor cells were cultured for 48 h in the presence of IFN-γ (1000 U/ml; R&D Systems).

Western blot analysis

Two million cells from tumor cell lines were washed twice with TBS and lysed directly in the culture flask by adding 1 ml of TRIzol reagent (Invitrogen Life Technologies), and 50–100 mg of fresh lung tumor tissue were homogenized in 1 ml of TRIzol using a power homogenizer (PBI International). Proteins from the lysates were obtained after DNA precipitation in 95% ethanol. Samples were electrophoresed in 7% polyacrylamide gel and then transferred to nitrocellulose paper. Immunoblotting was performed as described by Towbin et al. (28). The blot was incubated with 5% nonfat dry milk in TBS buffer, then with 1/1000 dilution of anti-CEA IgG Ab conjugated to peroxidase (a generous gift from Dr. C. Rosa, Sorin, Saluggia, Italy) for 1 h and processed for ECL according to the supplier's instructions.

In vitro restimulation assay

CD4$^+$ T cells were purified from total PBMC by positive magnetic selection (Miltenyi Biotec) and cultured in tissue culture medium with irradiated (4000 rad) CD4$^+$-depleted PBMC as APC at a 1:3 ratio in 96-well plates in six replicates for each condition as described elsewhere (29). Stimuli were PHA (1 μg/ml), as a positive control, CD4$^+$ T cells in the presence of the APC only, as baseline, and each single peptide (10 μg/ml). At day 7, IL-2 (25 U/ml) was added without further Ag stimulation. At day 14, 150 μl of supernatant was removed from each well for cytokines detection as described above. In the case of patient (Pt) 15, we repeated the experiment at 6 mo after surgery. Moreover, to verify whether peptide-specific CD4$^+$ T cells recognize the native CEA protein, at day 14 CD4$^+$ T cells stimulated with peptide CEA$_{99-111}$ were collected, washed, and rechallenged with the CEA protein or normal human IgG in the presence of autologous irradiated CD4$^+$-depleted PBMC as APC. After 2 days of culture, GM-CSF release in the supernatant was detected, as described above.

Table I. *Repertoire of CEA sequences recognized by polyclonal CD4$^+$ T cells propagated in vitro with the CEA subdominant pool*[a]

	Peptide Sequence	Donor 2	Donor 3
B + APC	None	2 ± 0.2	0.6 ± 0.1
CEA$_{13-25}$	IPWQRLLLTASLL	0.7 ± 0	0.8 ± 0
CEA$_{51-63}$	VLLLVHNLPQHLF	1.9 ± 0.4	0.8 ± 0
CEA$_{99-111}$	EIIYPNASLLIQN	7.2 ± 0.9***	24 ± 0.2***
CEA$_{117-129}$	TGFYTLHVIKSDL	2.3 ± 0.2	0.8 ± 0
CEA$_{425-437}$	TYYRPGVNLSLSC	7.6 ± 0.7***	1 ± 0.1
CEA$_{447-459}$	YSWLIDGNIQQHT	2.2 ± 0	0.6 ± 0
CEA$_{533-545}$	LWWVNGQSLPVSP	2.4 ± 0.3	0.6 ± 0
CEA$_{568-582}$	AYVCGIQNSVSANRS	4.4 ± 0.6***	0.7 ± 0.1
CEA$_{652-666}$	TYACFVSNLATGRNN	0.5 ± 0	0.6 ± 0
CEA$_{666-678}$	NSIVKSITVSASG	1.9 ± 0	6.2 ± 0.3***

[a] Polyclonal CD4$^+$ T cell lines from the two healthy donors (donors 2 and 3), propagated in vitro with the CEA subdominant pool, were tested with each single peptide (10 μg/ml) forming the pool in 2-day microproliferation assays. The data are expressed as cpm $\times 10^{-3}$ and are means of triplicate determinations ± SD. Responses significantly higher than the blanks (i.e., the basal level of proliferation of CD4$^+$ T cells in the presence of autologous PBMC as APC: B + APC) were determined by unpaired, one-tailed Student's t test and indicated as: ***$p < 0.001$.

Results

Repertoire of subdominant epitopes recognized by CD4$^+$ T cell lines and clones after in vitro priming with the CEA peptides

We previously showed that the CEA sequence repeated at position 177–186 and 355–367 is predominantly recognized by CD4$^+$ T cells from healthy donors and colon cancer patients, revealing a dominance over the other 10 selected peptides we used for in vitro priming (19). To identify subdominant CEA sequences able to form MHC class II epitopes, PBMC from two of the responsive healthy donors (donors 2 and 3 of Ref. 19) were stimulated with a pool of CEA peptides (CEA subdominant pool) (Table I), in which the immunodominant sequence was not included to prevent the dominant expansion of $CEA_{177-186/355-367}$-specific CD4$^+$ T cells. After 7 days, the activated cells were expanded in the presence of IL-2-containing medium and propagated by weekly restimulation with irradiated peptide-pulsed autologous PBMC as APC. After two round of peptide stimulation, T cells were 98% CD4$^+$ (data not shown). We determined the repertoire of peptides recognized by CD4$^+$ T cells by testing their proliferation to each single peptide forming the CEA subdominant pool (Table I). CD4$^+$ T cells from donor 2 significantly recognized CEA_{99-111}, $CEA_{425-437}$, and $CEA_{568-582}$, while CD4$^+$ T cells from donor 3 significantly recognized CEA_{99-111} and $CEA_{666-678}$. Several clones, specific for each peptide recognized, were then obtained by limiting dilution from polyclonal CD4$^+$ T cell lines from both donors. CEA_{99-111} and $CEA_{425-437}$ raised the largest number of clones. We demonstrated that peptide recognition by the clones was HLA-DR restricted by inhibition of CD4$^+$ T cell proliferation to the peptides in the presence of an anti-HLA-DR mAb (Fig. 1*A*). To identify the HLA-DR-restricting alleles, peptide-specific CD4$^+$ T cell clones were challenged with LCL, expressing each of the two HLA-DRβ1 alleles of the donor (donor 2, *0301, *0701; donor 3, *0701, *1401), pulsed with the peptides, and ^{3}H incorporation was tested. CEA_{99-111} was recognized in association with DR*07 by donor 2 (clone 7) and DR*14 by donor 3 (clone 27); $CEA_{425-437}$ with DR*07 (clone 16) and $CEA_{568-582}$ with DR*03 (clone 33) by donor 2; $CEA_{666-678}$ with DR*14 by donor 3 (clone 1) (Fig. 1*B*). Peptide titration curves for all of the representative clones are also shown in Fig. 1*C*. The concentration of peptide requested to reach the half maximal stimulation for the several clones was comprised

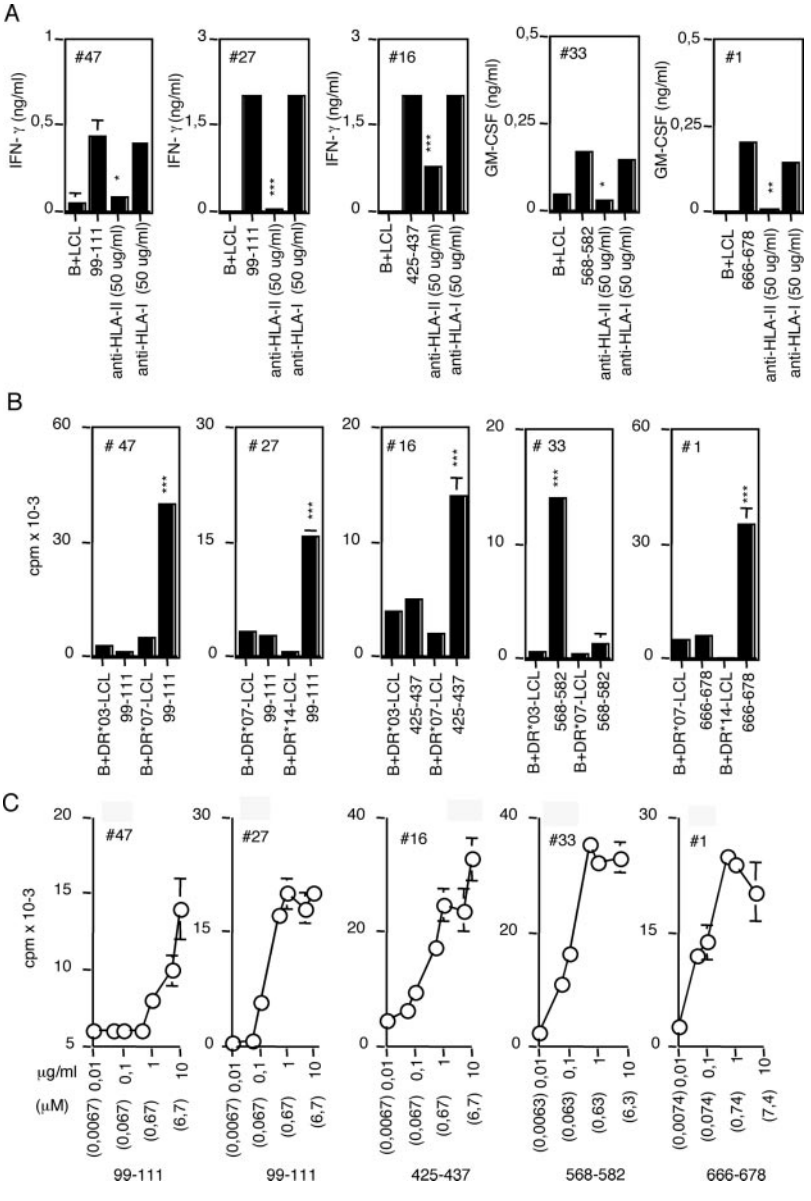

FIGURE 1. HLA-DR restriction and peptide titration curves of CD4$^+$ T cell clones specific for the CEA subdominant peptides. *A,* CD4$^+$ T cell clones were cultured with the relevant peptide in the presence of autologous LCL and tested for cytokine release. The blanks (i.e., the basal level of proliferation of CD4$^+$ T cells in the presence of the LCL only) are expressed as B+LCL. The data are means of triplicate determinations ± SD. Responses significantly higher than the blanks are indicated as: *, $p < 0.05$; **, $0.001 < p < 0.05$; ***, $p < 0.001$ (determined by unpaired, one-tailed Student's *t* test). *B,* CD4$^+$ T cell clones were tested in 2-day microproliferation assays with peptides in the presence of LCL expressing each of the HLA-DRβ1 alleles of the donor. The blanks (i.e., the basal level of proliferation of CD4$^+$ T cells in the presence of the LCL only) are expressed as B+LCL. The data are means of triplicate determinations ± SD and are representative of several experiments. Responses significantly higher than the blanks are indicated as: ***, $p < 0.001$ (determined by unpaired, one-tailed Student's *t* test). *C,* CD4$^+$ T cell clones were tested in 2-day microproliferation assays in the presence of titrated doses of the corresponding active peptides. Peptide concentrations are expressed both as micrograms per milliliter and micromolar. The data are means of triplicate determinations ± SD and are representative of at least three experiments.

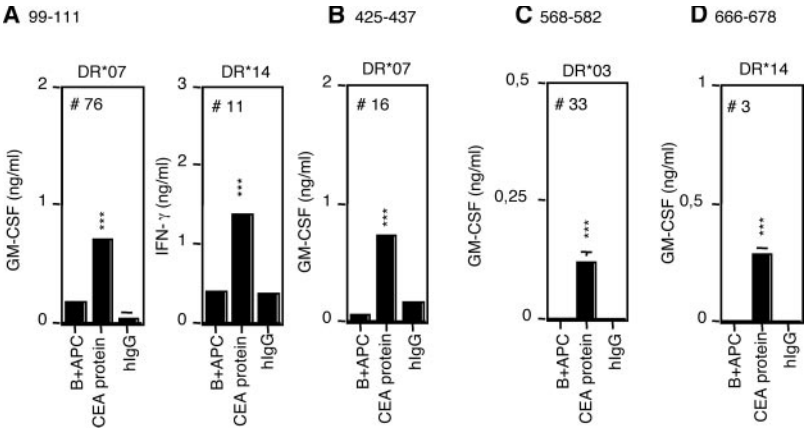

FIGURE 2. Recognition of the native CEA protein by peptide-specific CD4+ T cell clones. *A,* CEA_{99-111}-specific CD4+ T cells: DR*07-restricted clone 76 from donor 2 and DR*14-restricted clone 11 from donor 3. *B,* $CEA_{425-437}$-specific DR*07-restricted clone 16 from donor 2. *C,* $CEA_{568-582}$-specific DR*03-restricted clone 33 from donor 2. *D,* $CEA_{666-678}$-specific DR*14-restricted clone 3 from donor 3. Cells were challenged in vitro with the purified CEA protein (20 μg/ml) or human IgG (20 μg/ml) in the presence of autologous PBMC as APC and tested for cytokines release. The blanks (i.e., the basal level of GM-CSF or IFN-γ release of CD4+ T cells in the presence of irradiated autologous PBMC only) are expressed as B+APC.

in a range concentration as follows: 0.67–3.3 μM (clone 47), 0.067–0.33 μM (clone 27), 0.067–0.33 μM (clone 16), 0.063–0.31 μM (clone 33), and 0.0037–0.074 μM (clone 1).

CD4+ T cells specific for the CEA subdominant sequences recognize the native protein

To verify whether the recognized CEA sequence contains naturally processed epitope(s), we tested the recognition by peptide-specific CD4+ T cell clones of the native CEA protein after processing and presentation by autologous APC (Fig. 2). The results of representative clones are reported. CD4+ T cell clones were challenged with the CEA protein or normal human IgG, as a negative control, and assayed for GM-CSF and/or IFN-γ release. We chose as control protein normal human IgG both because of structural similarity with CEA and its proven immunogenicity (30). All clones, although to a different extent, significantly produced proinflammatory cytokines in the presence of the CEA protein but not of the normal human IgG, thus demonstrating that CEA_{99-111}, $CEA_{425-437}$, $CEA_{568-582}$, and $CEA_{666-678}$ contain naturally processed epitopes. In addition, to exclude that CEA protein recognition by peptide-specific CD4+ T cells was attributable to a contaminant Ag purified from the same tissue, clone 27 was also challenged with the cathepsin L protein purified from human liver, and no specific cytokine release was detected (data not shown).

We also tested the direct recognition of carcinoma cells expressing endogenous CEA and MHC class II molecules. CEA_{99-111}-specific CD4+ T cell clones 47 and 11, $CEA_{425-437}$-specific clone 16, and $CEA_{666-678}$-specific clone 3 significantly produced cytokines in the presence of the DR-matched lung adenocarcinoma cell line Calu-1 (DR*07, DR*14), while they did not recognize the HLA unrelated colon carcinoma cell line (Lovo) (Fig. 3, *A-C*). Moreover, the IFN-γ-producing DR*14-restricted CEA_{99-111}-specific clone 11 from donor 3 exerted specific killing activity against CEA_{99-111}-pulsed DR14-LCL and, most importantly, against the Calu-1 cells (Fig. 3*D*). The levels of expression of CEA as well as of surface MHC class II molecules, after 48 h of culture in the presence of IFN-γ, by all tumor cells were verified by Western blotting and flow cytometry, respectively (data not shown).

Cross-recognition experiments with analog self-sequences present on homologous protein of the CD66 family

Since CEA, or CD66e, belongs to a family of highly homologous proteins that are expressed at high levels in normal neutrophils, we verified the sequence similarity of the CEA subdominant sequences among the different CD66 molecules. We found CEA_{99-111} analogs on the CD66a, b, c, and d molecules (Fig. 4*C*) and $CEA_{425-437}$ analogs on the CD66a, b, and c molecules (Fig. 4*F*). We then tested the cross-reactivity of CEA_{99-111}- and $CEA_{425-437}$-specific CD4+ T cells for the analog peptides (Fig. 4).

The DR*14-restricted clone 27 proliferated in the presence of CEA_{99-111} and analogs $CD66a/c_{99-111}$ and $CD66b_{99-111}$, but not in the presence of analog $CD66d_{99-111}$ (Fig. 4*A*). To discriminate

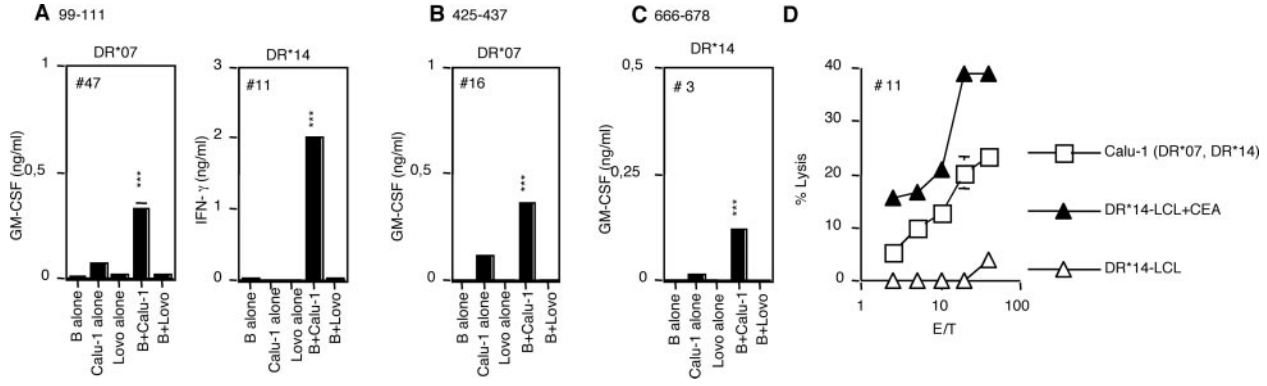

FIGURE 3. Recognition of CEA-expressing carcinoma cells by peptide-specific CD4+ T cell clones. *A,* CEA_{99-111}-specific CD4+ T cells (DR*07-restricted clone 47 from donor 2 and DR*14-restricted clone 11 from donor 3). *B,* $CEA_{425-437}$-specific CD4+ T cells (DR*07-restricted clone 16 from donor 2). *C,* $CEA_{666-678}$-specific CD4+ T cells (DR*14-restricted clone 3 from donor 3). Cells were challenged in vitro with HLA-DR-matched and -unmatched tumor cells expressing CEA and tested for cytokine release. The data are means of triplicate determinations ± SD and are representative of at least two experiments for each clone. Responses significantly higher than the blanks are indicated as: ***, $p < 0.001$ (determined by unpaired, one-tailed Student's *t* test). *D,* Lytic activity, measured in a ^{51}Cr release assay, of CEA_{99-111}-specific clone 11 against peptide-loaded LCL and CEA-expressing lung carcinoma cells. Targets used and their HLA-DR types are indicated. The results are representative of three experiments.

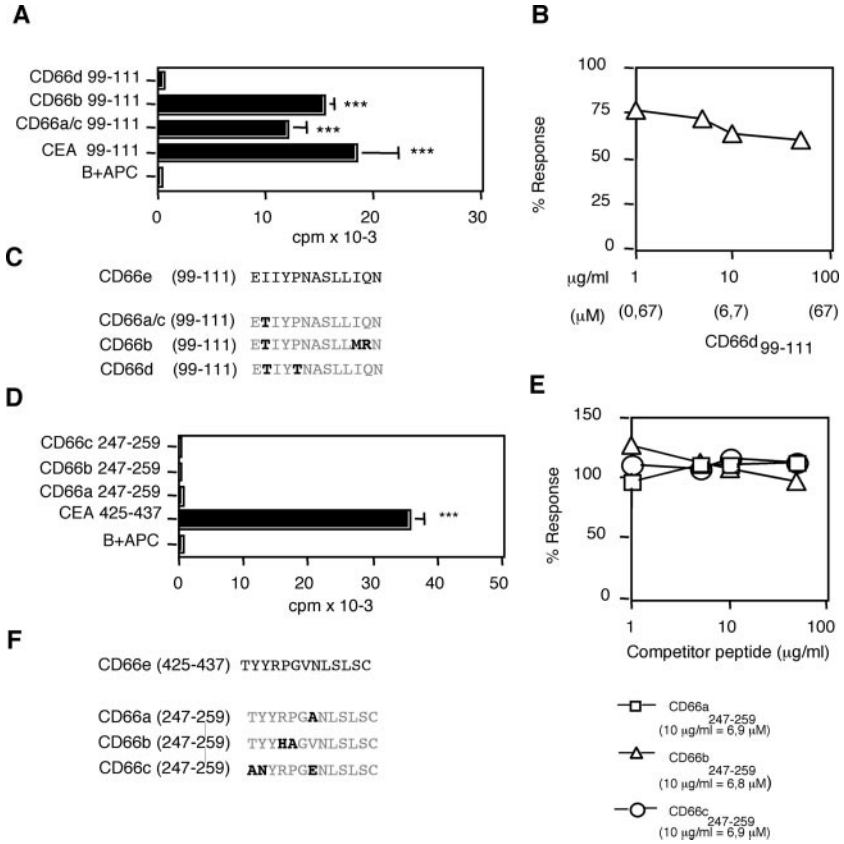

FIGURE 4. Cross-reactivity of CEA-specific $CD4^+$ T cells for CD66 analog sequences. CEA_{99-111}-specific $CD4^+$ T cells from donor 3 (DR*14-restricted clone 27) (*A*) and $CEA_{425-437}$-specific $CD4^+$ T cells from donor 2 (DR*07-restricted clone 16) (*D*) were challenged with autologous LCL as APC, pulsed with the relevant peptides or synthetic peptides corresponding to the analogs belonging to different members of the CD66 family (*C* and *F*), and tested in a 2-day microproliferation assay. *B* and *E,* Competition experiments: APC were pulsed for 2 h with increasing amounts of competitor peptides (1, 5, 10, and 50 μg/ml) and $CD4^+$ T cells were then added in the presence of suboptimal concentrations of relevant peptides. Competitor peptides were: *B,* $CD66d_{99-111}$; *E,* $CD66a_{247-259}$ (□), $CD66b_{247-259}$ (△), and $CD66c_{247-259}$ (○). The data are representative of at least three experiments. Responses significantly higher than the blanks are indicated as: ***, $p < 0.001$ (determined by unpaired, one-tailed Student's *t* test). *C* and *E,* Alignment of the CD66 analogs with the relevant CEA sequences.

whether the latter lack of cross-recognition was due to poor binding to the DR molecules or to lack of TCR stimulation, we performed competition experiments in which APC prepulsed with increasing amounts of competitor peptide (1, 5, 10, and 50 μg/ml) were used in microproliferation assays to stimulate clone 27 in response to a suboptimal dose (5 μg/ml) of CEA_{99-111}. The response of clone 27 to CEA_{99-111} was only marginally affected in the presence of peptide $CD66d_{99-111}$, which therefore appears to be a poor binder (Fig. 4*B*).

Concerning cross-reactivity of the DR*07-restricted clone 16 for $CEA_{425-437}$ analogs, we found no proliferation of the clone in the presence of the analogs, demonstrating that in this case the recognition of $CEA_{425-437}$ is CEA specific (Fig. 4*D*). In competition experiments, the response of clone 16 to $CEA_{425-437}$ was not affected in the presence of any of the analog peptides, which also appear to be poor binders (Fig. 4*E*).

Recognition of CEA epitopes by $CD4^+$ T cells from lung cancer patients

PBMC from eight recently diagnosed lung cancer patients, whose characteristics are described in Table II, were obtained before surgery and in the case of Pt 15 also at the 6-mo follow-up. All patients' tumors expressed CEA, as verified by Western blotting (data not shown), while none had soluble CEA above the normal range. $CD4^+$ T cells were purified and cultured in six replicates in 96-well plates in the presence of irradiated autologous $CD4^+$-depleted PBMC alone (APC), as baseline, or PHA, as positive control, or the peptides corresponding to the CEA sequences identified. At day 7 of culture, IL-2 was added without further Ag-specific stimulation, and at day 14 the supernatant was removed for the cytokine release assay. We previously reported (29) that this assay allows detection of in vivo-primed $CD4^+$ T cells even when

Table II. *Patients' characteristics*

Pt	Sex	Age (years)	Hystotype	Grading	T	N	Stage	Soluble CEA[a]	Tumor CEA[b]
4	M	47	ADC[c]	G3	1	0	IA	8.4	+
5	M	70	Meta-colon carcinoma					1.6	+
6	F	66	ADC	G2	2	1	IIB	1	+
7	F	52	ADC	G2	1	0	IA	5.3	+
9	M	65	ADC		2	0	IB	2.1	+
11	M	69	ADC	G3	4	2	IIIB	5	+
15	M	62	ADC	G3	2	0	IB	1.3	+
16	M	77	SQLC	G2	2	0	IB	1.9	+

[a] (Normal values <7–10 ng/ml).
[b] Determined by Western blot as described in *Materials and Methods.*
[c] ADC, adenocarcinoma; SQLC, squamous cell lung cancer.

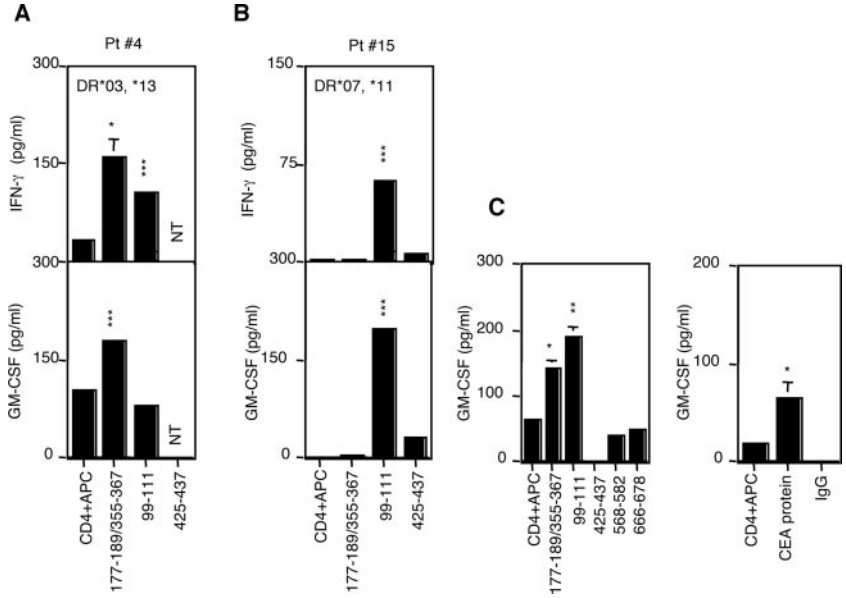

FIGURE 5. Detection of CEA-specific CD4+ T cells in lung cancer patients. CD4+ T cells were purified from total PBMC at surgery (*A* and *B*) or, in the case of Pt 15 at the 6-mo follow-up (*C, left panel*) and cultured in several replicates in 96-well plates in the presence of the indicated stimuli plus autologous irradiated CD4+-depleted PBMC as APC. At day 7, IL-2 (20 U/ml) was added in each well without further Ag stimulation. At day 14, 150 μl of supernatant was removed from each well, pooled, and used for cytokine release assays. *C, right panel,* CD4+ T cells from Pt 15 purified at the 6-mo follow-up were stimulated with CEA_{99-111} as described above. At day 14, CD4+ T cells were collected, washed, and rechallenged with the CEA protein or human IgG. After 2 days of culture, GM-CSF release in the supernatant was measured. Responses significantly higher than the blanks are indicated as: *, $p < 0.05$; **, $0.001 < p < 0.05$; ***, $p < 0.001$ (determined by unpaired, one-tailed Student's *t* test).

they are present in the blood at low frequency. As shown in Fig. 5, CD4+ T cells from Pt 4 (Fig. 5*A*) and 15 (Fig. 5*B*) secreted significant amounts of cytokines in the presence of CEA_{99-111}, and CD4+ T cells from Pt 4 also in the presence of the $CEA_{177-189/355-367}$. In the same experimental conditions, CD4+ T cells from healthy donors 2 and 3, as well as from five additional normal subjects, did not produce cytokines in the presence of any CEA peptides (data not shown). IFN-γ and GM-CSF secretion in the supernatant of PHA-stimulated CD4+ T cells was >2000 pg in all donors. In the case of Pt 15, we repeated the experiment at the 6-mo follow-up (Fig. 5*C*). Recognition of CEA_{99-111} was confirmed; moreover, specific cytokine release became evident also in the presence of $CEA_{177-189/355-367}$ (Fig. 5*C, left panel*). To verify whether recognition of peptide CEA_{99-111} was CEA specific, at day 14 after supernatant removal, CD4+ T cells stimulated with the peptide CEA_{99-111} were collected, washed, and rechallenged with the CEA protein or normal IgG and tested for cytokine release. As shown in Fig. 5*C* (*right panel*), CEA_{99-111}-specific CD4+ T cells secreted significant amounts of GM-CSF in the presence of the CEA protein, but not of normal IgG, thus demonstrating that indeed Pt 15 did contain CEA-specific CD4+ T cells in the blood.

Discussion

In this study, we first identified four subdominant naturally processed CEA epitopes by in vitro priming of CD4+ T cells from healthy donors, and, second, we demonstrated the presence of spontaneous CD4+ T cell responses against CEA in the blood of early-stage lung cancer patients.

These results are the first demonstration of the potential for CD4+ T cells to recognize the CEA in vivo and suggest that tolerance toward CEA or cross-reactive CD66 homologous molecules might be either not absolute or be overcome in the neoplastic disease. Indeed, at difference with lung cancer patients, CD4+ T cells from the healthy donors did not recognize the CEA peptides in the in vitro restimulation assays, suggesting the existence of a repertoire in their blood of anti-CEA naive CD4+ T cells, which need to be primed in vitro. Recently, it has been reported (4) that human medullary thymic epithelial cells express CEA, suggesting that thymocytes expressing CEA-specific TCR would be subjected to central tolerance. Our results are not in contrast with these findings: although high-affinity CEA-specific CD4+ T cells may have been deleted, CD4+ T cells with intermediate to low-affinity TCR may have escaped deletion. Indeed, although we could not directly investigate this feature of the patients' anti-CEA CD4+ T cells, the dose-response curves of the CD4+ T cell clones from the healthy donors (Fig. 1*C*) have shown for their TCR an intermediate affinity for the MHC class II-peptide complexes (mostly with EC_{50} in the range of 10^{-7} M). It should also be stressed that the level of soluble CEA in the patients was in the normal range (Table II), therefore reducing the possibility of peripheral tolerance induction. The patients tested suffered from early stages of neoplastic disease; therefore, it will be interesting to evaluate the anti-CEA CD4+ T cell response in a cohort of metastatic patients whose level of circulating CEA is increased above normal.

Another important issue raised by our results is the cross-reactivity for the analogs on the CD66 homologous molecules. We previously demonstrated (19) that recognition by CD4+ T cells of $CEA_{177-186/355-367}$ is CEA specific. The same applies for recognition of $CEA_{425-437}$ in the present study (Fig. 4*D*). On the contrary, CEA_{99-111}-specific CD4+ T cells showed cross-reactivity with the analogs (Fig. 4*A*), suggesting that these potentially autoreactive CD4+ T cells could be responsible for autoimmune phenomena against the hemopoietic self.

The demonstration of the spontaneous CD4+ T cell response against this epitope in the blood of lung cancer patients has two important implications: first, that the potential tolerance induced by the homologous molecules expressed on hemopoietic cells seems not to affect the repertoire of CEA-specific CD4+ T cells and, second, that CEA_{99-111}-specific CD4+ T cells should not recognize naturally processed epitopes on the homologous CD66 molecules. Indeed, the two responding patients did not have alterations in the white blood cell count and formula. An anti-CEA response potent enough to induce antitumor therapy in the absence of autoimmunity was demonstrated in CEA-transgenic mice vaccinated with recombinant vaccinia virus-expressing CEA (31). Nonetheless, our data recommend that the possible induction of autoimmunity needs to be carefully and specifically addressed in therapeutic settings.

The strategy we used for in vitro priming with pools of peptides with different binding affinities to the MHC class II molecules should favor the expansion of CD4+ T cells specific for peptides

with higher affinity. By this approach, we previously (19) identified an immunodominant CEA sequence recognized by normal donors and colon cancer patients in association with seven HLA-DR alleles. By stimulation of $CD4^+$ T cells, in the absence of the immunodominant peptide, in this study, we identified four new epitopes; three of them ($CEA_{425-437}$, $CEA_{568-582}$, and $CEA_{666-678}$) are recognized by a single donor, while CEA_{99-111} still represents a promiscuous epitope recognized in association with three to four alleles (DR*07 and DR*14 by the normal donors, and either DR*03, or DR*13, and either DR*07 or DR*11 by the two patients, respectively). From the algorithm data, the other subdominant CEA epitopes also were predicted as promiscuous MHC class II binders; therefore, their possible association with other DR alleles is worthwhile to be investigated.

CEA_{99-111} was recognized by both patients; it will be interesting to increase the number of patients tested to evaluate whether subdominant vs immunodominant epitopes are more frequently recognized. Indeed, the priming conditions in vivo may differ from the in vitro, resulting in the stimulation of a larger repertoire of CEA-specific $CD4^+$ T cells where immunization may develop at different times with the possible occurrence of epitope spreading (32).

We show that the newly identified CEA sequences contain naturally processed epitopes. This is proved by the specific production of proinflammatory cytokines by peptide-specific $CD4^+$ T cells in the presence of APC after processing and presentation of purified CEA and of CEA-expressing tumor cells: CEA_{99-111}-specific $CD4^+$ T cells also exerted killing activity. Definitive demonstration that this epitope is formed in vivo is its recognition by $CD4^+$ T cells from the patients.

In conclusion, we have identified four new CEA subdominant sequences, one of which is recognized in association with several HLA-DR alleles, and found spontaneous anti-CEA $CD4^+$ T cell responses in lung cancer patients. The identification of these new sequences will be highly instrumental for future studies covering a larger cohort of patients at different stages of neoplastic disease, addressing the relationship between the presence and quality of CEA-specific $CD4^+$ T cell responses and disease progression in cancer patients.

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Disclosures

The authors have no financial conflict of interest.

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