Different Forms of Helper Tolerance to Carcinoembryonic Antigen: Ignorance and Regulation

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Abstract Purpose: Understanding the mechanisms of immune tolerance to tumor-associated antigens (TAA) is an important step in the design of cancer immunotherapy. The aim was to determine how T helper (Th) cell tolerance is mediated for a prototypic TAA, carcinoembryonic antigen (CEA).

Experimental Design: Peripheral blood mononuclear cells from 50 healthy volunteers were stimulated with CEA, and the type and fine specificity of any Th cell responses were identified. The inhibitory effects of T regulatory (Tr) populations were determined by depleting "natural" CD25+ Tr cells or neutralizing cytokine produced by the "induced" Tr form.

Results: Proliferative Th cell responses were consistently induced by CEA in 22 of 50 individuals. Responding cells were drawn from the CD45RA+ "naive" or quiescent population. Depleting the CD25+ fraction did not enhance CEA responsiveness. However, CEA elicited secretion of the Tr cytokine interleukin-10 (IL-10) in 23 of 50 donors, including 20 of 22 where no proliferation was induced. Neutralizing IL-10 revealed previously unseen proliferation to CEA by CD45RO+ "memory" Th cells. Epitope maps revealed differences in the fine specificities of Th cells capable of proliferating or secreting IL-10.

Conclusions: There are at least two major forms of CEA tolerance in different individuals. One is "ignorance," a failure of specific Th cells to respond to antigen presented in vivo. The other, seen when ignorance is lost, is mediated by IL-10-secreting Tr cells that recognize CEA. TAA tolerance, for example to colorectal carcinoma cells expressing CEA, may be overcome by peptide vaccines that exploit the differences in epitopes recognized by effector and Tr responses.

The rational development of specific immunotherapy for cancer depends on the identification of tumor-associated antigens (TAA) as targets and on overcoming the mechanisms of immune evasion that prevent or blunt protective responses. Tumor evasion strategies include the production of immuno-regulatory factors, expression of FAS (CD95) and inhibitors of apoptosis, down-regulation of MHC and costimulatory molecules on antigen-presenting cells, and induction of host immune tolerance to TAA (1–4). It is now clear from experimental animal models that tolerance can result from several mechanisms, such as the purging of specific lymphocytes from the repertoire by deletion or anergy (5), potentially responsive T cells ignoring presented antigen (6), and active suppression by regulatory cells (7–10). An important question for cancer vaccine design will be to establish which of these processes establish tolerance to human TAA.

Carcinoembryonic antigen (CEA) is a prototypic TAA in one of the most common human cancers, colorectal carcinoma. A heavily glycosylated 180-kDa protein, CEA, comprises an NH2-terminal domain of 108 residues, 3 internal repeats each of 178 amino acids with a high degree of sequence homology, and a 26–amino acid hydrophilic COOH terminus (11). CEA is highly expressed by the majority of colorectal cancers, as well as most other gastrointestinal tract neoplasms and pancreatic carcinomas, 50% of breast cancers, and 70% of non–small cell lung carcinomas (11–14). It is also abundant in colon epithelial cells during embryonic development and detectable, but at much lower levels, in normal adult colonic tissue (15) and shares homology with the CD66 family expressed in lymphoid tissues (11).

The focus of much recent research on tumor immunity has been the critical roles played by different subsets of CD4+ T helper (Th) cells in initiating, maintaining, and regulating responses (7–10, 16–19). It became clear some years ago that the effectiveness of immune responses can be determined by mutual antagonism between different helper subpopulations (20), initially categorized as Th1 or Th2 cells that produce IFN-γ or interleukin (IL)-4, respectively (20). The Th1 subset is potentially important for tumor immunity by providing help to cytotoxic CD8+ lymphocytes (CTL; ref. 19). More recently, further, T regulatory (Tr) CD4+ cell subpopulations with important contributions to tolerance have been described, including two major forms defined as "natural" or "antigen
induced” (7–10). Although the relationship between the two forms is not clear, they have different functional characteristics. “Natural” Tr seem to differentiate into a distinct suppressor lineage during thymic development, mediate inhibition via undefined mechanisms of direct cell-cell contact, rather than by secreted cytokine, and constitutively express activation markers, classically CD25 (8, 9). “Induced” Tr are believed to be generated in the periphery and include Th3 cells (10) that produce transforming growth factor-β (TGF-β) and Tr1 cells that predominantly secrete the inhibitory cytokine IL-10 (7). The Tr subsets control a wide range of rodent adaptive and innate immune responses to foreign, self, and tumor antigens (7–10). However, little is known about the different forms of CD4+ T cell that recognize human TAA and their functions in tolerance or immune evasion.

The cellular immune response to CEA has been partially characterized as a potential target for therapeutic vaccination, despite early suggestions that T-cell repertoire was profoundly tolerant to the antigen (21). CTL responsive in vitro to epitopes derived from CEA have now been shown, both in cancer patients and in normal individuals (22–26), and a growing number of helper epitopes have also been described (27–31). Four studies, in which potential Th epitopes were initially predicted from the sequence of CEA by computer algorithms, each identified a different peptide that was recognized by Th cells in functional assays (27–31). When a recombinant CEA vaccine was tested in colon cancer, Th cells from 6 of the 10 immunized patients proliferated in vitro against up to six CEA peptides (32), but only two of these sequences showed any correspondence with the epitopes reported by others.

The above studies were based on relatively small numbers of individuals and limited analyses of the CEA-specific cells. Questions that are important for vaccine design therefore remain unanswered, including how commonly Th responsiveness to CEA occurs in the general population, whether there are also Tr cells capable of inhibiting such reactivity, and how extensive are the numbers of epitopes recognized. The aims of the current work were to answer these questions. The results reveal that virtually all individuals maintain an extensive repertoire of circulating Th cells specific for multiple CEA epitopes but that these cells remain unstimulated or “immunologically ignorant” in approximately half of the donors. In the remaining donors, Th cells that recognize CEA epitopes have been activated in vivo, but their responses are held in check by IL-10–secreting Tr cells specific for the antigen. The widespread presence of CEA-reactive Th cells raises the prospect that responses to such TAA can be manipulated to confer protective immunity.

Materials and Methods

Donors. Blood samples from 50 healthy control donors (27 male and 23 female, 22-45 years old) were obtained by venipuncture and drawn into tubes containing citrate or preservative-free heparin anticoagulant.

Antigens. The control antigen Mycobacterium tuberculosis purified protein derivative (Statens Seruminstitut) was dialyzed extensively against PBS (pH 7.4) and filter sterilized before it was added to cultures (10 μg/mL). Purified protein derivative readily provokes recall T-cell responses in vitro (33, 34) because most citizens of the United Kingdom have been vaccinated with Bacillus Calmette-Guerin. The primary antigen keyhole limpet hemocyanin (Calbiochem) was also used to stimulate control cultures at 10 μg/mL after extensive dialysis against PBS. Purified CEA was obtained from Calbiochem (preservative and reductant free) and was dialyzed extensively against PBS (pH 7.4) and filter sterilized before use in cultures at concentrations of 0.05 to 10.0 μg/mL as described below.

Peptides. A panel of 33 peptides derived from the CEA sequence was synthesized by the Department of Biochemistry, University of Bristol, Bristol, United Kingdom (Table 1). These 13- to 15-mer peptides spanned the NH2 terminus (NT), a long chain terminal repeat (LTR) region, and the COOH terminus (CT) of CEA, with overlaps of at least five amino acids. To ensure purity, peptides were screened by high-pressure liquid chromatography and amino acid analysis. As in previous studies (35), peptides were used to stimulate cultures at 15 μg/mL.

Isolation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMC) were separated from fresh blood samples by density gradient centrifugation (Lymphoprep, Nycomed; refs. 33, 35–37). Cell viability was >90% in all experiments, as indicated by trypan blue exclusion.

Depletion of CD45RO+ or CD45RA+ cells. Depletion of CD45RO+ or CD45RA+ cells from cultures was done based on a previously described method (33, 34). Briefly, PBMC were incubated with murine monoclonal antibody UCHL1, specific for human CD45RO, or SN130, specific for human CD45RA (gifts from Profs. P.C.I. Beverley, ICRF Human Tumour Immunology Group, Faculty of Clinical Sciences, University College, London, United Kingdom and G. Janossy, Department of Immunology, Royal Free Hospital School of Medicine, London, United Kingdom, respectively). Cells that bound monoclonal antibody were removed by immunomagnetic separation using ferrous beads coated with antibody specific for mouse IgG (Biomag, PerSeptive Biosystems). Each depleted population contained <10% cells expressing the respective CD45 isoform, as determined by flow cytometry (FACSCalibur, Becton Dickinson).

Depletion of CD25+ T cells. CD25+ cells were removed from cultures using a commercially available kit according to the manufacturer's instructions (Miltenyi Biotec). Briefly, PBMC were incubated with ferrous beads coated with anti-CD25 antibody, and the bound cells were removed by passage through magnetic columns. Thorough removal of the CD25+ fraction was ensured by repeating each separation twice, and flow cytometry (FACS Calibur) confirmed that depleted cultures contained essentially no CD3+CD4+CD25hi T cells, which represent the natural Tr population (8, 9).

Cell culture and proliferation assays. Culture conditions were used that support both primary and recall Th cell responses (33, 34). Cells were cultured in 2 mL volumes at a concentration of 1.25 × 106 cells/mL in the Alpha Modification of Eagle's Medium (Life Technologies) supplemented with 5% autologous serum, 4 mmol/L L-glutamine (Sigma), 100 units/mL sodium benzylpenicillin G (Sigma), 100 μg/mL streptomycin sulphate (Sigma), and 20 mmol/L HEPES (pH 7.2; Sigma). All plates were incubated at 37°C in a humidified atmosphere of 5% CO2/95% air. Cellular proliferation in cultures was estimated from the uptake of [3H]thymidine over the period of 5 to 8 days after stimulation (33, 34). On each of these days, triplicate 100 μL samples were withdrawn from the cultures into the wells of round-bottomed microtiter plates. The cells in each microtiter well were pulsed with 1 μCi [3H]thymidine (Amersham) for 6 h and then harvested onto glass fiber mats (LKB-Wallac) using a multisample harvester (Mauch III TomTech). Mats were treated with Meltilex A melt-on scintillant sheets (LKB-Wallac) and the radioactivity incorporated into newly synthesized DNA was measured using a Trilux 1450 Microbeta scintillation counter (LKB-Wallac). Results are presented either as mean counts per minute ± SD of the triplicate samples or as a stimulation index (SI), expressing the ratio of mean counts per minute in stimulated versus unstimulated control cultures. A SI > 3 is interpreted as representing a significant positive response (33–37).

Blocking HLA class II restricted proliferation. Blocking antibodies specific for HLA class II molecules (Becton Dickinson) were dialyzed thoroughly against PBS before addition to cultures at the previously determined optimum concentration of 2.5 μg/mL (33, 37).

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Neutralization of IL-10. To inhibit the effects of the regulatory cytokine IL-10 on cell responses, as in previous studies (35, 37), a specific neutralizing antibody (PharMingen) was added to freshly established cultures at 1 ng/mL.

Measurement of cytokine production. The production of the cytokines IFN-γ, IL-4, IL-10, and TGF-β in cultures was measured by a highly sensitive cellular ELISA (35–37). Briefly, 5 days after stimulation, cell cultures were transferred into duplicate wells in microtiter plates (Nunc) coated with monoclonal anti-cytokine capture antibody (PharMingen). After incubation of PBMC for 24 h at 37°C, the plates were developed with the appropriate biotinylated monoclonal detection antibody (PharMingen), ExtrAvidin-alkaline phosphatase conjugate (Sigma), and p-nitrophenyl phosphate substrate (Sigma). The absorbance at 405 nm was measured using a multiscan plate reader (Labsystems). Cytokine secretion was calculated by interpolation from a standard curve generated by incubating duplicate wells with doubling dilutions of recombinant human, IFN-γ, IL-4, IL-10, and TGF-β (PharMingen).

Results are presented either as the mean cytokine concentration in duplicate wells or as SI, expressing the ratio of mean concentration in stimulated versus unstimulated control cultures. An SI > 2.0 is interpreted as representing a significant positive response (35–37).

Characterization of responding cells. The phenotypes of cultured cells that proliferate or secrete cytokine after stimulation were determined by flow cytometry. As described previously (35, 37), cells from unstimulated or responding cultures were analyzed for expression of the T-cell marker CD3, the Th marker CD4, and the activation markers CD69 or CD71 by three-color flow cytometry. Cells synthesizing IL-10 were labeled by incubating with anti-IL-10 after inhibition of protein secretion with brefeldin A (Sigma) and permeabilization with Intraprep (Beckman Coulter). All antibodies and control immunoglobulins were supplied by Beckman Coulter. A total of 10,000 cells per sample was counted using an Epics XL cytometer (Beckman Coulter) and the results were analyzed with Expo 32 software (Beckman Coulter).

Statistical analysis. Nonparametric tests, the $m^2$ and Spearman’s rank correlation, were used, with the level for significance taken as $P < 0.05$.

Table 1. The sequences of the CEA peptides and the type of response mounted against each peptide by PBMC from a panel of healthy donors

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Peptide sequence</th>
<th>Location within CEA protein</th>
<th>Residues</th>
<th>Percentage of donors with PBMC responding to each peptide (n = 42)</th>
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<td>NT1</td>
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*$^*$PBMC mount proliferative response with IFN-γ.

$^1$PBMC mount IL-10 response but no proliferation.
purified CEA under conditions that support both recall Th proliferation and primary responses by previously inactive Th cells. The kinetics of any responses were followed from days 5 to 8 after stimulation of cultures. PBMC from 23 (46%) of the donors proliferated strongly against CEA, and Fig. 1A and B shows representative results from this responsive group. Optimal proliferation was induced by CEA at concentrations of 1 to 5 μg/mL, and so 1 μg/mL of the antigen was used to stimulate cultures in subsequent experiments. All the responses to CEA peaked relatively late, at day 7 of culture or afterwards, kinetics that are typical of slow-developing, primary responses, mounted by Th cells that have been inactive previously or naive in vivo (33, 34).

The inability of PBMC samples from the remaining 27 (54%) donors to proliferate after stimulation with CEA was specific because all mounted strong responses to the control antigens purified protein derivative (mean SI, 23; range 6-81) and keyhole limpet hemocyanin (mean SI, 29; range 9-78) and to the mitogen Con A (data not shown). The distinction between donors whose PBMC proliferated or were nonresponsive to CEA was reproducible when repeat samples were tested.

**Phenotype of PBMC proliferating in response to CEA.** To confirm that the PBMC proliferating against CEA were of the CD3^+CD4^+ Th phenotype, selected cultures were analyzed by multicolor flow cytometry. Responding cells were labeled with antibody to the activation markers CD69 or CD71, and the Th subset was identified by counterstaining with anti-CD3 and anti-CD4. Representative results from one donor (n = 4) whose PBMC proliferate against CEA are shown in Fig. 1C. It can be seen that, as expected, the background level of CD69 expression in control, resting cultures was low, and there was a small increase (up to 6%) in numbers of activated CD69^+ cells after stimulation with CEA. The size of this expansion is typical of the responses to antigen made by specific lymphocytes within a polyclonal population, and the vast majority (>80%) of the cells that up-regulated CD69 as a result of the peptide stimulation were CD3^+CD4^+. In control cultures that showed no proliferative response to CEA, there was no such increase in CD69 staining (n = 3 donors; changes in CD69^+ cell numbers, -0.1%, 0.3%, and -1.8%). To further show that the responses were mediated by Th cells, blocking antibodies specific for HLA class II molecules were tested for the ability to inhibit proliferation (30). When PBMC were stimulated with CEA, proliferative responses were abrogated by the addition of an antibody against all HLA class II types (100% inhibition; n = 3 donors; n = 4 replicate experiments per donor). A similarly potent inhibitory effect (100% inhibition; n = 3 donors; n = 4 replicate experiments per donor) was also obtained using anti-HLA-DR, but not anti-DQ, antibody. Together, the results show that the proliferative responses to CEA were mediated by Th cells restricted predominantly by HLA-DR.

The kinetics of the proliferation against CEA seen in responsive donors suggested that it was mediated by Th cells that had previously been quiescent or naive. To confirm that the Th cells proliferating in vitro had not been activated in vivo, depletion experiments determined the isoform of the CD45 molecules they express because primary and recall Th responses are mediated by cells bearing CD45RA and CD45RO, respectively (33, 34). Responses against CEA by PBMC from five healthy donors were analyzed (Fig. 1D). In all cases, the proliferating cells were drawn from the CD45RA^+ subset containing previously inactive Th cells, rather than the activated or memory CD45RO^+ fraction. The CD45RO^+ fraction was responsive, as expected (33, 34), to the control recall antigen purified protein derivative (data not shown).

![Fig. 1. Frequent proliferative responses to CEA by PBMC from healthy donors.](www.aacjournals.org)
Effect of natural Tr cells on Th responsiveness to CEA. Natural CD25+ Tr cells in the peripheral repertoire can suppress responses to a wide variety of antigens, including those associated with tumors (8, 9, 16–19). Experiments were therefore set up to determine whether the natural Tr cells present in PBMC partially suppressed Th proliferation to CEA in donors where it was detected and whether the lack of responsiveness in other individuals could be attributed to this regulatory population. CD25+ cells were depleted from the PBMC of both CEA responsive and nonresponsive donors, and the ability of the remaining CD25- population to proliferate after CEA stimulation was compared with that of the unfractionated cells. Figure 2A shows that removal of the CD25+ subset neither enhanced existing Th proliferation to CEA nor revealed any further responses. In contrast, proliferation against the control antigen purified protein derivative was increased when the CD25+ population containing Tr cells was depleted (data not shown).

Regulatory cytokines and Th responses to CEA. The induced forms of Tr cell secrete regulatory cytokines, such as IL-10 or TGF-β, when stimulated with antigen, rather than proliferating. Th2 cells also proliferate poorly, and the IL-4 they produce can regulate effector Th1 responses. The possibility was therefore tested that PBMC contain CEA-specific T cells that secrete regulatory cytokines. PBMC from 23 (46%) of the healthy donors secreted IL-10 when stimulated with CEA. Strikingly, there is a highly significant reciprocal relationship ($\chi^2 = 16.2; P < 0.001$) between the ability to secrete IL-10 or proliferate against CEA (Fig. 2B), with 20 of the 23 (87%) donors whose PBMC mounted IL-10 responses showing no proliferation. Figure 2C and D shows the results from typical cultures where either proliferative or IL-10 responses predominated. No TGF-β was elicited by CEA throughout the study, but PBMC from six donors, including four with no other form of response, secreted IL-4 when stimulated with the antigen. The PBMC from only 2 of the 50 (4%) individuals tested made no detectable response of any type to CEA.

Phenotype of PBMC secreting IL-10 in response to CEA. Because a variety of cell types can secrete IL-10, the phenotype of the PBMC producing this cytokine in response to CEA was determined by flow cytometry ($n = 4$; representative result depicted in Fig. 3A). As expected when a polyclonal, nonproliferative population responds to specific antigen, a small proportion (up to 2%) of PBMC up-regulated IL-10 after stimulation with CEA. The vast majority (>95%) of these responsive cells expressed the CD3+CD4+ phenotype, consistent with belonging to the Tr1 subset.

Fig. 2. Natural Tr cells have no effect on the ability of PBMC to proliferate in response to CEA, but the induced regulatory cytokine IL-10 is associated with lack of proliferation. A, PBMC from donors HC004 and HC002, as shown previously to proliferate in response to CEA, or from unresponsive donors HC014, HC034, HC037, HC044, and HC050 were stimulated with CEA, either as unfractionated PBMC (black columns) or after depletion of CD25+ cells (white columns). Line, the level of response taken as representing a positive response (SI > 3).

B, comparison of proliferative and IL-10 responses to CEA by PBMC from healthy donors. Points representing proliferative and IL-10 responses for each donor ($n = 50$) are joined by a solid line. Line, the level of response taken as representing a positive response (SI > 2 for IL-10 production). C and D, comparison of proliferative and IL-10 responses to unstimulated cells (white columns) and CEA-stimulated cells (black columns) by PBMC from HC002, representative of the 23 donors that showed proliferative responses, and from HC010, representative of the 23 donors that did not. Line, the level of response taken as representing a positive response (SI > 3 for proliferation; SI > 2 for IL-10 production).
**Inhibitory effects of IL-10.** To determine whether the IL-10 secreted by Tr cells when stimulated with CEA was suppressing proliferative responses by potential effector T cells, neutralizing antibody specific for the cytokine was added to cultures. The results, shown in Fig. 3B, show that blocking the activity of IL-10 secreted in response to CEA allowed PBMC that had previously seemed anergic to proliferate when stimulated with the antigen. The neutralizing antibody had no effect on existing proliferative responses to CEA where no IL-10 was produced. Where donors’ T cells produced IL-10 in response to CEA, but failed to proliferate unless the cytokine was blocked, CD45RA+ or CD45RO+ fractions were depleted to test whether the suppressed cells that were capable of proliferation had been previously activated in vivo (Fig. 3C). In contrast to the naïve CD45RA phenotype of the T cells that proliferated to CEA where no IL-10 was secreted, those mediating proliferation that was suppressed by IL-10 were drawn from the CD45RO+ activated or memory fraction.

**Mapping T-cell epitopes on CEA.** To further characterize the different forms of CD4+ T cell that recognize CEA, the epitopes that they recognize were mapped in representative donors whose PBMC showed either proliferation or IL-10 secretion. It was important to determine the breadth of the responses, and to avoid selection pressures inherent in cloning, and so we took advantage of techniques successfully developed for studying polyclonal T cells (33, 35, 37). PBMC from 42 individuals were screened for the ability to proliferate or secrete cytokine when stimulated with a panel of peptides spanning the NH2 terminus, one LTR, and the COOH terminus of CEA. The results are summarized in Table 1, and representative epitope maps from the two groups of donors whose PBMC proliferated or secreted IL-10 in response to CEA are shown, respectively, in Figs. 4 and 5. It can be seen that T cells from each donor were able to proliferate in response to peptides from the CEA panel and that, typically, multiple sequences were stimulatory. IFN-γ responses to the peptide panel were also common and significantly associated (P < 0.05, Spearman rank correlation) in every donor with proliferation. In the donors whose T cells secreted IL-10 against purified CEA, multiple peptides also elicited this cytokine. However, in all such individuals, these sequences that stimulated IL-10 differed from those that induced proliferation, with a strong negative correlation (P < 0.05, Spearman rank correlation) between the two forms of response across the peptide panel. For both effector type and IL-10 responses, the patterns of stimulatory peptides varied between different individuals, but particular sequences were promiscuous, with NT2 (amino acids 41-53), NT5 (amino acids 65-79), LTR4 (amino acids 168-182), and LTR9 (amino acids 218-232) each stimulating proliferation and/or IFN-γ production and LTR5 (amino acids 178-192), LTR14 (amino acids 268-282), and LTR15 (amino acids 278-292) eliciting IL-10, in a third or more of the donors (Table 1).

**Inhibitory effects of CEA peptides that induce IL-10.** The epitope mapping experiments indicated that, in those individuals with T cells that secrete IL-10 when stimulated with CEA, particular peptides preferentially elicit this response. Furthermore, the Th cells capable of proliferation that are also present in these donors, and which are suppressed by the IL-10, recognize a different set of epitopes. To confirm that the CEA epitopes inducing IL-10 were indeed activating a regulatory mechanism, PBMC were copresented with CEA and selected peptides that elicit the cytokine. The proliferation against CEA was consistently suppressed by the response to IL-10–inducing peptides (n = 2 donors, n = 6 peptides; mean percentage inhibition, 57.6% ± 27.3 SD). This inhibitory effect is specific because the same peptides had no inhibitory effect in donors where they did not induce IL-10 (n = 3 donors, n = 8 peptides; mean percentage inhibition, 2.5% ± 4.06 SD).
Discussion

This study shows that helper tolerance to CEA in the vast majority of the healthy population is not profound. Instead, tolerance in different individuals is due either to ignorance of the antigen or to active suppression, and, in either case, multiple specificities of Th cells responsive to CEA survive in the peripheral repertoire. The 50 healthy donors studied can be divided into two main groups based on their T-cell responses to CEA. In the first, circulating T cells are capable of proliferating against CEA in vitro, but these are naive or previously quiescent cells that ignore (6, 33) the antigen in vivo. In contrast, the second group harbors T cells that have been activated in vivo and are capable of mounting memory proliferative responses in vitro, but these are held in check by CEA-specific Tr cells that secrete IL-10 (7). These results are relevant to the development of future vaccines against TAA, such as CEA, because they reveal the existence of pools of potentially responsive Th cells and suggest approaches to overcome their tolerance.

CEA would be expected to be subject to the mechanisms of self-tolerance that prevent autoreactivity in the healthy individual, and early indications (21) were that the T-cell repertoire was purged of CEA-specific cells by deletion and/or anergy (5). However, it is now recognized that such censorship of the repertoire is very incomplete for many autoantigens of pathogenic relevance (33, 38, 39), and the current work shows that there is a similar potential for the Th repertoire to be stimulated by CEA. The forms of peripheral tolerance that prevent activation of autoaggressive Th cells include...
“immunological ignorance” (6, 33) and suppression by Tr cells (7–10, 40), and we now show that these mechanisms can also restrain responsiveness to CEA.

“Immunologically ignorance” is a term coined by Ohashi et al. (6), who reported that, in double transgenic mice, T cells bearing TCR specific for viral antigen were neither deleted, nor anergic, nor stimulated, despite the expression of the corresponding antigen on pancreatic β cells. Such ignorance of autoantigens also occurs in humans and is common (33). The explanations for the phenomenon are either that antigen-presenting cells are insufficiently activated in vivo to provide costimulation to the specific T cells (6) or that the epitopes recognized by the T cells are poorly displayed due to inefficient processing from the antigen (41, 42) or low affinity for the restricting MHC molecules (43). The first possibility is most likely for CEA because the Th cells identified were reactive with the purified antigen, and predictions using a web-based algorithm3 indicate that many of the CEA peptides that stimulate Th cell responses show high affinity for common MHC class II molecules. Previous studies (27, 28, 30, 31) of Th cells reactive with epitopes on CEA did not address ignorance as a mechanism of tolerance because they did not determine whether the responsive population had previously been activated in vivo. Here, we characterize CEA-specific Th cells that mediate proliferation with primary kinetics and express the CD45RA isoform that is characteristic of naive or quiescent Th cells (33, 34) and which have therefore remained ignorant in vivo despite the presence of antigen. In the context of CEA vaccination, it is noteworthy that such ignorance can be overcome experimentally given sufficient immunogenic stimulation because the mice in the original model develop a

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response to the transgenically expressed viral antigen and diabetes after infection with the virus (6).

Active suppression by Tr cells is the second mechanism of tolerance to CEA, which we identify in virtually all the donors in whom there was no evidence of ignorance. It is now generally accepted that Tr populations are crucial in preventing and inhibiting a wide range of unwanted or damaging immune responses in healthy individuals (7–10, 37, 40) and that they can also suppress beneficialantitumor immunity (1–3, 16, 17, 40, 44). Both the CD25+ "natural" and cytokine-secreting "induced" Tr forms may potentially be implicated in the inhibition of effector cells in cancer (7, 40, 44). However, the current work showed no effect of CD25+ populations on responsiveness to CEA but did reveal inhibitory, IL-10–secreting Tr1 cells that recognize the antigen. These "induced" regulatory cells were identified in approximately half of the donors, who were also the individuals characterized by the presence of CEA-specific Th cells bearing the activation or memory CD45RO marker, rather than expressing the ignorant CD44RA+ phenotype (33, 34). Our interpretation of these results is that regulation of CEA responsiveness does not arise naturally in the CD25+ Tr compartment during lymphocyte development but, instead, is induced in the periphery to control CEA-specific Th cells if they lose ignorance and become activated. This activation may be due to more immunogenic presentation of CEA (6), or exposure to cross-reactive antigen(s) in the environment (45), and in either case could be triggered by infection (6, 45). Any Tr1 cells that are specific for TAA in cancer patients may blunt protective responses, and successful vaccination will depend on overcoming the suppression they mediate.

The epitope mapping experiments revealed three features of the Th cell repertoire specific for CEA. First, irrespective of whether the Th cells were ignorant, or had been previously activated in vivo, multiple CEA peptides stimulated responses in each donor and, second, these sequences varied markedly between individuals. This diversity explains the lack of agreement between previous, smaller studies as to the CEA peptides that are recognized by Th cells (27–31). The third characteristic is that, in those individuals who exhibited both activated Th and suppressive Tr1 responses to CEA, the fine specificities of the two types of cell differed significantly. This divergence has been observed previously for other antigens (35, 37) and may reflect variation in the affinity of TCR preferred by effector and regulatory cells, or different rates of clonal exhaustion and epitope spreading in the two populations (35, 37). Whatever the cause, the result opens up the possibility that the balance between immunity and regulation against CEA may be manipulated therapeutically by selectively targeting T cells with different fine specificities.

The finding that normal immune system is not purged of all Th cells potentially responsive to CEA, but contains abundant specificities for this antigen, encourages the search for effective TAA vaccines. Furthermore, the difference in the specificities of effector and regulatory cells, where suppression occurs, could be exploited to stimulate the immune system with the CEA peptides that preferentially induce Th cell proliferation and IFN-γ secretion. However, the peptides used for any such vaccines may need to be tailored for different individuals. We are now extending our study to patients with colorectal cancer to assess the potential for manipulating their responses against CEA toward protective immunity.

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