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ORIGINAL ARTICLE

Low TCR avidity and lack of tumor cell recognition in CD8$^+$ T cells primed with the CEA-analogue CAP1-6D peptide

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Abstract The use of "altered peptide ligands" (APL), epitopes designed for exerting increased immunogenicity as compared with native determinants, represents nowadays one of the most utilized strategies for overcoming immune tolerance to self-antigens and boosting anti-tumor T cell-mediated immune responses. However, the actual ability of APL-primed T cells to cross-recognize natural epitopes expressed by tumor cells remains a crucial concern. In the present study, we show that CAP1-6D, a superagonist analogue of a carcinoembriyonic antigen (CEA)-derived HLA-A*0201-restricted epitope widely used in clinical setting, reproducibly promotes the generation of low-affinity CD8$^+$ T cells lacking the ability to recognized CEA-expressing colo-rectal carcinoma (CRC) cells. Short-term T cell cultures, obtained by priming peripheral blood mononuclear cells from HLA-A*0201$^+$ healthy donors or CRC patients with CAP1-6D, were indeed found to heterogeneously cross-react with saturating concentrations of the native peptide CAP1, but to fail constantly lysing or recognizing through IFN- γ release CEA$^+$CRC cells. Characterization of anti-CAP1-6D T cell avidity, gained through peptide titration, CD8-dependency assay, and staining with mutated tetramers (D227K/T228A), revealed that anti-CAP1-6D T cells exerted a differential interaction with the two CEA epitopes, i.e., displaying high affinity/CD8-independency toward the APL and low affinity/CD8-dependency toward the native CAP1 peptide. Our data demonstrate that the efficient detection of self-antigen expressed by tumors could be a feature of high avidity CD8-independent T cells, and underline the need for extensive analysis of tumor cross-recognition prior to any clinical usage of APL as anti-cancer vaccines.

Keywords Altered Peptide ligand · Tumor Antigens · T cells · Tumor immunity · Vaccination

Abbreviations

APL	Altered peptide ligands
CEA	Carcinoembriyonic antigen
CRC	Colo-rectal carcinoma

Introduction

Most tumor antigens recognized by T cells derive from non-mutated self-proteins commonly expressed in normal tissue counterparts and up-regulated in tumors [26]. The self-origin of these tumor Ags may be responsible for a state of immune tolerance toward cancer cells, which can

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not only limit the ability of the immune system to counter tumor growth in vivo but also reduce the potential efficacy of anti-cancer vaccines. Indeed, both the thymic control exerted on TCR repertoire and the extrathymic mechanisms active on the low affinity self-reactive T cell fraction that survived in the periphery, maintain T cell unresponsiveness or anergy to avoid the occurrence of auto-immune phenomena [23]. However, unresponsiveness of T cells to self-peptides determines, in a tumor context, a condition known as "tumor ignorance" that has been hypothesized to play a key role in the defective interactions occurring between the immune system and cancer cells [28].

Several studies have recently focused on breaking immune tolerance toward self-tumor antigens by the usage of altered peptide ligands (APL). APL, which represent a useful approach to analyze the variable behavior of T cells in response to subtle alterations in peptide sequence [39], are characterized by changes at peptide positions critical either for HLA binding [29, 44, 47] or for TCR interaction [32, 42, 49]. As a result of the high flexibility of the TCR structure, these modifications may trigger a broad range of responses in T cells [42], which could be either enhanced (as in the case of APL utilized in cancer vaccines) [12] or reduced (as for peptides used to down-modulate T cell reactivities in auto-immune diseases) [2], as compared with native peptides.

Carcinoembryonic antigen (CEA) is a 180 kDa glycoprotein expressed in normal tissues, during fetal development and at low level in adult gastrointestinal tract. This protein is typically overexpressed in tumor cells of different histotypes including colorectal, gastric, pancreatic, lung and breast carcinomas [11]. Because of this expression pattern in a broad range of tumors, CEA represents a crucial tumor marker and potentially an optimal target of anti-tumor immune response. However, the self-nature of this antigen implies the existence of an immune tolerance state that could hamper the ability of CEA-based vaccines to promote tumor regression in vaccinated patients. The CAP1-6D peptide was derived from the native HLA-A*0201-binding peptide CAP1 ($CEA_{605-613}$) [45], by introducing an Asp residue at position 6, which generates an analogue (defined as superagonist) with potentially stronger ability to interact with the TCR as compared with the native peptide, without affecting HLA binding [36, 49]. The CAP1-6D analogue was tested in cancer vaccine trials, using DC as antigen presenting cells, either expanded in vivo by Flt3 ligand administration [10] or derived from $CD14^+$ sorted monocytes [1]. However, both clinical studies reported a minimal number of tumor regressions, not always associated with immunological responses.

One of the mechanisms responsible for the limited ability of superagonist peptide analogues to promote robust immunological responses, resulting in enhanced clinical efficacy, may be represented by the reduced capacity of T cells raised in the presence of the modified epitope to cross-recognize the native determinant endogenously expressed by tumor cells [8]. Indeed, vaccinations based on APL have been reported to expand a wide range of T cell reactivities not necessarily efficient in targeting tumor cells [5, 41], a phenomenon that could either nullify or profoundly reduce the benefits of the increased immunogenicity promoted by APL.

With the aim of investigating the possible large-scale in vitro generation of tumor-specific T lymphocytes for adoptive cell transfer, we analyzed the immunological outcome of T cells raised in the presence of CAP1-6D analogue from PBMC of healthy donors and CRC patients. CAP1-6D-specific T cells were extensively evaluated for their functional properties, including their ability to cross-react with the native peptide CAP1 and, most importantly, to recognize tumor cells expressing endogenously processed CEA derived peptides.

The data here reported indicate that CAP1-6D peptide, despite its strong immunogenicity, primes T cell responses displaying low avidity for the native peptide, which results in the lack of CEA^+ tumor recognition.

Materials and methods

Peptides

The peptides used in this study were CAP1 (YLSGANLNL, $CEA_{605-613}$), CAP1-6D (YLSGADLNL, $CEA_{605-613/D}$) and HIV-Nef (VLEWRFDSRL), the latter utilized as negative control. All peptides were provided by Neosystem (Strasbourg, France) as >95% pure preparations. Mass spectrometry analysis (by a Perseptive Biosystems Voyager MALDI-TOF STR system) performed in our facility showed that CAP1-6D preparation contained exclusively a peak of 963.76 molecular mass (calculated molecular mass of 964.49) plus salt adducts of the same peptide, and CAP1 preparation contained also a single peak of 962.77 molecular mass (calculated, 963.50) plus salt adducts of the same peptide. To minimize the risk of potential undetectable peptide contaminants altering immunogenicity profiles, four different peptide batches of CAP1 and CAP1-6D peptides were used throughout the study. Additionally, to check for possible vial-peptide contaminants, each batch was tested for recognition by T cells specific for M1-Flu_{58-66} or CEF (cocktail peptides from influenza virus, EBV and CMV, Mabtech, Nacka, Sweden) and found to trigger no IFN-γ release by these cells even at 10 μM concentration (data not shown).

PBMC and T cell priming

PBMC for in vitro priming studies were obtained from buffy coats or blood samples collected from HLA-A*0201$^+$

healthy donors or CRC patients (Dukes' stages B and C) by Ficoll-Paque density gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden). Cells were cultured in RPMI 1640 medium (BioWhittaker, Walkersville, MD, USA) supplemented with 2 mM glutamine (BioWhittaker Europe, Verviers, Belgium), 100 U/ml penicillin (Penicillina G sodica; Pharmacia, Milan, Italy), 100 mg/ml gentamycin (Gentalyn 80; Schering-Plough, Milan, Italy), 1% HEPES buffer (Bio-Whittaker Europe) and 10% human serum (AB serum pool). To prime Ag-specific $CD8^+$ T cells, dendritic cells (DC) were generated from adherent PBMC by in vitro differentiation either with IFNα (Alfa Wassermann, Alanno, Italy) [43], or with the conventional IL-4 and GM-CSF (Research Diagnostic, Concord, MA, USA) cocktail for DC differentiation [37] and a standard cytokine cocktail for DC maturation [15]. DC were pulsed for 2 h at 37°C with 5 μg/ml CAP1-6D or CAP1 peptide, irradiated at 3,000 rad and co-cultured in 24-well plates with autologous PBMC (at 1:4 DC:PBMC ratio) in RPMI 1640 medium supplemented with 10% human serum. After 24 h co-culture, IL-2 was added at 60 IU/ml (Proleukin; Chiron, Milan, Italy). Cloning of anti-CAP1-6D was performed by limited dilution. Briefly, anti-CAP1-6D T cells were seeded as 0.3 cell per well in 96 well/plate, in the presence of irradiated (3,000 rad) feeder cells consisting of 1×10^5 allogeinic PBMC (pool of three healthy donors) and 2×10^4 B LCL cells/well. T cell clones were expanded in RPMI 1640 supplemented with 10% of human serum, 900 IU/ml IL-2 (Proleukin) and 1 μg/ml PHA (Sigma-Aldrich, Deisenhofen, Germany). This study was approved by the Independent Ethics Committee of the Istituto Nazionale Tumori of Milan and informed consent was obtained from all subjects.

CRC lines

The CRC cell lines SW403 (HLA-A*0201$^+$ CEA$^+$), SW480 (HLA-A*0201$^+$ CEA$^+$), SW1463 (HLA-A*0201$^+$ CEA$^+$), SW1116 (HLA-A*0201$^+$ CEA$^+$), Colo206 (HLA-A*0201$^+$ CEA$^+$) [purchased from ATCC (Manassas, VA, USA)]; CG705 (HLA-A*0201$^+$ CEA$^+$), (both kindly provided by Dr T. Schweighoffer), 1869col (HLA-A*0201$^+$ CEA$^-$, kindly provided by Dr C. Maccalli), SW707 (HLA-A*0201$^+$ CEA$^+$), were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (BioWhittaker, Europe). CEA expression was confirmed by Western blot and cytofluorimetric analysis using Col-1 mAb (Abcam, Cambridge, UK), while HLA-A2 surface expression was verified by staining with BB7.2 mAb (Biosource, Camarillo, CA, USA). The two HLA-A2$^+$CEA$^+$ lines Colo206 and CG705 were also demonstrated to express significant levels of HLA-A2/CAP1 peptide complexes on their cell surface, as they were recognized by CAP-1 specific T cells in an HLA-restricted fashion [33].

Cytokine secretion by specific T cells and blocking experiments

At culture times indicated in the different experiments, antigen recognition by T cells was determined by IFN-γ ELISPOT (Mabtech, Nacka, Sweden) according to the manufacturer's instructions. Briefly, T cell cultures (at the indicated numbers) were plated with 16.7×10^3 cells of T2 cells (TAP-deficient lymphoma cell line), alone or previously pulsed with 10 μg/ml peptide (CAP1, CAP1-6D or Nef), or with HLA-A*0201$^+$ CEA$^+$ CRC cells. To evaluate HLA class I-restriction, target cells were pre-treated for 30 min at 37°C with the W6/32 mAb, recognizing a non-polymorphic epitope of HLA-A, -B, and -C molecules associated with beta 2 m. In order to verify CD8 dependency, T cells were pre-incubated for 30 min at 37°C with anti-CD8 blocking mAb (DK25 clone, DAKO Italia S.p.A., Milan, Italy). After 18 h of incubation at 37°C, IFN-γ-secreting cells were identified as spot-forming cells by a computer-assisted system ELISPOT Reader (Bioline, Torino, Italy). Data were analyzed with 3.1 ELISPOT AID software (Autoimmune Diagnostika, Strasburg, Germany). Assay of peptide titration was performed pulsing T2 cells with different peptide concentrations, as specified for each experiment. Multiple cytokine detection in supernatants from overnight co-culture of anti-CAP1-6D T cells with specific targets (at 1:1 ratio) was performed by CBA assay (BD Biosciences), according to the manufacturer's instructions.

Assessment of cytotoxic activity

Lytic activity of anti-CAP1-6D T cells was evaluated by a standard 4 h ^{51}Cr release assay, using as target cells, T2 cells loaded with CAP1, CAP1-6D or irrelevant peptide (Nef) at a final concentration of 10 μg/ml, or alternatively HLA-A*0201$^+$CEA$^+$ CRC cells. One thousand ^{51}Cr-labeled target cells (T) per well were co-cultured for 4 h with effectors (E) at different E:T cell ratios. Spontaneous target cell lysis was evaluated by incubating target cells with medium, whereas total target cell lysis was determined by treatment with 2% Nonidet P-40 detergent-supplemented medium. The percentage of specific lysis was calculated as follows: [(experimental cpm − spontaneous cpm)/(total cpm − spontaneous cpm)] × 100. The assay was performed in triplicate. For cold target inhibition assay, T2 cells were labeled (hot), or not (cold) with ^{51}Cr and pulsed with CAP1, CAP1-6D and Nef peptides at 10 μg/ml for 2 h at 37°C. Anti-CAP1-6D T cells were incubated with "cold" targets for 30 min at 37°C, and then "hot" target cells were added. The effector-"hot" target cell ratio was 50:1, and the "cold"–"hot" target cell ratios were 20:1 and 5:1. Specific lytic activity was obtained as described above. Lytic potential

was also evaluated as CD107a mobilization and intracellular staining. For this assay, anti-CAP1-6D T cells were analyzed for ability to up-regulate CD107a molecules after degranulation in response to T2 pulsed with peptide 5 μg/ml or CRC cells. This staining was combined with the analysis of intracellular IFN-γ production in response to the same target cells. T cells were co-incubated with target cells at 5:1 E:T ratio for 4 h at 37°C in the presence of 1 μl of CD107a-PE antibody (BD Pharmingen, San Diego, CA, USA), and blocking drugs, monensin and brefeldin A (Sigma-Aldrich) were added after 1 h. Subsequently, T cells were harvested, washed with PBS supplemented with 1% FCS, labeled with FITC-conjugated anti-CD8 mAb (BD PharMingen), and then analyzed for IFN-γ intracellular staining by permeabilization with Cytofix/Cytoperm kit (BD Biosciences) and labeling with allophycocyanin-conjugated anti-IFN-γ mAb (BD Pharmingen).

HLA-A*0201/peptide Tetramer (multimers) staining and TCR down-modulation

T cell staining with HLA-A*0201/peptide tetramers was performed with wild type or modified in HLA α3 domain PE-conjugated molecules, synthesized as previously described [30]. Cells were stained for 1 h at room temperature with 1 μl tetramers (10^3 ng/ml concentration), washed with PBS+1% FCS and then labeled for 20 min at 4°C with FITC-conjugated anti-CD8 mAb (BD Pharmingen). Binding was evaluated by FACSCalibur and Cell-Quest software (BD Biosciences). The percentage indicates the tetramer and CD8 double positive cells among $CD8^+$ T lymphocytes. For titration experiments, anti-CAP1-6D T cells were stained in the presence of different concentrations of either wild-type or α3-mutated tetramers, as specified in the different experiments. For sorting experiments, anti-CAP1-6D T cells were labeled with PE-HLA-A*0201 tetramers carrying CAP1 peptide (Beckman Coulter, San Diego, CA, USA), and then isolated by immunomagnetic selection with anti-PE mAb-coated beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the sorted subpopulation was then checked by flow cytometry. TCR down modulation analysis was performed by co-incubating anti-CAP1-6D T cells with different target cells (T2 cells pulsed with 5 μg/ml CAP1, CAP1-6D, Nef, or CRC cells) at E:T ratio of 1:1 for 18 h at 37°C. TCR expression was then assessed by flow cytometry, by staining T cells with HLA-A*0201/CAP1-6D tetramers (Beckman Coulter) and FITC-conjugated anti-CD8 mAb (BD PharMingen).

Statistical analysis

Statistical analysis was performed by *t* test for unpaired samples. $P < 0.05$ was considered as statistically significant.

Results

Efficient in vitro generation of CAP1-6D-specific T cells lacking recognition of CEA^+ HLA-A*0201^+ CRC cells

CAP1-6D-specific T cell bulk cultures were generated from PBMC of HLA-A*0201^+ healthy donors (HD, $n = 10$) and CRC patients (Pt, $n = 5$) by in vitro peptide sensitization, and weekly monitored by IFN-γ release (measured by ELISPOT) in response to T2 cells loaded with CAP1-6D, CAP1 or irrelevant (Nef) peptide. As depicted in Fig. 1a (referring to data obtained in eight representative cases tested after four stimulation rounds), generation of T cells recognizing CAP1-6D peptide in a class I-HLA-restricted fashion could be observed in the totality of PBMC analyzed from both healthy donors and CRC patients, although with heterogeneous activity (Fig. 1a). In addition, relatively high frequencies of CAP1-6D specific $CD8^+$ T cells (mean % in CD8+ cells = ±36; mean % in total PBMC = ±9) were detected in these cell cultures by CAP1-6D/tetramer staining (Fig. 1b). Recognition of CAP1-6D epitope could also be detected as early as after 2 weeks in vitro culture in five of the cases analyzed (four HD and one Pt; data not shown), confirming the significant immunogenicity of the APL. However, all anti-CAP1-6D bulk T cell cultures appeared to cross-recognize the native epitope CAP1 with a lower efficiency, even at the saturating peptide concentration of 10 μg/ml (Fig. 1a). The differential recognition of the two peptides was confirmed by analyzing the release of other Tc1 cytokines, such as IL-2 and TNF-α, which were predominantly produced in the presence of CAP1-6D, with little or no secretion after stimulation with the native peptide (Fig. 1c).

The same T cell cultures were analyzed for recognition of CRC cells, expressing CEA epitopes from endogenous processing [33]. Unexpectedly, no recognition of CEA^+ HLA-A*0201^+ CRC cells (CG705 line) could be detected with anti-CAP1-6D T cells generated from healthy donors or CRC patients, either in terms of class I-HLA-mediated IFN-γ release (Fig. 2a, left panel) or lysis (Fig. 2a, right panel). The lack of tumor cell recognition by anti-CAP1-6D T cells was confirmed with two additional HLA-A*0201^+CEA^+ CRC lines (Fig. 2b), and could not be improved by target pre-treatment with IFN-γ (data not shown) which is known to ameliorate endogenous presentation by up-regulating HLA-class I and antigen expression. To further assess any potential interaction occurring between anti-CAP1-6D T lymphocytes and CRC cells, we investigated the ability of these T cells to down-modulate TCR expression (and thus lose the capacity to bind to HLA/peptide tetramers) as a result of the encounter with HLA/peptide complexes exposed on target cells. As depicted in Fig. 2c, no TCR down-modulation (and thus no change in

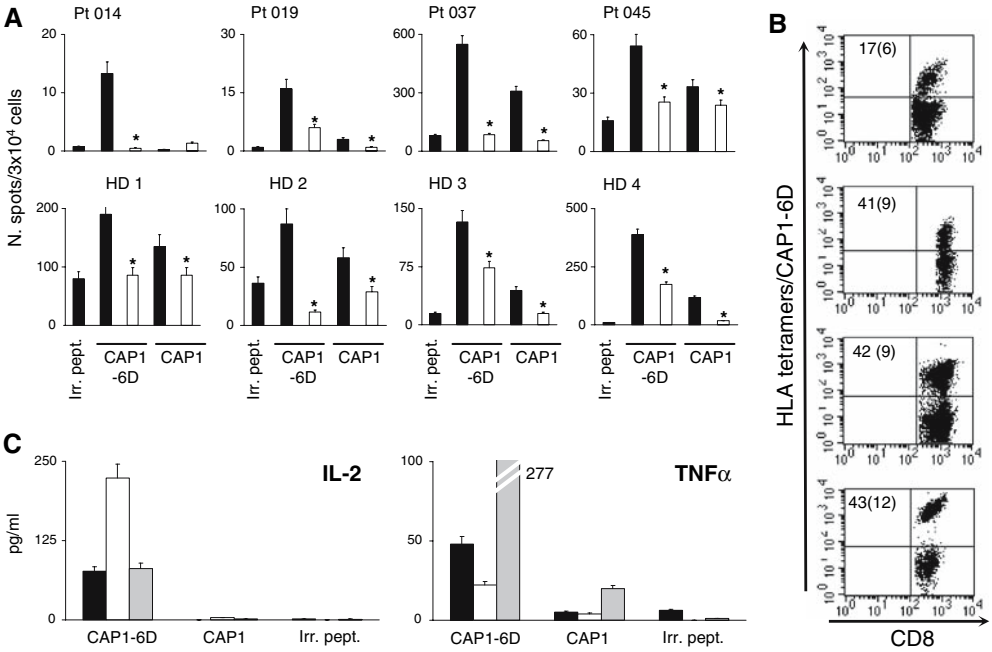

Fig. 1 Limited cross-recognition of native peptide by CD8$^+$ T cells raised by the superagonist CEA-analogue CAP1-6D. **a** Anti-CAP1-6D specific T cells were generated from PBMC of HLA*0201$^+$ healthy donors (HD) or CRC patients (Pt) by in vitro sensitization with CAP1-6D-pulsed autologous DC, and then monitored for specific peptide recognition and cross-reactivity against native peptide by IFN-γ ELISPOT, using as target T2 cells pulsed with 10 μg/ml CAP1, CAP1-6D or irrelevant peptide (Irr. Pept. = Nef). Data refer to 4-week cultures; spot numbers were normalized referring to the CD8$^+$ percentuage in T cells bulk culture. Target cells were pre-treated (*white bars*) or not (*black bars*) with anti-HLA class I mAb (W6/32) to verify specific TCR involvement. **b** Staining with HLA-A2/CAP1-6D tetramers of CAP1-6D-specific T cell cultures raised from three healthy donors (*upper panel* HD1, HD2, HD4, respectively) and 1 CRC patient (Pt037, *lower panel*). Numbers indicate percentage of positive cells within CD8+ (in parenthesis within the lymphocyte-gated region). **c** Supernatants recovered from overnight co-incubation (at 1:1 E:T ratio) of anti-CAP1-6D T cells with T2 target cells loaded with different peptides were analyzed with CBA assay in order to detect cytokines (including IL-2 and TNFα) released in response to native and modified peptides. Data refer to representative results obtained with anti-CAP1-6D T cells derived from healthy donor HD1 (*black bars*), HD4 (*white bars*) and CRC patient Pt037 (*gray bars*). *$P < 0.05$ (evaluated by *t* test for unpaired samples), as compared with the recognition of the same target in the absence of anti-HLA I mAb. Results are representative of three independent experiments

tetramer staining) occurred when anti-CAP1-6D were co-cultured with three different CEA$^+$ HLA-A*0201$^+$ CRC cell lines. In contrast, tetramer staining was completely abrogated after co-incubation with CAP1-6D-pulsed T2 cells, while a relevant percentage of the same cells retained tetramer binding after co-culture with CAP-1-loaded targets.

These data suggest that the superagonist peptide CAP1-6D is able to efficiently trigger in vitro a specific CD8$^+$ T cell population that only partially cross-reacts with the native epitope and, most importantly, is unable to recognize the endogenous epitope presented by CEA$^+$ tumor cells. The evidence that anti-CAP1 CD8$^+$ T cells, obtained after prolonged in vitro peptide stimulation due to the poor immunogenicity of the native epitope, were indeed able to recognize native CAP1 with high avidity (Fig. 2d, left panel) and to produce IFN-γ in response to CRC CEA$^+$ cells (Fig. 2d, right panel) suggests that the lack of tumor recognition by anti-CAP1-6D may be ascribed to the low affinity of the effectors, rather than to deficient epitope processing and presentation by the target cell.

Anti-CAP1-6D T cell avidity for the superagonist and parental peptides

The reduced ability of anti-CAP1-6D to cross-recognize CAP1 epitope and CRC cells could indeed stem from a differential T cell avidity for the superagonist as compared with the parental peptide. Avidity is actually a complex parameter influenced by peptide affinity, TCR expression levels, requirement for co-stimulatory molecules, and microenvironment conditions [13, 21]. However, information about T cell avidity can also be obtained in vitro by evaluating the peptide amounts required to activate a certain T cell response [40]. In this view, we performed peptide titration analysis with anti-CAP1-6D T cells in the presence of the superagonist and the native peptide. As depicted in Fig. 3a, showing data concerning T cell cultures from three cases representative of the different patterns observed, CAP1-6D-specific T lymphocytes released IFN-γ in response to CAP1-6D peptide with heterogeneous efficiency, displaying 50% activity at peptide concentrations

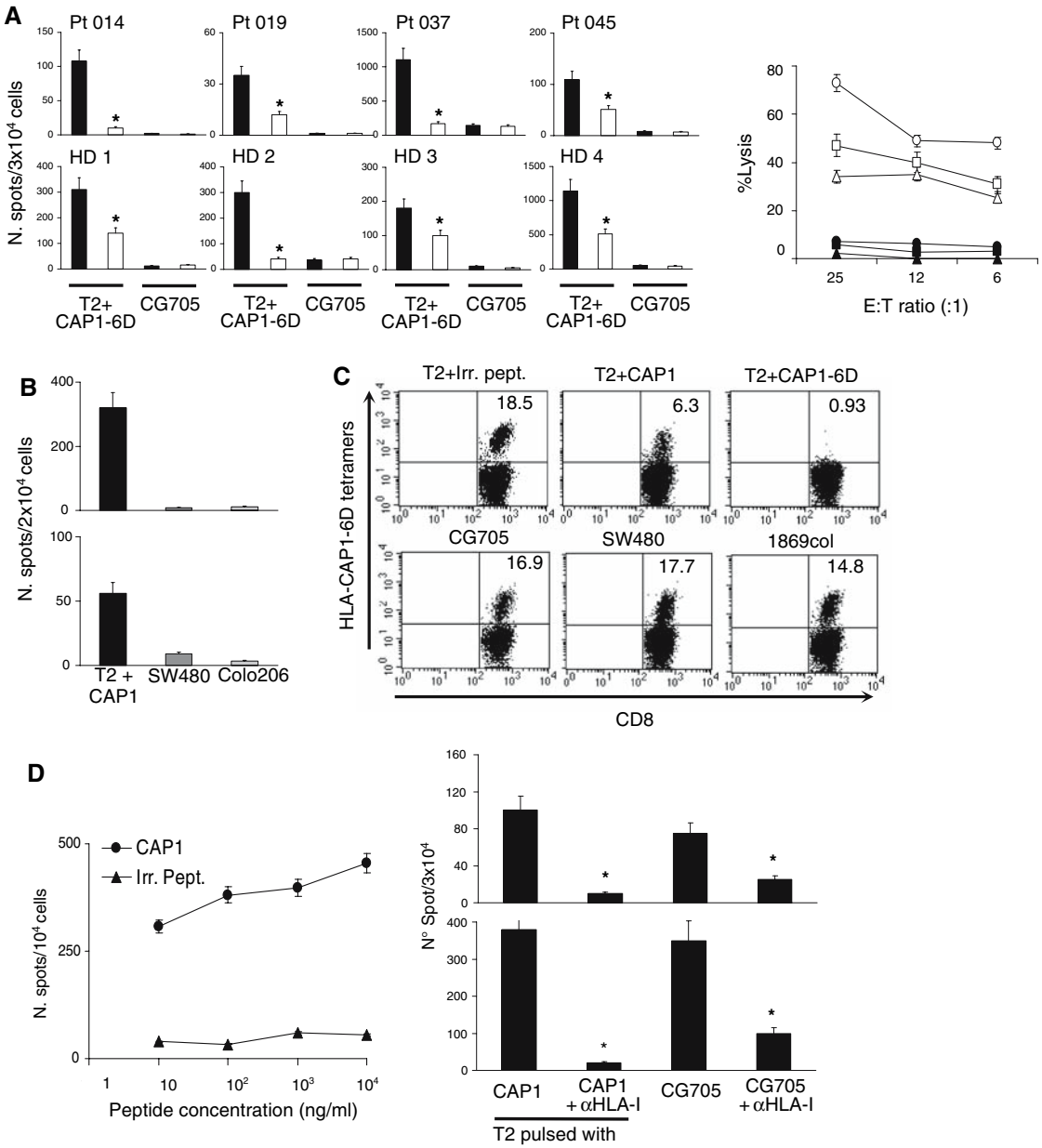

Fig. 2 Lack of recognition of CEA$^+$ HLA-A*0201$^+$ CRC cell lines by anti-CAP1-6D T cells. **a** Anti-CAP1-6D T cells derived from healthy donors or CRC patients by in vitro peptide sensitization (4 week culture) were tested for IFN-γ release (by ELISPOT) in response to T2 cells pulsed with CAP1-6D peptide or CEA$^+$ HLA-A*0201$^+$ CRC cells (CG705 cell line) (*left panel*). Targets were pre-treated (*white bars*) or not (*black bars*) with the anti-HLA class I mAb W6/32, to assess TCR involvement. Lytic activity of anti-CAP1-6D T cells was assessed by ^{51}Cr release assay (*right panel*). Anti-CAP1-6D T cells, obtained from HD1 (*square*), HD2 (*circle*), and CRC patient Pt037 (*triangle*) against T2 cells pulsed with 10 μg/ml of CAP1 peptide (*open symbols*) or CG705 cells (*filled symbols*). **b** Anti-CAP1-6D T cells (obtained from HD2, *upper pane*, and HD4, *lower panel*) were analyzed for IFN-γ release in response to T2 cells loaded with CAP1 peptide and two CRC cell lines CEA$^+$ HLA-A*0201$^+$ SW480 and Colo206 cells. **c** Anti-CAP1-6D T cells were plated at 1:1 ratio with T2 cells pulsed peptides, HLA-A*0201$^+$CEA$^+$ CRC lines (SW480 and CG705), or HLA-A*0201$^+$CEA$^-$ CRC line (1869 col). After 18 h co-culture, anti-CAP1-6D T cells were harvested and stained with tetramers containing CAP1-6D peptide to assess TCR down-modulation. *Numbers* indicate the percentage of CD8$^+$/tetramer$^+$ T cells in the lymphocyte gate, as assessed by flow cytometry. **d** CAP1 T cells were generated from PBMC of healthy donors by in vitro sensitization with CAP1-pulsed autologous DC and then evaluated for recognition decreasing amounts of CAP1 peptide (*circle symbol*) or irrelevant peptide (*triangle symbol*) loaded on T2 cells (*left panel*). Recognition of CEA$^+$ HLA-A*0201$^+$ CRC line CG705 by anti-CAP1 T cells (right panel, HD1 upper and HD3 lower, respectively) was assessed in IFN-γ-ELISPOT. *$P < 0.05$ (evaluated by *t* test for unpaired samples), as compared with the recognition of the same target in the absence of anti-HLA I mAb. Results are representative of three independent experiments

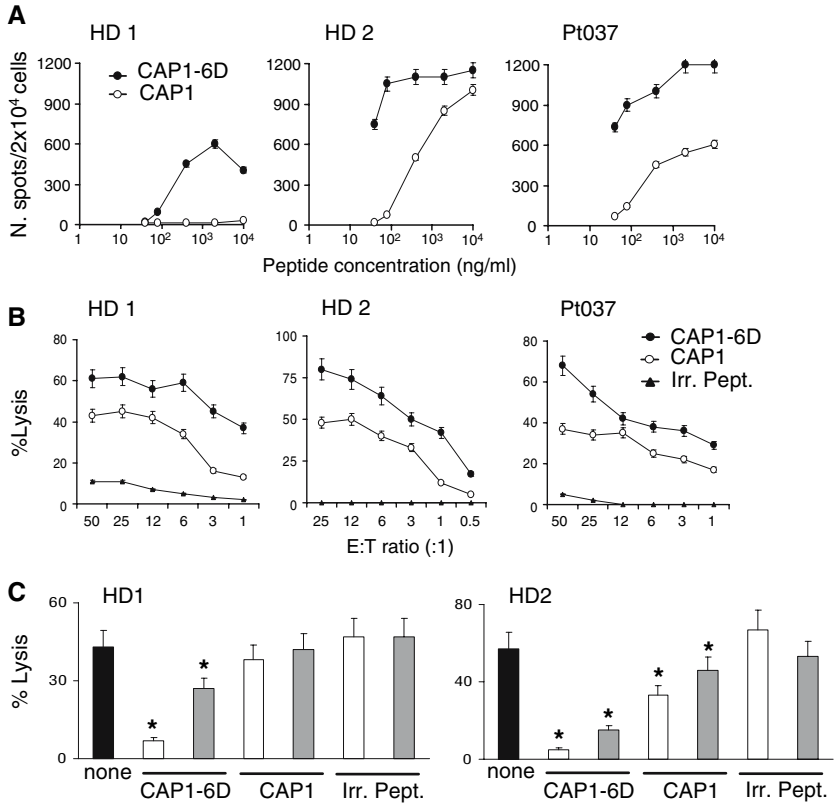

Fig. 3 Different TCR avidity in CAP1 recognition by anti-CAP1-6D T cells. **a** TCR avidity was evaluated by peptide titration in terms of IFN-γ release, detected by ELISPOT. T2 cells were pulsed with decreasing peptide concentrations of CAP1 and CAP1-6D, and then co-incubated with anti-CAP1-6D T cells obtained from two healthy donors (*HD1* and *HD2*), and one CRC patient (*Pt037*). CAP1-6D tetramer$^+$ T cell fraction, detected in each anti-CAP1-6D culture, was respectively 9.5% (HD1), 63% (HD2) and 15.5% (Pt037) (percentage refers to CAP1-6D tetramer$^+$CD8$^+$ T cells on lymphocyte gate). **b** Anti-CAP1-6D T cells were analyzed for cytotoxic activity in a standard 4 h ^{51}Cr release assay against T2 cells loaded with 10 µg/ml CAP1-6D, CAP1 or irrelevant peptide (Nef), at different E:T ratios. **c** Cold target inhibition assay was performed on "hot" T2 cells loaded with CAP1-6D peptide. Effectors from two HD were co-incubated with ^{51}Cr labeled-T2 cells loaded with CAP1-6D either in the presence or in the absence of "cold" T2 cells pulsed with CAP1-6D, CAP1 or Nef (at hot:cold ratio of 1:20, *white bars*; and 1:5, *gray bars*). *$P < 0.05$ (evaluated by *t* test for unpaired samples), as compared with the recognition of the same target in the absence of "cold" target cells. Results are representative of three independent experiments

ranging from 40–200 ng/ml. However, the avidity for CAP1 native peptide was remarkably lower, with few cases (4 out of the 15 analyzed) recognizing the parental epitope similarly to CAP1-6D but only at 10 µg/ml saturating peptide concentration (Fig. 3a and data not shown). Comparable results were obtained when the ability of anti-CAP1-6D T cells to lyse at different E:T ratios T2 target cells loaded with native and altered peptide, was analyzed in a standard 4 h ^{51}Cr release assay (Fig. 3b). Indeed, CAP1-6D-pulsed target cells were efficiently lysed at E:T ratios lower than those required when target cells were loaded with the parental CAP1 peptide. Furthermore, cold-target inhibition assay, using excess of cold T2 cells pulsed either with the superagonist or with the native peptide to inhibit lysis of ^{51}Cr-labeled CAP1-6D-pulsed targets, showed that anti-CAP1-6D T cell activity was completely abrogated by the CAP1-6D-pulsed cold cells, but only minimally or partially impacted by cold targets loaded with CAP1 (Fig. 3c). This confirmed the evidence that the superagonist peptide CAP1-6D triggered the generation of T cells displaying limited TCR affinity for the native epitope.

CD8-dependency of CAP1-6D-specific T cells

The requirement of CD8 co-receptor binding is known to be an hallmark of low avidity T cells [6, 7]. We thus investigated whether anti-CAP1-6D T cells necessitate CD8 involvement when interacting with the native peptide CAP1. As shown in Fig. 4a, CD8-blocking mAb did not affect anti-CAP1-6D T cell activation when target cells were pulsed with the superagonist peptide, but significantly impaired recognition of targets loaded with the parental epitope. To confirm these functional results, we stained T cells with HLA/tetramers bearing mutations in the α3 domain, which abrogate TCR binding to CD8 and allow the detection of high avidity and CD8-independent T cells [4, 30, 31]. As

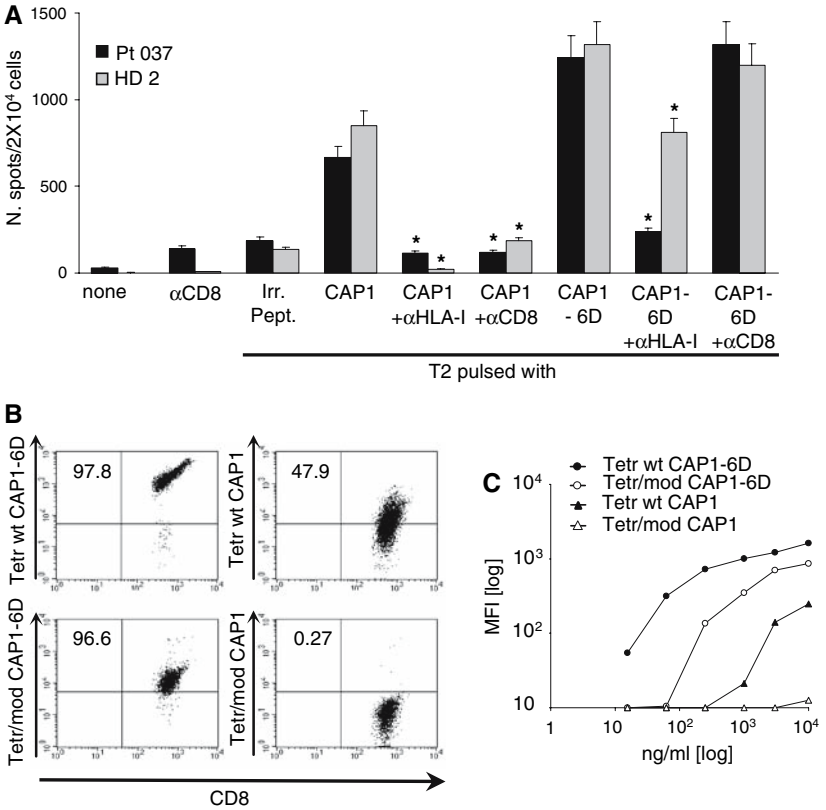

Fig. 4 Low avidity recognition can be linked to CD8-dependent CAP1 recognition. **a** Anti-CAP1-6D T cells from HD2 or Pt037 were tested for IFN-γ ELISPOT in response to T2 cells pulsed with CAP1, CAP1-6D or irrelevant peptide (Nef) (2 μg/ml), or to CG705 CRC cells. T cells or targets were pre-treated or not with anti-CD8 (T cells) or anti-HLA-class I (targets) mAb to prove TCR involvement and HLA-I-restriction**. b** CD8 dependency was also assessed by staining with wild-type or mutated tetramers in the α3 HLA domain, abrogating CD8-binding. Anti-CAP1-6D T cells were labeled with wild type (Tetr wt) and mutated tetramers (Tetr/mod) at 10^3 ng/ml and co-stained with the anti-CD8 FITC conjugated mAb. *Numbers* indicate the percentage of CD8/tetramer double positive cells. **c** Tetramer titration assay was performed using serial dilutions of the indicated tetramers. Anti-CAP1-6D T cells were labeled with tetramers containing either CAP1 or CAP1-6D peptide 1 h at room temperature, and then stained with anti-CD8 FITC conjugated mAb. Mean intensity fluorescence (MFI) was evaluated on tetramer$^+$CD8$^+$ stained T cells. *$P < 0.05$ (evaluated by *t* test for unpaired samples), as compared with the recognition of the same target in the absence of blocking mAb. Results are representative of three independent experiments

depicted in Fig. 4b, staining with wild-type tetramers was observed in the presence of both CAP1-6D and CAP1 peptides, although with significantly binding of different affinity. In contrast, α3-mutated tetramers stained anti-CAP1-6D T cells when loaded with the superagonist peptide, but failed to engage the TCR of these cells in the presence of CAP1 peptide (Fig. 4b). These data were confirmed by titration assays, showing that anti-CAP1-6D T cells bound to decreasing concentrations of wild-type HLA/peptide tetramers with higher efficiency (as indicated by the mean fluorescence intensity) when tetramers were loaded with CAP1-6D with respect to CAP1 peptide. Again, efficient staining with α3-mutated tetramers was observed only in the presence of CAP1-6D (Fig. 4c). Altogether, these data suggest that anti-CAP1-6D T cells display low affinity and CD8-dependency, thus, when interacting with the native CAP1 peptide, while recognition of CAP1-6D, occurring with high affinity, does not required CD8 involvement.

Low affinity and CD8-dependency of tetramer/CAP1-sorted T cells and clones

As the functional analysis of anti-CAP1-6D specific T cells was performed in lymphocyte bulk cultures likely containing polyclonal peptide-specific T cell responses expressing different TCRs, cells cross-reacting with native peptide CAP1 were sorted from anti-CAP1-6D T lymphocytes and functionally analyzed. T cells binding to HLA-A*0201$^+$/CAP1 tetramers were thus purified by immunosorting, achieving a >95% mean purity of tetramer$^+$CD8$^+$ T cells (a representative case is shown in Fig. 5a). Nevertheless, this selection did not enhance the ability of T cells (either sorted T cell bulk cultures or clones, obtained by limiting dilution and selection for CAP1 reactivity) to cross-react with native CAP1 peptide. In fact, T cells retained their limited affinity for the native epitope, as evaluated by IFN-γ ELISPOT in response to CAP-1-pulsed T2 cells (Fig. 5b, left

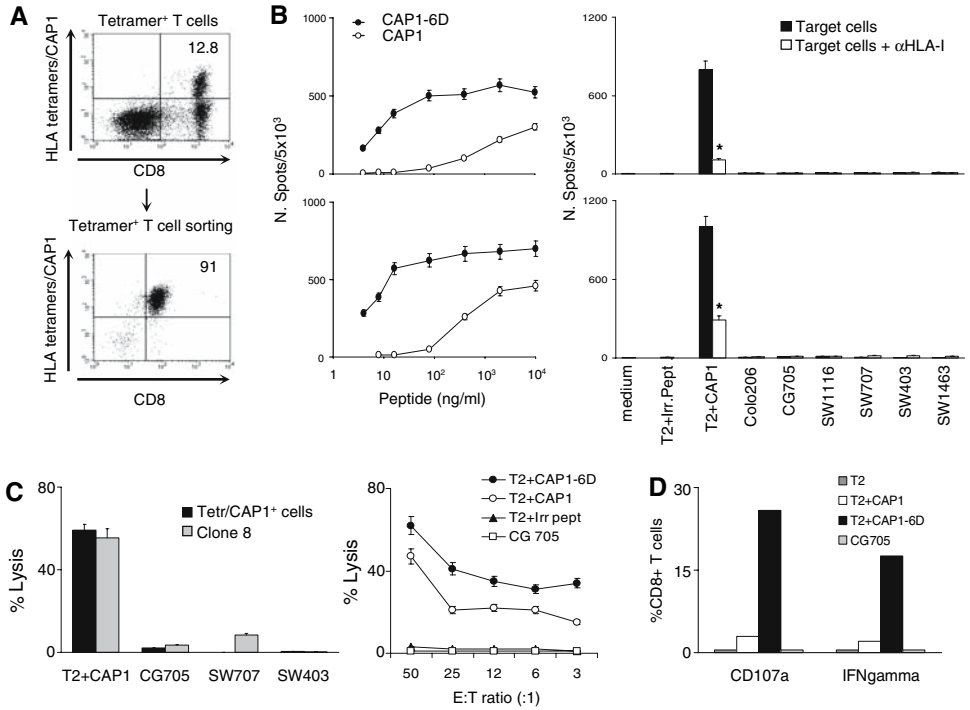

Fig. 5 Functional avidity of anti-CAP1-6D T cells. **a** Anti-CAP1-6D cells (at 4 week culture) were enriched for T cells recognizing the parental peptides by immuno-magnetic sorting of CAP1/tetramer$^+$ cells. *Numbers* indicate the percentage of CAP1-tetramer$^+$ population before (*upper panel*) and after (*lower panel*) immuno-sorting. T cells were then further expanded in vitro, preserving their peptide specificity. **b** IFN-γ release by CAP1/tetramer-sorted T cells (*upper panel*) and Clone 8, a clone obtained by limiting dilution of T cells sorted for CAP1/tetramer binding (*lower panel*), in response to T2 cells loaded with increasing concentrations of native CAP1 and analogue CAP1-6D peptides. CAP1/tetramer-sorted T cells, or the T cell Clone 8, were evaluated for IFN-γ ELISPOT in the presence of a panel of CEA$^+$HLA-A*0201$^+$ CRC cell lines or T2 cells pulsed with native peptide CAP1. Targets were pre-treated (*white bars*) or not (*black bars*) with the anti-HLA class I mAb W6/32. **c** CAP1/tetramer sorted T cells and Clone 8 were tested for the ability to lyse T2 cells pulsed with CAP1 peptide at 10 μg/ml and CRC CEA$^+$ cell lines (CG705, SW707, SW403). Cytotoxic activity of CAP1-6D T cells was evaluated in 4 h ^{51}Cr release assay, using T2 cells pulsed with CAP1, CAP1-6D or irrilevant peptide (Nef) at 10 μg/ml peptide concentration or CG705 cells as target cells. **d** Anti-CAP1-6D T cells were also tested for CD107a mobilization against target cells pulsed with CAP1, CAP1-6D or CG705 cells at *E*:*T* ratio of 5:1. At the same time IFN-γ production was detected by intracellular staining. *$P < 0.05$ (evaluated by *t* test for unpaired samples), as compared with the recognition of the same target in the absence of anti-HLA I mAb. Results are representative of three independent experiments

panel). Most importantly, they did not acquire the ability to release IFN-γ in response to a large panel of HLA-A*0201$^+$CEA$^+$ CRC cell lines (Fig. 5b, right panel), to lyse these targets in a 4 h ^{51}Cr-release assay (Fig. 5c) or to mobilize in their presence the lytic granule marker CD107a (Fig. 5d).

In line with these functional data, experiments of tetramer titration using wild type and α3-mutated HLA-A2 tetramers showed that CAP1/tetramer$^+$-sorted T cells retained their CD8-dependency when interacting with the parental peptide. In fact, binding was completely abrogated when CAP1 peptide was presented in the context of α3-mutated HLA-A2 tetramers (Fig. 6a, b). Furthermore, and similarly to data achieved with unsorted cells, anti-CD8 mAb significantly reduced IFN-γ release in response to T2 cells loaded with CAP1, but not with CAP1-6D peptide (Fig. 6c). These results indicate that low affinity and CD8-dependency may be intrinsic features of T cells generated in the presence of the superagonist CAP1-6D analogue, and could justify the inability of CAP1-6D specific T cells to efficiently interact with the natural epitope endogenously presented by CEA$^+$ CRC cells.

Discussion

Altering peptide sequence to improve either HLA binding or TCR interaction is presently considered to be a valid strategy to ameliorate vaccine-induced T cell responses against human tumors [29, 32, 39, 42, 44, 47, 49]. Indeed, specific immunotherapy performed with native tumor antigens has thus far led to limited immune responses and, most importantly, has shown poor clinical efficacy [14, 17, 18, 22, 24, 35, 38, 46].

The idea of breaking tolerance toward tumor antigens, thanks to the in vivo triggering of TCR repertoires different

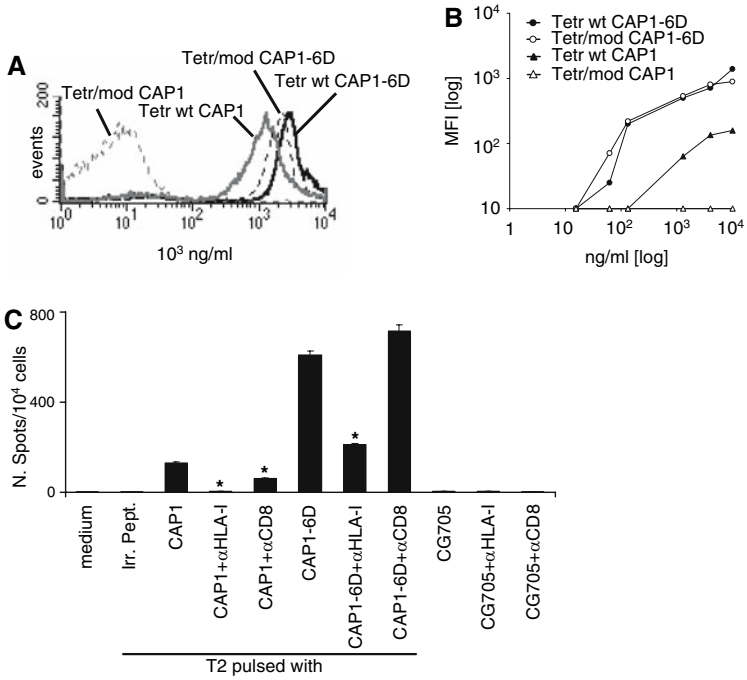

Fig. 6 CD8 involvement in recognition of native and APL. **a** CD8 dependency was assessed using tetramer staining with wild-type or mutated tetramers in α3 domain of HLA, abrogating CD8-binding. Anti-CAP1-6D T cells, sorted for CAP1/tetramer staining, were labeled with tetramers containing either CAP1 or CAP1-6D, showing a CD8 dependency when stained with CAP1/tetramer. MFI was evaluated on tetramer+CD8+ stained T cells. **b** Tetramer titration assay was performed using serial dilutions of the indicated tetramers. **c** CD8 dependency was evaluated by IFN-γ release of CAP1-6D T cells in response to different target cells: T2 cells pulsed with CAP1, CAP1-6D or irrelevant peptide (Nef) and CG705 cells. Alternately, target cells were pre-treated with anti-HLA class I antibody W6/32 to prove TCR involvement and CAP1-6D T cells were pre-incubated with anti-CD8 to verify the CD8 dependency. $^*P < 0.05$ (evaluated by *t* test for unpaired samples), as compared with the recognition of the same target in the absence of blocking mAb. Results are representative of three independent experiments

from those naturally involved in spontaneous anti-tumor responses, is presently encouraging a large number of clinical trials based on the usage of mutated antigenic epitopes, or APL, for in vivo immunization. This is particularly true for those tumor histologies, such as CRC, in which natural antigens capable of boosting potent T cell reactivities are still unavailable for clinical use.

However, APL-based vaccine trials performed in melanoma and CRC patients have so far provided disappointing results, showing indeed improved immunological responses but no equivalent increase in tumor regressions [10, 41]. Although multiple mechanisms could be responsible for the relative failure of APL as cancer vaccines, such as the usage of ineffective immunization strategies or the presence of regulatory/suppressive mechanisms in the host [19, 34], it cannot be denied that the inability of APL-primed T cells to effectively cross-recognize native epitopes expressed by tumor cells could actually represent an easy explanation.

Indeed, given the high complexity of the TCR structure, it is conceivable that alterations in the epitope sequence, even with subtle and conservative aminoacid changes, may produce a different structure of the HLA/peptide complex that may be no longer recognized by the whole TCR repertoire directed toward the original epitopes [8]. While examples of efficient cross-reactivity with native antigens have been reported for specific APL-induced T cells [3], low efficiency and even lack of tumor recognition by APL-induced T lymphocytes has been observed as well, especially at clonal level even for highly immunogenic analogues such as those derived Melan A/Mart-1 and gp100 [5, 41].

CAP1-6D was identified as a superagonist analogue of the native CEA-derived epitope CAP1, hypothesized to mediate a better TCR binding, introducing of an Asp residue in position 6 [49]. Although some evidence about the ability of CAP1-6D to generate T cells displaying a certain degree of recognition of CEA-expressing tumor cells has been reported [49], it should be underlined that these initial studies were indeed performed with T cells raised in the presence of robust co-stimulatory conditions and by multiple in vitro re-stimulations.

With the final aim of using CAP1-6D as an immunogen for clinical protocols of adoptive or active immunotherapy, we performed an extensive analysis of the immunogenicity of this peptide in PBMC from both healthy donors and CRC patients. Unexpectedly, we found that CAP1-6D

specific T cells that could be reproducibly generated and efficiently expanded in all cases analyzed, displayed reduced avidity for the native epitope and, most importantly, constantly lacked reactivity against CEA^+ CRC cells. In this regard, signs of tumor recognition were extensively probed utilizing a broad array of functional assays, i.e. testing lytic activity (by ^{51}Cr-release), production of IFN-γ and other cytokines, CD107a mobilization and TCR down-modulation. Furthermore, the CRC targets used in the study were carefully selected for adequate levels of CEA and HLA-A2 expression and for recognition by CAP-1-specific T cells as an index of HLA/CAP1 peptide complex expression on their cell surface [40]. Through a more detailed characterization of T cell reactivity, we observed that anti-CAP1-6D T cells interact with the natural CAP1 epitope with a limited TCR affinity and in a CD8-dependent fashion. This evidence clearly suggests that the inability of anti-CAP1-6D T cells to recognize CEA-expressing tumors could be ascribed to the limited T cell avidity that these cells display for the natural ligand, and that, conversely, high avidity T cells may hence be required for CRC cell recognition. This observation is in line with several reports generally showing a direct correlation between T cell avidity and recognition of suboptimal targets such as tumor cells, known to express limited amounts of HLA/peptide complexes and lack co-stimulatory molecules [21].

It could be hence hypothesized that, for achieving clinical efficacy, anti-tumor vaccines should be aimed at triggering high avidity T lymphocytes [13, 21]. Indeed, although most high affinity self-reactive T cells should have been deleted in the thymus [23], it has been recently demonstrated that cells with these features and capable of recognizing self-tumor antigens are indeed conserved in the T cell repertoire of healthy donors as well as cancer patients [50]. In vivo expansion of high avidity tumor-specific T cells could then be achieved by several strategies, providing for instance strong co-stimulation during in vivo priming, as recently described for vaccines based on the usage of CAP1-6D combined to TRICOM adjuvant [20, 24, 27]. In addition, immunization with low amounts of peptide, in the presence of robust co-stimulation, could favor the in vivo expansion of high avidity T cells, as described in murine models [16] and in vitro settings [48]. Sorting anti-tumor T cells for their CD8-independency, by α3-mutated HLA tetramers, may instead represent a valid option for adoptive immunotherapy [30].

Although modified T cell epitopes with point mutations enhancing binding to HLA or TCR, still remains a valid strategy for priming T cells against the original non-mutated antigen expressed by tumor cells, as recently underlined by the elegant studies of Engelhorn et al. [9], our data warn of the importance of performing extensive immunological monitoring when APL are used as anti-cancer vaccines. In fact, as an increase in the frequency of APL-specific T cells may not be indicative of the concrete anti-tumor activity induced by vaccination, T cell recognition of native Ag-expressing tumor cells should be a mandatory evaluation in the immunological monitoring of APL-based vaccines.

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