Difficulties and dangers of CEA-targeted immunotherapy against colorectal cancer

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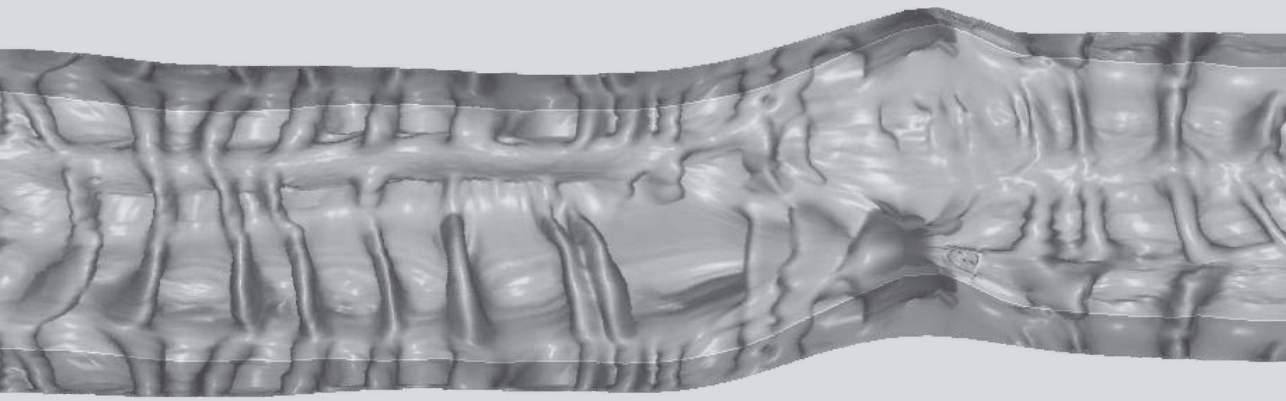

Expression of a natural tumor antigen by thymic epithelial cells impairs the tumor-protective CD4+ T-cell repertoire

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Abstract – A variety of antigens that display a highly tissue-specific expression pattern, have recently found to be also expressed in medullary thymic epithelial cells (mTEC). This unique feature of mTEC plays an important role in preventing hazardous autoimmune responses through thymic tolerization of T-cell subsets directed against auto-antigens, but could also limit the possibility of exploiting tumor-associated antigens for immune-mediated targeting of cancers. Our present study shows that expression of carcinoembryonic antigen (CEA) in thymic epithelial cells of CEA-tg mice results in tolerization of a major fraction of the CD4+ T-cell repertoire against this antigen, thereby markedly limiting the effect of CEA-specific immunization against CEA-overexpressing tumors. The expression of CEA in mTEC of CEA-tg mice is mirrored by its expression in human mTEC, arguing that promiscuous gene expression in these thymic stromal cells needs to be considered as a potential hurdle for immunotherapies of cancer that target tissue-specific auto-antigens.

Introduction

Development of effective immunotherapies against cancer crucially depends on the availability of suitable target antigens that allow the immune system to discriminate between tumorigenic versus normal somatic cells. Ideal targets are the tumor-specific antigens as expressed by, for instance, virus-induced tumors. For such antigens, the potency of the immune repertoire is not restricted by self-tolerance. These restrictions may, however, apply to the development of immunotherapies for cancers that lack tumor-specific antigens, in that the immune attack in these cases needs to be targeted against tumor-associated auto-antigens. Of the many antigens belonging to this latter category, carcinoembryonic antigen (CEA) was one of the first to be discovered. Over the past years, CEA has been intensively studied for its potential in the immunological targeting of cancers of epithelial origin, in particular colorectal cancers [1,2 and references therein]. Although much research has been conducted to study immunity against this antigen in humans and in mouse models, convincing evidence that CEA-specific immunity is effective in preventing tumor growth and metastasis in colorectal cancer patients is still missing. The potential of CEA as a target antigen for immunotherapy of cancer could be restricted by the fact that CEA is expressed in several large and vital epithelial tissues, including gut and lung, and is even routinely found in the serum of healthy individuals. In view of these issues, we have done a detailed analysis of the specificity and anti-tumor efficacy of CEA-specific immunity in a transgenic mouse model in which the expression of CEA closely resembles that in man [3]. Intriguingly, our data suggest that it is not the expression of CEA in the periphery, but rather that in the thymic epithelial cells that restricts the anti-tumor potential of the CEA-specific immune response.

Materials and Methods

Mice. C57BL/6 Kh (B6, H-2^b) and CEA-tg mice were bred in our own facilities (Leiden, The Netherlands). CEA-tg mice were originally obtained from Dr. John Thompson (Freiburg, Germany). Mice were analyzed for CEA genotype by PCR analysis as described previously [4]. C57BL/6 *nu/nu* mice were purchased from Taconic (Ry, Denmark). The experiments were approved by the animal experimental commission (UDEC) of Leiden University.

Immunizations and tumor challenge experiments. ALVAC-CEA (provided by Aventis Pasteur, Toronto, Ontario, Canada) was diluted in PBS and mice were injected i.v. with 3×10^7 plaque-forming units (pfu) in 200 µl. Two weeks after the last vaccination, spleens were isolated for in vitro tests or mice were used for tumor challenge experiments where 250.000 MC38-CEA tumor cells were injected s.c. in 200 µl PBS/0.5% bovine serum albumin. MC38-CEA cells (obtained from Dr. James Primus, Nashville, TN) [5] were cultured in Iscove's modified Dulbecco's medium (Life Technologies, Rockville, MD) supplemented with 10% FCS, 50 µmol/L 2-mercaptoethanol, 2 µmol/L L-glutamine, 1 unit/ml penicillin and 300 µg/ml geneticin. CEA expression by MC38-CEA was regularly examined by cell surface staining with an anti-CEA antibody (PARLAM4, Monosan, The Netherlands). Depletions of

CD4+ or CD8+ cells were done by i.p. injection of 100 μg GK1.5 or 2.43 respectively, starting one week before the tumor challenge and was continued during the experiment.

In vitro analysis of CD4+ T-cell responses. Splenocytes from immunized mice were cultured (4×10^6 cells per well in 24-well plates) in the presence of D1 cells (1×10^5 cells per well) and CEA protein (5 μg/ml). After 4 days of culture, cells were harvested with EDTA and viable cells were isolated by centrifugation over a Ficoll gradient. Cells were plated at 2×10^6 per well in 24-wells plates in the presence of 10 IU/ml interleukin 2 (IL-2) (Chiron BV, Amsterdam, The Netherlands). At day 7, cells were tested for their responsiveness against an overlapping set of 25-mer CEA-peptides covering the entire CEA-protein sequence. 1×10^5 T cells were incubated with 1×10^5 irradiated spleen cells in the presence of 5 nmol/ml peptide in 150 μl in 96-wells plates. After 24 hours, the supernatant was tested for IFN-γ by sandwich ELISA (BD Pharmingen, Alphen aan den Rijn, The Netherlands). For intracellular cytokine staining we used the same culture protocol as described above. At day 7.2 $\times 10^5$ T cells were incubated with 2×10^5 irradiated spleen cells in the presence of 5 nmol/ml peptide in 150 μl in 96-wells plates. Fixation and staining procedures were performed as described previously [6].

Thymus transplantation. Thymic lobes were isolated from newborn mice and stored in PBS on ice to allow for genotyping of the donor mice for the CEA transgene. Two lobes were transplanted under the kidney capsule of each of the recipient C57BL/6 *nu/nu* mice, and 10 weeks later, recipients were vaccinated thrice with 3×10^7 pfu ALVAC-CEA i.v. with a 2-week interval. Two weeks after the last vaccination, spleens were isolated for in vitro testing of CD4+ T-cell responses as described above.

Bone marrow transplantation. Wild-type recipient mice were irradiated to ablate their hematopoeitic system (9.5 Gy) and reconstituted with T-cell depleted bone marrow (7×10^6 cells) derived from wild-type or CEA-tg donor mice. Seven weeks after bone marrow engraftment, recipient mice were immunized thrice with ALVAC-CEA with a 2-week interval. Two weeks after the last vaccination, spleens were isolated to measure IFN-γ production by CD4+ T cells.

Analysis of carcinoembryonic antigen mRNA expression by reverse transcription-PCR and microarray analysis. Murine and human thymic stromal cell purification, RNA isolation, cDNA synthesis, PCRs and microarray analysis were done as described previously [7,8]. Quantative reverse transcription PCR (RT-PCR) was done with the SYBR Green I kit (Eurogentec, Liege, Belgium). Primers used to amplify specific gene products from human cDNA were: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense, 5'-TCGACAGTCAGCCGCATCT-3'; GAPDH antisense, 5'-CCGTTGACTCCGACCTTCA-3'; CEA sense, 5'-TCCAGAACTCAGTGAGTGCAAAC-3'; and CEA antisense, 5-'CTCCCGAAAGGTAAGACGAGTC-3'. Primers used for murine cDNA were: CEA sense, 5'-GCCTGTTTTGTCTCTAACTTGGCTACTGG-3'; CEA antisense, 5'-TTGGCTAGGATGGTCTCGATCTCTGGT-3'; β-actin sense, 5'-TGGAATCCTGTGGCATCCATGAAAC-3'; and β-actin antisense, 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'.

Results

CEA-specific anti-tumor immunity in wild-type and CEA-tg mice

Although several members of the CEACAM gene family are quite conserved between man and mouse, a true homologue for human CEA (CEACAM-5) is missing from the mouse. In the case where mice are challenged with CEA-overexpressing tumor cells, CEA can therefore be considered a foreign, tumor-specific antigen. Accordingly, immunization of C57BL/6 (wild-type) mice with recombinant canarypox virus encoding CEA (ALVAC-CEA) induced strong protective immunity capable of rejecting an otherwise lethal dose of the syngeneic, CEA-expressing tumor cell MC38-CEA (Fig. 1A). To study the effector mechanism responsible for the tumor eradication, we depleted CD4+ or CD8+ cells in vaccinated mice during the effector phase of the anti-tumor response. The majority of the tumors grew out after CD4+ depletion, whereas CD8+ depletion had no significant effect on tumor development (Fig. 1A). This indicates that CEA-specific CD8+ T cells have no major role in the effector phase of the MC38-CEA-specific anti-tumor response in wild-type mice, whereas CD4+ cells are essential for tumor eradication. This is somewhat unexpected in view of the fact that MC38 is MHC class II negative. Notably, crucial importance of CD4+ T-helper cells, and even a dominance of this T-cell subset over the CD8+ cytotoxic T-cell response in protective immunity against class I-positive, class II-negative tumors has been reported in several other tumor models, including those involving challenge with RMA leukemia and B16 melanoma cells [9-12]. Because CD4+ T cells, although indispensable for immune defence against MC38-CEA, cannot directly attack this MHC class II-negative tumor, it is conceivable that the CEA-specific CD4+ T-cell response orchestrates other effector mechanisms. Indeed, we found that depletion of natural killer cells in vaccinated wild-type mice resulted in reduced protection (50%) against tumor outgrowth and vaccination failed to protect Fcγ receptor knockout mice (data not shown), indicating that innate and humoral effector mechanisms contributed to the tumoricidal immune attack.

To evaluate the effect of CEA-specific immunization on the outgrowth of CEA-overexpressing tumors in a setting where CEA represents a tumor-associated auto-antigen, we also did tumor challenge experiments in CEA-tg mice. These mice exhibit a tissue-specific expression of the CEA-transgene essentially identical to that found in humans [3]. Repeated vaccination with ALVAC-CEA, for up to three sequential doses, failed to elicit sufficient protective immunity capable of even delaying outgrowth of MC38-CEA (Fig. 1B). To further enhance T-cell responses, we also tested the efficacy of heterologous prime boost protocols in these mice, because several reports have shown these to be superior in triggering immunity compared with repeated vaccination with the same vaccine construct [13,14]. However, none of various prime boost protocols, involving sequential administration of DNA, protein, ALVAC and/or NYVAC based in different combinations and order, succeeded in inducing an immune response sufficient to protect mice against a challenge with the CEA-positive tumor (data not shown), indicating that tolerance to CEA somehow seemed to prevent the induction of effective anti-tumor immunity.

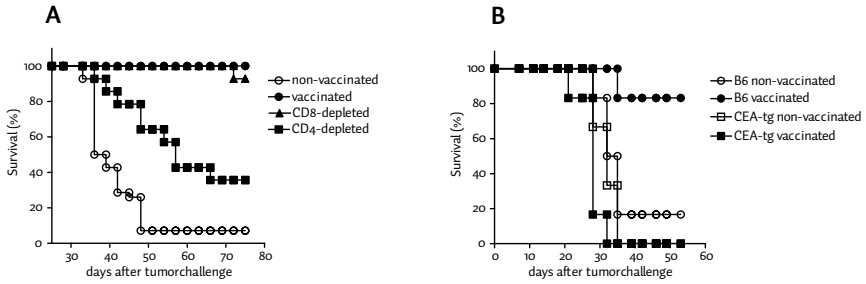

FIGURE 1. Anti-tumor effect of CEA-specific immunization in wild-type and CEA-tg mice. A. Wild-type B6 mice (14 per group) were vaccinated twice i.v. with a 2-week interval with 3×10^7 pfu of ALVAC-CEA (●) and were, in addition, depleted for CD8+ T cells (▲) or CD4+ T cells (■), starting depletion 5 days before tumor challenge. Two weeks after the last vaccination all mice were challenged s.c. with a lethal dose of 0.3×10^6 MC38-CEA cells. Tumor growth was also monitored in non-vaccinated mice (○). Tumor size was measured every 3 days and mice were sacrificed when tumor size exceeded 1000 mm^3. **B**. The same tumor challenge experiment was done in groups (6 mice per group) of wild-type mice, either non-vaccinated (○) or vaccinated twice with ALVAC-CEA (●), as well as in groups of CEA-tg mice, either non-vaccinated (□) or vaccinated thrice with ALVAC-CEA (■). Each of the experiments in A and B was done twice with comparable outcome.

Charting of CEA-specific CD4+ T-cell responses in wild-type and CEA-tg mice

Because CD4+ cells play a crucial role in the protection against MC38-CEA in wild-type mice, we assessed the magnitude and specificity of the CEA-specific CD4+ T-cell response in CEA-immunized wild-type and CEA-tg mice. T-cell reactivity was tested against an array of 69 overlapping 25-mer peptides covering the entire CEA-protein sequence. CEA-specific IFN-γ production by splenocytes from wild-type mice was detected against five distinct epitopes that we numbered 1 to 5 based on their position in the CEA protein sequence (Fig. 2A, Table I). Epitope 1 is comprised by peptide 18 as well as by peptide 36 and the adjacent, overlapping peptides 53 and 54. The repeated occurrence of this epitope is due to the fact that CEA is built up from several repetitive domains, including the A domain that comprises epitope 1. Each of the other four epitopes occurs only once in the CEA sequence. Epitopes 2 and 3 are each covered by sets of two adjacent overlapping peptides (42/43 and 57/58 respectively), whereas epitopes 4 and 5 are each comprised by a single peptide (62 and 65 respectively). Immunized CEA-tg mice also displayed CEA-specific T-cell responses, but these were directed against epitopes distinct from those recognized by wild-type mice, in that reactivity was directed against two epitopes that were comprised in peptides 44 and 61 (Fig. 2B; Table I, epitopes 6 and 7). Although these peptides overlap with peptides 43 and 62 respectively, which were recognized by wild-type mice, CEA-tg mice did not show any reactivity against these latter peptides. Both wild-type and CEA-tg splenocytes also reacted against APC loaded with the CEA-protein (Fig. 2C), showing their capacity to respond against physiological quantities of naturally processed antigen. CEA specific T-cell immunity in wild-type and CEA-tg mice showed marked differences in magnitude, in that wild-type splenocytes produced high levels of IFN-γ (2-8 ng/ml) that were already detectable after a single vaccination with ALVAC-CEA, whereas at least three vaccinations were required to trigger modest levels of CEA-specific T-cell immunity in

TABLE 1. Sequences of CEA peptides recognized by CD4+ T cells in wild-type versus CEA-tg mice.

epitope no.	peptide no.	amino acid no.	Ig-domain	amino acid sequence
wild-type				
1	18	170-195	A1	TQDA**TYLWWVNNQSLPVS**PRLQLSN
1	36	350-375	A2	NT**TYLWWVNNQSLPVS**PRLQLSNDN
1	53	520-545	A3	FTCEPEAQNT**TYLWWVNGQSLPVS**
1	54	530-555	A3	**TYLWWVNGQSLPVS**PRLQLSNGNRT
2	42	410-435	B2	VLYGPDDPTI**SPSYTYYRPGVNLSL**
2	43	420-445	B2	**SPSYTYYRPGVNLSL**SCHAASNPPA
3	57	560-585	A3	VTRNDARAYV**CGIQNSVSANRSDPV**
3	58	570-595	A3	**CGIQNSVSANRSDPV**TLDVLYGPDT
4	62	610-635	B3	LNLSCHSASNPSPQYSWRINGIPQQ
5	65	640-665	B3	FIAKITPNNNGTYACFVSNLATGRN
CEA-tg				
6	44	430-455	B2	VNLSLSCHAASNPPAQYSWLIDGNI
7	61	600-625	B3	PDSSYLSGANLNLLSCHSASNPSPQY

Listed are the amino acid sequences of the 25-mer peptides from the overlapping peptide array that were recognized, along with their position in the CEA protein. Sequences shared by recognized peptides are depicted in bold type.

CEA-tg mice (0-2 ng IFN-γ/ml) (Fig. 2B). Detection of CEA-reactive T cells by fluorescence-activated cell sorting (FACS) through intracellular IFN-γ staining of the reactive T cells in combination with CD4/CD8 staining, confirmed that only CD4+ T cells were responsible for IFN-γ production. These data furthermore illustrate the marked qualitative and quantitative difference between CEA-specific T-cell immunity in wild-type and CEA-tg mice (Fig. 2D).

In addition to the differences in CD4+ T-cell responses, immunized CEA-tg mice showed approximately 10-fold lower CEA-specific IgG antibody titers compared with wild-type mice (data not shown). This difference is most likely caused by the lower CEA-specific CD4+ T-cell responses in CEA-tg mice and/or by restrictions in the CEA-reactive B-cell repertoire of CEA-tg mice. The importance of both CD4+ T-cell and IgG-mediated immunity in protecting wild-type mice from outgrowth of CEA-positive tumors, together with the considerably reduced breadth and/or magnitude of these responses in CEA-tg mice, can readily account for the failure of CEA-tg mice to reject CEA-positive tumors even after extensive CEA-specific vaccination schemes.

Partial tolerance is induced by thymic epithelial cells

Our results indicate that a major part of the CEA-specific CD4+ T-cell repertoire, directed against immunodominant CEA epitopes, is tolerized in CEA-tg mice, leaving only weak responses against other, subdominant CEA epitopes. This might be due to peripheral tolerance, established by the presence of circulating CEA and/or to the expression of CEA by several epithelia in these mice. Alternatively, the thymus could express CEA protein, which would result in central tolerance for this antigen. Thymic expression of CEA in the CEA-tg mice used for these experiments could previously not be shown by northern blot or immunohistochemical analysis as shown by Eades-Perner et al. [3]. Nevertheless, several tissue-specific auto-antigens, previously considered absent from the thymus, were recently found to be expressed by medullary thymic

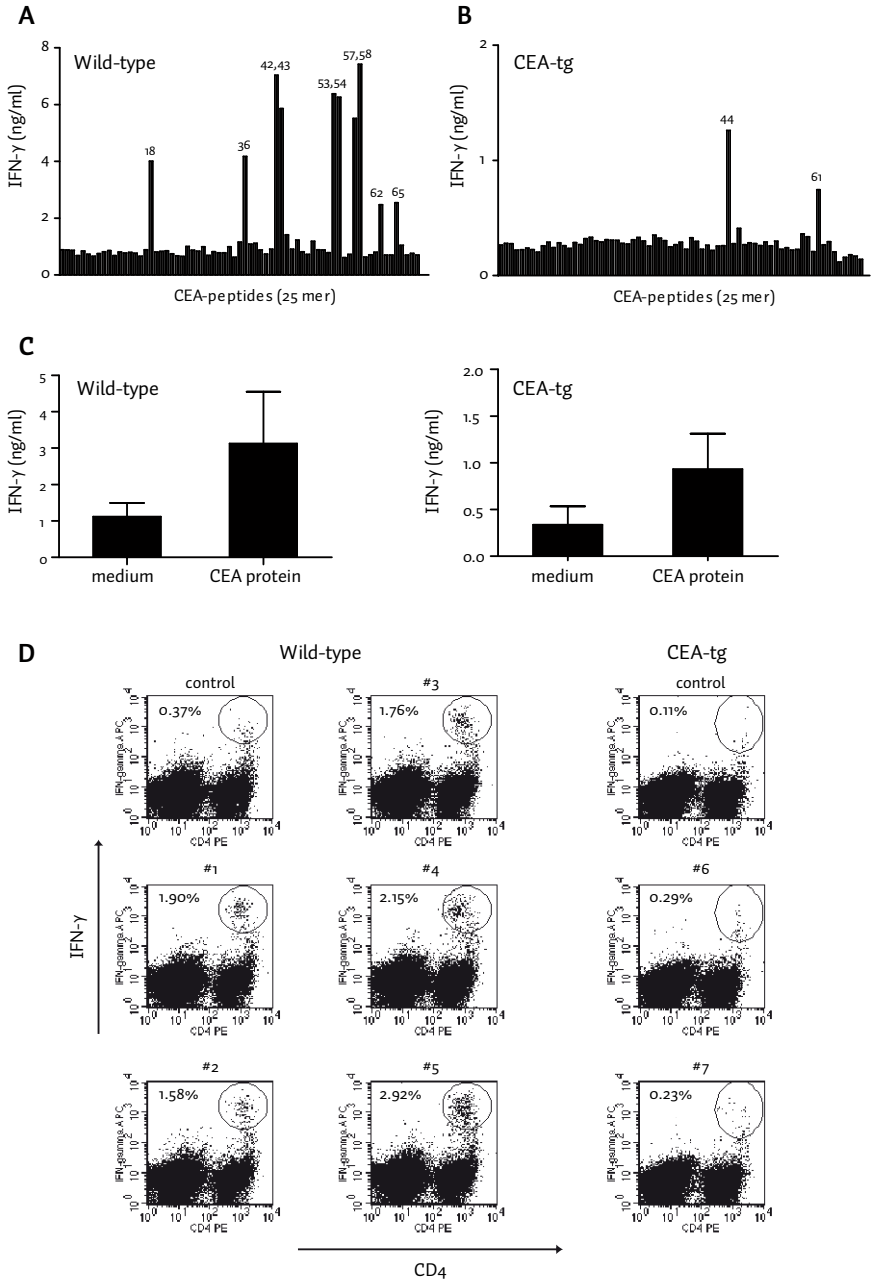

FIGURE 2. Distinct CEA-specific CD4+ T-cell responses in wild-type versus CEA-tg mice. Wild-type (**A**) and CEA-tg (**B**) mice were immunized respectively two and three times with 2-wk intervals by i.v. injection of 3×10^7 pfu ALVAC-CEA. Splenocytes were isolated 2 weeks after the last vaccination, after which they were cultured in the presence of CEA-loaded D1 cells for 1 week. Viable cells from these cultures were assayed for IFN-γ production in the presence of overlapping 25-mer peptides covering the CEA protein sequence (69 peptides). Each figure depicts a representative example of the response observed in at least 10 individual mice. **C**, in addition, the reactivity of wild-type and CEA-tg splenocytes against APC loaded with the full length CEA protein was tested. Columns, means of six mice per group; bars, ± SD. **D**, Epitope-specific IFN-γ production by CD4+ T cells was also measured by intracellular cytokine staining.

epithelial cells (mTEC) [7]. In fact, these mTEC were found to express a wide range of tissue-specific antigens and to be able to present these antigens to thymocytes in the context of both class I and class II MHC [15,18].

To address the potential role of the thymus in restricting the CEA-specific CD4+ T-cell repertoire in CEA-tg mice, we transplanted the thymic lobes from newborn CEA-tg and from wild-type donor mice under the kidney capsule of T-cell deficient nude mice and studied the T-cell responses in the grafted animal after CEA-specific immunization. The panel of epitopes recognized by mice that had received thymic lobes from non-transgenic mice was identical to that found for wild-type mice (Fig. 3A). By contrast, recipients of CEA-tg thymic lobes vaccinated thrice with ALVAC-CEA displayed CD4+ T-cell responses closely resembling those in CEA-tg mice (Fig. 3B). This shows that the origin of the thymus dictates the specificity of the CD4+ T-cell response against CEA as observed in CEA-tg mice.

Several cell populations in the thymus might be involved in establishing CD4+ T-cell tolerance, such as dendritic cells, macrophages and epithelial cells, including the aforementioned mTEC. Therefore, CEA-expression of these thymic subsets isolated from CEA-tg mice was analyzed by RT-PCR. Indeed, a high level of CEA mRNA was detected in mTEC from CEA-tg mice. In addition, approximately 10 fold lower levels of CEA mRNA were found in macrophages and cortical epithelial cells (cTEC), whereas CEA expression was not detected in thymic dendritic cells. Purity of epithelial cell subsets was confirmed by detection of GAD67 expression in mTEC only (Fig. 4). To investigate whether the expression of CEA in thymic macrophages would contribute to tolerance induction, we generated bone marrow-chimeric animals in which there was no potential source of CEA other than the cells from the CEA-tg bone marrow-derived lineage. Mice were vaccinated thrice with ALVAC-CEA, starting 7 weeks after the transplantation. CD4+ T-cell responses resembled those in wild-type mice, clearly showing that the presence of CEA-tg bone marrow-derived cells, including the thymic macrophages, did not affect the CEA-specific CD4+ T cells (Fig. 3C,D). To confirm that the hematopoietic system, including T cells and APC, of the bone marrow-chimeric mice was effectively reconstituted by the donor graft, and that the IFN-γ production we measured in vitro was produced by donor CD4+ T cells, we used Ly5.1 donor mice and Ly5.2 recipients. FACS analysis showed that reactive CD4+ T cells were indeed all of Ly5.1 donor origin (data not shown). Apart from confirming efficient reconstitution of the immune system of graft recipients, this experiment showed that T-cell progenitors from CEA-tg mice do have the potential of developing into a full CEA-specific CD4+ T-cell repertoire in the absence of a CEA-tg thymus. The results of these experiments in conjunction with the expression pattern of CEA further support the notion that thymic epithelial cells are prominently involved in restricting the CEA-specific CD4+ T-cell repertoire in CEA-tg mice.

CEA is expressed by human mTEC

To assess the relevance of our findings in CEA-tg mice for the human setting, we analyzed the expression pattern of CEA (CEACAM 5) in human TEC isolated from three different human thymus biopsies by real-time PCR. In agreement with our findings in the CEA-tg

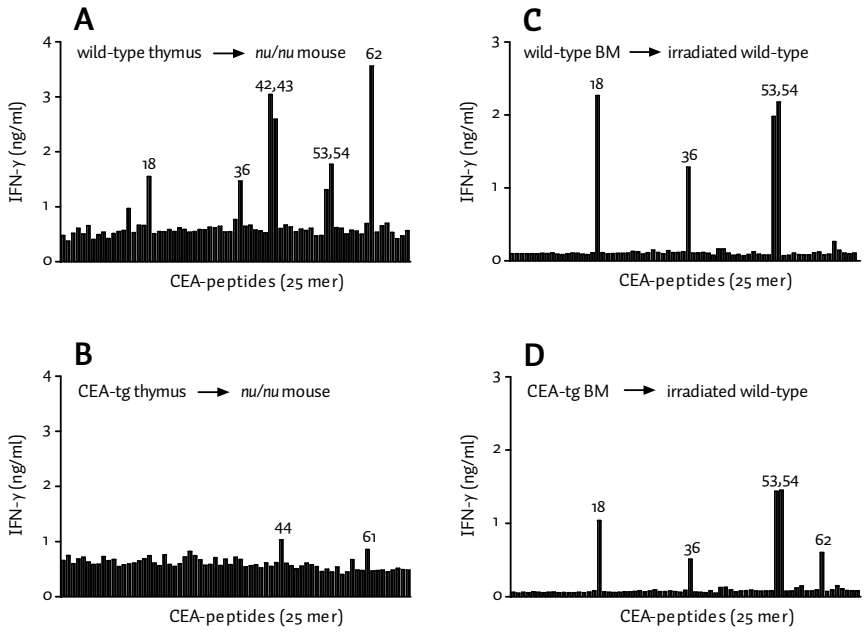

FIGURE 3. Specificity and magnitude of the CEA-specific T-cell response are dictated by the thymus. Thymic lobes from a newborn wild-type (**A**) and CEA-tg (**B**) mice were transplanted under the kidney capsule of nude mice. After reconstitution, recipient mice were vaccinated thrice at 2-week intervals with ALVAC-CEA. Splenocytes were isolated 2 weeks after the last vaccination and restimulated in vitro in the presence of CEA-loaded D1 cells. Of the 14 mice that received a wild-type thymus, 12 showed $CD4+$ T-cell responses (IFN-γ concentration > 1.5 × medium control) against epitopes 1 to 5. Two mice did not show a response, most likely as a result of inefficient thymus grafting. Of the 11 mice that received a CEA-tg thymus, three showed a $CD4+$ T-cell response (IFN-γ concentration > 1.5 × medium control) against epitope 6 to 7. In the other mice, detection of this relatively weak CEA-specific response is most likely hampered by the fact that the immunocompetence of thymus-engrafted nude mice is generally decreased compared with mice with a fully intact immune system. Representative examples of responding mice for each group. Lethally irradiated wild-type mice were reconstituted with T-cell depleted wild-type (**C**) or CEA-tg bone marrow (**D**). IFN-γ production by splenocytes was measured after the third vaccination with ALVAC-CEA. For each group, one representative example out of seven mice is shown.

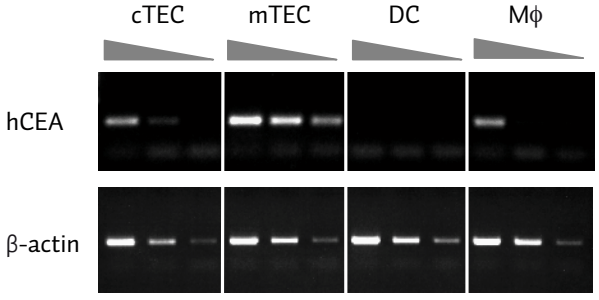

FIGURE 4. Expression of CEA by different thymic stromal cell subsets isolated from CEA-tg mice. Expression of CEA in purified cTECs, mTECs, thymic dendritic cells (DC), and macrophages from CEA-tg mice was assessed by RT-PCR. Purity of epithelial cell subsets was confirmed by detection of GAD67 expression in mTEC only. The amount of input cDNA was normalized according to signals obtained for actin.

mice, we found CEA to be expressed in mTEC (Fig. 5). This suggests that human thymocytes expressing CEA-specific T-cell receptors, like those in CEA-tg mice, will encounter their target antigen in the thymus and therefore will be subject to tolerization.

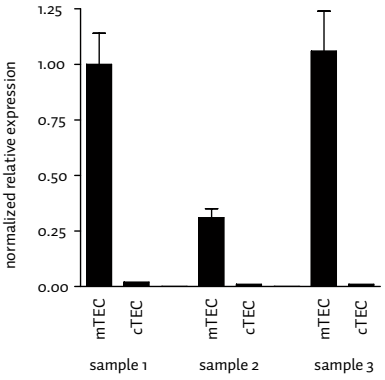

Figure 5. Expression of CEA in human medullary thymic epithelial cells. Expression of CEA in purified cTECs and mTECs of three human thymi was assessed by quantitative RT-PCR. Purity of cell isolates was >99% and verified by PCR expression analysis of indicator genes as described previously [6]. CEA expression was normalized to the GAPDH signal in each sample.

Discussion

Since the discovery of promiscuous gene expression in thymic epithelial cells, it has been speculated whether this feature would limit the T-cell repertoire directed against tumor-associated auto-antigens and, thereby, the potential of this immune response for immunotherapeutic targeting of cancers [7,16-18]. Our work shows expression of a tumor-specific antigen considered for targeting of human cancers, CEA, in mTEC of both CEA-tg mice and of human beings, and that this expression in mice profoundly restricts the breadth and potency of the T-cell repertoire available for antigen-specific targeting of tumors. This expression in mTEC can result in central tolerance through encounter of the antigen by the thymocytes on mTEC directly, or on other thymic APC that cross-present the mTEC-derived antigen [15,18,19]. Although our experiments in the CEA-tg mice do not discriminate between these two mechanisms, our data do show that the CEA presented in the thymus is derived from the epithelial, non-bone marrow-derived cell subset in the thymus. Our data are in concordance with the previously reported observation that additional members of the CEACAM gene family, in particular CAECAM-1 and -6, are expressed by human mTEC [7].

T-cell precursors that recognize the dominant CEA epitopes are tolerized in the CEA-tg mice. This central tolerance induction of CEA-specific CD4+ T cells is not complete, as CEA-tg mice do display weak responses against two other, subdominant epitopes that are apparently not presented in sufficient quantities in the thymus to cause tolerization. The residual CEA-specific T-cell repertoire is, however, not capable of control-

ling tumor growth, even if boosted by multiple vaccinations. Others have reported that repeated CEA-specific immunizations, when combined with repeated systemic administration of granulocyte macrophage colony-stimulating factor and/or Il-2, can delay (and in some cases prevent) the outgrowth of CEA-overexpressing tumors in CEA-tg mice [20-24]. These data argue that, under conditions that provide a strong non-specific stimulation to the immune system, the limited CEA-specific T-cell repertoire in these mice can suffice. Experiments in mice with adoptively transferred CD8+ T cells directed against the murine melanocyte/melanoma antigen gp100 have similarly shown that clearance of B16 melanoma requires both antigen-specific vaccination and systemic administration of IL-2 [25].

Analysis of the CEA-specific T-cell response in humans has resulted in the identification of several CTL and T-helper epitopes [26-28]. Especially with respect to the T-helper epitopes, our data raise the question whether these would be equivalent to those identified in CEA-tg or to those in wild-type mice. Our finding of CEA expression in human mTEC would suggest that in man, like in CEA-tg mice, the CEA-specific CD4+ T-cell repertoire is blunted by central tolerance and therefore that the CEA-target peptides identified in man represent subdominant epitopes. This by no means implies that the CEA-specific T-helper cells found in human subjects would recognize their target sequence inefficiently. In fact, the human T-helper cells, like the CEA-specific CD4+ T cells found by us in the CEA-tg mice (Fig. 2C), do not only react against APC loaded with synthetic peptide, but also with APC loaded with the full-length protein, arguing that they can recognize physiologic quantities of naturally processed peptide antigen [26,27]. Currently ongoing phase II and III vaccination trials in non-terminal colorectal cancer patients will address the question whether the T-cell repertoire available in man will have tumoricidal potency and whether, as in CEA-tg mice [20,22-24], drastic immunomodulatory measures such as repeated administration of cytokines are required to potentiate CEA-targeted immune attack against the tumor.

In conclusion, we have found that CEA is expressed in thymic epithelial cells of both humans and CEA-tg mice, and that this expression markedly affects the T-cell repertoire available in CEA-tg mice for targeting CEA-expressing tumors. Our data suggest that the CEA-specific T-cell repertoire may be similarly restricted by self tolerance in humans, and argue that promiscuous expression of tumor-associated auto-antigens in mTEC should be taken into account when considering such antigens as targets for immunotherapy of cancer.

Acknowledgements

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