Expression of a Natural Tumor Antigen by Thymic Epithelial Cells Impairs the Tumor-Protective CD4+ T-Cell Repertoire

Rinke Bos,1 Suzanne van Duikeren,1 Thorbald van Hall,1 Patricia Kaaijk,1 Richard Taubert,2 Bruno Kyewski,1 Lodger Klein,1 Cornelis J.M. Melief,1 and Rienk Offringa1

Abstract
A variety of antigens that display a highly tissue-specific expression pattern have recently been 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 autoantigens 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-transgenic 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-transgenic 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 autoantigens. (Cancer Res 2005; 65(14): 6443-9)

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 autoantigens. 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 immunologic targeting of cancers of epithelial origin, in particular colorectal cancers (refs. 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 antitumor 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 antitumor potential of the CEA-specific immune response.

Materials and Methods
Mice. C57BL/6 Kh (B6, H-2b) and CEA-transgenic mice were bred in our own facilities (Leiden, The Netherlands). CEA-transgenic mice were originally obtained from Dr. John Thompson (Freiburg, Germany). Mice were analyzed for CEA genotype by PCR analysis as described previously (4).

MC38-CEA cells were injected i.v. with 3 × 105 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.

Depletions of CD4+ or CD8+ cells were done by i.p. injection of 100 μg GK1.5 or 2.43, respectively, starting 1 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 × 106 cells per well in 24-well plates) in the presence of D1 cells (1 × 105 cells per well) and CEA protein (5 μg/mL). After 4 days of culture, cells were harvested and cell viability was determined by trypan blue exclusion. Cells were incubated with 1 × 105 irradiated spleen cells in the presence of 5 mmol/mL peptide in 150 μL in 96-well 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 × 105 T cells were incubated with 2 × 105 irradiated spleen cells in the presence of 5 mmol/mL peptide in 150 μL in 96-well plates. Fixation and staining procedures were done as described previously (6).

Note: The authors have no conflicting financial interests.

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References
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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 hematopoietic system (9.5 Gy) and reconstituted with T-cell–depleted bone marrow (7 × 10^6 cells) derived from wild-type or CEA-transgenic 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 thymus stromal cell purification, RNA isolation, cDNA synthesis, PCRs, and microarray analysis were done as described previously (3, 7). Quantitative 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'-TCGACAGT-CAGGCGCATCT-3'; GAPDH antisense, 5'-CGGTTGACTCCGACCTTCA-3'; CEA sense, 5'-TCCGAACTCAGTGAGTGCAAAC-3'; and CEA antisense, 5'-TCGGAAATGTAAGCGAGTCT-3'. Primers used for murine cDNA were CEA sense, 5'-GGCTGTGGTTTGTCTCTAACTTGGCTACTGG-3'; CEA antisense, 5'-TTGGCATGGTTCTCTGATGCTTGCTGT-3'; β-actin sense, 5'-TGGGAAATGGAATGTTGGCTCAG-3'; and β-actin antisense, 5'-TAAACCGCAGCCTGATAACGTCG-3'.

Results

Carcinoembryonic antigen–specific antitumor immunity in wild-type and carcinoembryonic antigen–transgenic 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 antitumor 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 antitumor 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 RNA leukemia and B16 melanoma cells (8–11). Because CD4+ T cells, although indispensable for immune defense 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 autoantigen, we also did tumor challenge experiments in CEA-transgenic mice. These mice exhibit a tissue-specific expression of the CEA-transgene essentially identical to that found in humans (12). 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 on 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...
Distinct CEA-specific CD4+ T-cell responses in wild-type versus CEA-transgenic (CEA-tg) mice. Wild-type (A) and CEA-transgenic (B) mice were immunized respectively two and three times with 2-week intervals by i.v. injection of $3 \times 10^7$ 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). Representative example of the response observed in at least 10 individual mice. C, In addition, the reactivity of wild-type and CEA-transgenic 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.
the CEA-protein (Fig. 2C). CEA-transgenic splenocytes also reacted against APC loaded with any reactivity against these latter peptides. Both wild-type and recognized by wild-type mice, CEA-transgenic mice did not show overlap with peptides 43 and 62, respectively, which were splenocytes produced high levels of IFN-γ. Whereas at least three vaccinations were required to trigger protection against MC38-CEA in wild-type mice, we assessed the magnitude, in that wild-type mice showed marked differences in magnitude, in that wild-type mice were 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. 2; Table 1). 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 covered by sets of two adjacent overlapping peptides (42/43 and 53/54), respectively, whereas epitopes 4 and 5 are each comprised by a single peptide (62 and 65, respectively). Immunized CEA-transgenic 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 1, epitopes 6 and 7). Although these peptides overlap with peptides 43 and 62, respectively, which were recognized by wild-type mice, CEA-transgenic mice did not show any reactivity against these latter peptides. Both wild-type and CEA-transgenic splenocytes also reacted against APC loaded with the CEA-protein (Fig. 2C), showing their capacity to respond against physiologic quantities of naturally processed antigen. CEA-specific T-cell immunity in wild-type and CEA-transgenic 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 CEA-transgenic 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-transgenic mice (Fig. 2D).

In addition to the differences in CD4+ T-cell responses, immunized CEA-transgenic mice showed ~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-transgenic mice and/or by restrictions in the CEA-reactive B-cell repertoire of CEA-transgenic 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-transgenic mice, can readily account for the failure of CEA-transgenic 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-transgenic 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-transgenic mice used for these experiments could previously not be shown by Northern blot or immunohistochemical analysis as shown by Eades-Perner et al. (12). Nevertheless, several tissue-specific autoantigens, previously considered absent from the thymus, were recently found to be expressed by medullary thymic epithelial cells (mTEC; ref. 7). In fact, these mTEC were found to express a wide range of tissue-specific antigens and to be able to

<table>
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<tr>
<th>Epitope no.</th>
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<th>Amino acid no.</th>
<th>Ig domain</th>
<th>Amino acid sequence</th>
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<tr>
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<td>61</td>
<td>600-625</td>
<td>B3</td>
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</tbody>
</table>

NOTE: 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.
present these antigens to thymocytes in the context of both class I and class II MHC (15, 16).

To address the potential role of the thymus in restricting the CEA-specific CD4\(^+\) T-cell repertoire in CEA-transgenic mice, we transplanted the thymic lobes from newborn CEA-transgenic 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-transgenic thymic lobes vaccinated thrice with ALVAC-CEA displayed CD4\(^+\) T-cell responses closely resembling those in CEA-transgenic 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-transgenic 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-transgenic mice was analyzed by RT-PCR. Indeed, a high level of CEA mRNA was detected in mTEC from CEA-transgenic mice. In addition, ~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-transgenic 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-transgenic 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.
that the IFN-\(\gamma\) production we measured \textit{in vitro} was produced by donor CD4\(^+\) 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-transgenic mice do have the potential of developing into a full CEA-specific CD4\(^+\) T-cell repertoire in the absence of a CEA-transgenic 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-transgenic mice.

\textbf{Carcinoembryonic antigen is expressed by human medullary thymic epithelial cells.} To assess the relevance of our findings in CEA-transgenic 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-transgenic 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-transgenic mice, will encounter their target antigen in the thymus and therefore will be subject to tolerization.

\textbf{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 autoantigens 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-transgenic 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 \(15, 16, 19\). Although our experiments in the CEA-transgenic 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 CEACAM-1 and CEACAM-6, are expressed by human mTEC \(7\).

T-cell precursors that recognize the dominant CEA epitopes are tolerized in the CEA-transgenic mice. This central tolerance induction of CEA-specific CD4\(^+\) T cells is not complete, as CEA-transgenic 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 controlling 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-transgenic mice \(20–24\). These data argue that under conditions that provide a strong nonspecific 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 cytotoxic T-cell 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-transgenic or to those in wild-type mice. Our finding of CEA expression in human mTEC would suggest that in man, like in CEA-transgenic 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-transgenic 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 nonterminal colorectal cancer patients will address the question whether the T-cell repertoire available in man will have tumoricidal potency and whether, as in CEA-transgenic 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-transgenic mice and that this expression markedly affects the T-cell repertoire available in CEA-transgenic 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 autoantigens in mTEC should be taken into account when considering such antigens as targets for immunotherapy of cancer.

Acknowledgments


Grant support: Dutch Cancer Society grant UI.2000-2135.

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We thank S. van der Burg for critical review of the article, J. Thompson for originally providing the CEA-transgenic mice, N. Berinstein and M. de Benedette for providing ALVAC-CEA and helpful discussions, S. Zegveld for additional technical assistance, and P. Kuppen for helpful comments.

References


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