Difficulties and dangers of CEA-targeted immunotherapy against colorectal cancer

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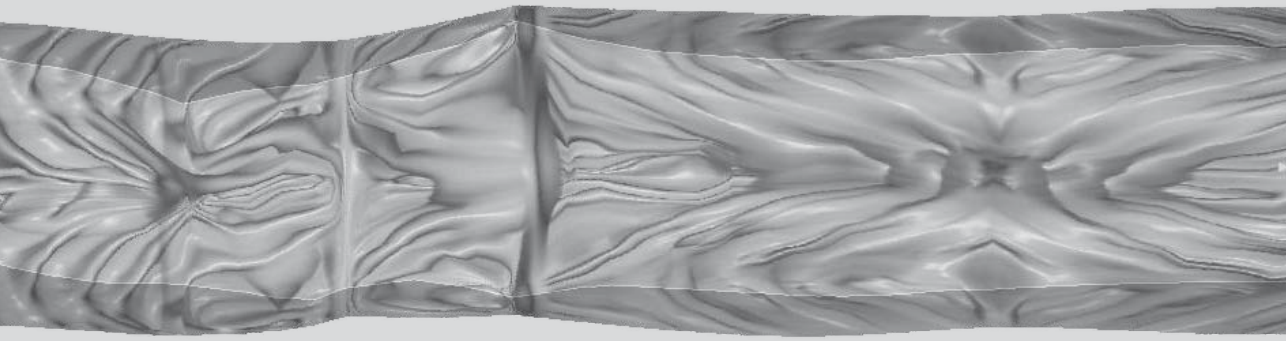

General discussion

Background

A wide range of strategies in cancer immunotherapy has been developed in the last decade, some of which are currently being tested in clinical settings. For a variety of tumors that do not express tumor-specific antigens, immunotherapy exploiting tumor-associated auto-antigens (TAAs) as targets for the anti-tumor immune response has been applied. It has been questioned whether this concept would be applicable to the treatment of human cancers, as the immune system was believed to strictly discriminate between self and non-self associated with profound immune tolerance for TAAs. Furthermore, when immune tolerance would be broken, this would induce a considerable risk of auto-immunity, self-antigen expression being widespread on normal tissues throughout the human body. The studies described in this thesis focus on the feasibility of using carcinoembryonic antigen (CEA) as a target for immunotherapy of colorectal cancer and on the balance between anti-tumor immunity and autoimmune pathology. Although a lot of effort has been put in the study of immunity against this antigen in mouse models and even in clinical trials, 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 is conceivably restricted by the fact that CEA is expressed in several abundant and vital tissues, including intestine and stomach, and is even routinely found in the serum of healthy individuals. In view of these issues we have performed a detailed and critical analysis of CEA-specific immunity in a transgenic mouse model in which the expression of CEA closely resembles that in man.

Summary

The character of the CEA-specific immune response was assessed by determining the specificity and magnitude of CEA-specific T-cell responses in immunized wild-type and CEA-tg mice. We demonstrate that the CEA-specific CD4+ T-cell repertoire in CEA-tg mice is severely limited compared to wild-type mice. This was studied by in vitro analysis of CD4+ T-cell responses and by in vivo experiments in which we determined the effect of the induced immune response on the outgrowth of a CEA-positive tumor. We analyzed the mechanisms by which this tolerance was induced and demonstrated that CD4+ T-cell tolerance for CEA was induced by the thymus. In addition we showed that CEA was expressed in medullary thymic epithelial cells (mTEC) in both mice and human beings. The latter suggests that the CEA-specific T-cell repertoire may also be tolerized in people.

Vaccination studies described above were all performed with ALVAC-CEA, which resulted in CEA-specific CD4+ T-cell responses. However, no CD8+ T-cell response was detected after ALVAC-CEA vaccination and tumor protection was only dependent on CD4+ T cells. In view of these data, we sought to understand the effector mechanisms induced by ALVAC-CEA vaccination. In addition, we compared tumor protection and T-cell responses after ALVAC-CEA with a DNA-based CEA vaccine in wild-type mice. These data showed that DNA-CEA vaccination induces CEA-specific CD8+ and CD4+ T cells and that tumor protection depends on both subsets of T cells. Further investigation of effector mechanisms induced by ALVAC-CEA revealed that Fc receptor γ-chains and NK cells play an important role in tumor eradication, suggesting that ADCC is the main effector mechanism induced by ALVAC-CEA.

In addition, our data show that immune responses directed against the ALVAC vector profoundly influence the type of immune response induced against the transgene-encoded antigen. ALVAC-CEA vaccination induced immunity against the CD4+ Th epitopes only, while DNA-CEA vaccination induced CEA-specific T-cell activity against both the CD4+ Th epitopes and the CD8+ CTL epitope. Furthermore, as anticipated, ALVAC-CEA induced very strong T-cell reactivity against ALVAC vector components that was associated with IFN-γ and IL-5 production. Interestingly, immunization with ALVAC encoding another antigen (OVA) resulted in a robust OVA-specific CTL response. These data indicate that the combination of a specific vector with an antigen will determine the eventual outcome of the immune response. Importantly, many clinical studies are performed with viral vectors encoding the antigen of interest and so far, it is not clear what the impact of the vector is on the immune response. To determine the mechanism by which the vector influences the immune response against different antigens, detailed analysis of immunity against the antigen and the vector is required.

In further studies we have focussed on possibilities to overcome tolerance of CEA by reconstituting the T-cell repertoire of CEA-tg mice by adoptive transfer of the T-cell repertoire of CEA-immunized wild-type mice into tumor-bearing CEA-tg mice. These experiments show that, in addition to central tolerance, peripheral mechanisms also contribute to tolerance induction in CEA-tg mice. Nevertheless, after testing different intervention strategies we conclude that tolerance in CEA-tg mice can be overcome. Notably, if adoptive transfer is combined with suppression of peripheral immune

regulation, either by a combination of 4.5 Gy TBI and IL-10 receptor blockade or myeloablative irradiation (9.5 Gy TBI) combined with reconstitution of the haematopoietic system through bone marrow transplantation, anti-tumor efficacy is invariably accompanied with autoimmune colitis. Importantly, this was not the case in one alternative treatment strategy that we have tested. Depletion of CD25 positive cells of the host before adoptive transfer resulted in a broad anti-tumor response that was effective in eradicating the tumor without the induction of auto-immunity. This response was not only CEA-specific, but also directed against other non-self tumor antigens. Also in human beings many unique antigens, like mutated genes, will be expressed by tumors and might get targeted by the immune system. We also explored whether this treatment would be sufficient in eradicating tumors that arise spontaneously in the intestine in the $APC^{1638N} \times$ CEA-tg mouse model. Our data show that this treatment indeed results in a significant reduction of the number and the size of the tumors in $APC^{1638N} \times$ CEA-tg mice compared to APC^{1638N} mice. These data were supported by in vitro analysis of T-cell responses and immunohistochemical analysis of T-cell infiltration in intestinal tumors. This indicates that reconstitution of the CEA-specific T-cell repertoire in CEA-tg mice can suppress the development of spontaneous, CEA-expressing intestinal tumors, in the absence of autoimmune pathology.

CEA-specific anti-tumor immunity in mice and men

To determine how our data concerning CEA-specific immunity relate to data from other research groups, I will now discuss the most informative studies performed in mouse models and compare these to the outcome of clinical trials. As mentioned before, many studies have addressed immunity against CEA in non-tolerant mouse models in which CEA is not a self-antigen, and these data do therefore not provide true information on the feasibility of CEA-targeted immunotherapy of colon cancer in human beings. For this reason, CEA-tg mouse models have been developed and used to investigate CEA-specific immunity. We have shown that CD4+ T cells recognizing dominant CEA epitopes are tolerized in the thymus in CEA-tg mice, but we also showed that this central tolerance induction was not complete, as weak CD4+ T-cell responses could be detected against subdominant epitopes. However, this repertoire was not sufficient in controlling tumor growth, even if boosted by multiple vaccinations.

Others have reported that heterologous prime boost vaccination with a plasmid (pVIJ/CEA) and a recombinant adenovirus carrying a codon usage optimized CEA cDNA (Ad-CEA) could overcome tolerance in CEA-tg mice [1]. In contrast to our data, this vaccine induced CEA-specific IFN-γ production by CD8+ T cells, but no CD4+ T-cell response or CEA-specific antibodies could be detected. These conflicting data might be explained by the effect of different vectors on the immune response and/or by the use of two different mouse models. One CEA-tg strain was generated by the group of W. Zimmerman and the other one by the group of J. Primus. Although both strains were made in the same manner, by using the complete CEA gene, the expression pattern and the levels of CEA differ significantly between the two strains. CEA-tg mice from the Zimmerman lab that we used for our experiments, have a similar expression pattern as human beings, but show higher concentrations of CEA in faeces, colonic

tissue and serum (Table I, Introduction). Therefore, this model might provide a somewhat pessimistic view on issues like tolerance and auto-immunity. However, in CEA-tg mice from the Primus lab, used for the experiments described above, CEA expression is restricted to only two tissues and concentrations in faeces and colon are much lower compared to humans. This will definitely lead to an underestimation of the level of tolerance and the risk for the induction of autoimmune responses. Nevertheless, even in this model in which it was possible to induce CEA-specific CD8+ T cells, 5 immunizations with pVIJ/CEA followed by a boost with Ad-CEA merely resulted in a small delay of tumor outgrowth, indicating that CD8+ T cells alone were not sufficient in eradicating the tumor. Another study of this group showed that a combination of Ad-CEA with CpG resulted in IFN-γ production by CD4+ T cells, but this vaccine induced only small numbers of CD8+ specific T cells [2], and the vaccine was not tested for impact on tumor outgrowth. Using these same CEA-tg mice from the Primus lab, Xiang et.al. [3,4] reported the induction of protective immunity by a CEA-based DNA vaccine. However, the tumor cells used for these experiments were not only expressing CEA, but were in addition co-transfected with a non-self antigen for these mice (Ep-cam) and the vaccine was targeting both antigens. Therefore, it is very unlikely that the response directed against the self-antigen will be dominating, as immunity against foreign antigens will be much stronger. However, immunological analysis of the induced response was not performed in sufficient detail and did not delineate effector mechanisms.

Studies performed by other research groups were performed in CEA-tg mice from the Zimmerman lab and these groups conducted an extensive set of anti-tumor vaccination experiments over the years, using many different vaccination strategies. In 2002, the identification of a CTL epitope in CEA-tg mice was reported, which mediated killing of a CEA-positive tumor [5]. Detailed analysis by our group and others did not identify this epitope to be involved in the CEA-specific response. Moreover, we found a distinct epitope involved in this reaction [1]. This supports our notion that the epitope described, does not truly take part in the CEA-specific response. Therefore, the anti-tumor response must be induced by other immune responses. Furthermore, this study showed that CD8+ T cells specific for this peptide did not have a striking effect on survival of CEA-tg mice challenged with a CEA-positive tumor. A better effect on tumor growth was accomplished when mice were vaccinated with recombinant vaccinia encoding the CEA transgene as well as a triad of costimulatory molecules (B7-1, ICAM-1 and LFA-3/TRICOM) [6]. However, this vaccine was combined with repeated systemic administration of granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-2 (IL-2), which are known to have an effect on the anti-tumor response [7], and the accompanying analysis of antigen-specific immunity did not provide direct evidence that the CEA-specific immune response is the main effector mechanism. It is therefore not clear what the contribution of CEA-specific immunity to the anti-tumor effect is in these experiments. Effective anti-tumor responses were also reported in CEA-tg mice after multiple intratumoral vaccinations [8,9] with recombinant fowlpox-CEA-TRICOM combined with GM-CSF. A major side effect of intratumoral injections is that this will also lead to the activation of the innate immune system. It has been shown that one single injection of CpG at the tumor site resulted in complete clearance

of the tumor [10] and we have shown in our experiments that after this treatment CEA-positive tumors were eradicated, but no CEA-specific responses could be detected [data not shown]. This implies the induction of a strong innate immune response. However, evaluation of innate immune responses has not been taken into account in the experiments described above. Furthermore, their in vitro data showed that immune responses were not only induced against CEA but also to other (non-self) tumor antigens, like an endogenous retroviral epitope of gp70 that is expressed by MC38 tumor cells. Importantly, the predominant T-cell population that infiltrated the regressing CEA+ positive tumor was mainly specific for gp70. Together, these data indicate that CEA-specific immunity does play a role in vaccine-induced tumor eradication, but it is also clear that this is only achieved when additional factors such as GM-CSF, IL-2 and immunity against other tumor antigens contribute to the immune response. The effects of immune responses other than CEA-specific induced by these vaccinations can readily explain why anti-tumor immunity was never found to be accompanied by autoimmune pathology. In our studies, we also detected immune responses against an endogenous retroviral gene product of Mulv that has been found to be expressed by MC38 (M8), but this response was only observed after CD25 depletion. Strikingly, this was the only treatment regimen that, of those used by us, showed anti-tumor efficacy without the induction of autoimmune pathology. Our data indicated that DNA-CEA induced CEA-specific immunity was important, but mainly in the initiation of the anti-tumor response and that other reactivities could take over. On the basis of these results one would not expect that the CEA-specific immune response would be effective to naturally arising tumors. Nevertheless, we demonstrated that this treatment was CEA-specific and effective in $APC^{1638N} \times$ CEA-tg mice by showing a reduction in intestinal tumor load and infiltration of CEA-specific T cells only in CEA+ tumors without the induction of colitis. Anti-tumor efficacy can be explained by the effects of CD25 depletion, that is in particular depleting regulatory T cells, but is also known to stimulate the innate immune system such as NK cells [11]. Furthermore, irradiation of recipient mice stimulated homeostatic proliferation of donor cells and depleted additional host regulatory mechanisms. However, it is rather strange that the infiltrating CEA-specific T cells in the CEA+ intestine do not induce colitis. T cells homing to the intestine might need to get triggered by an inflammatory environment to induce colitis. This does happen when intestinal damage is induced by myeloablative irradiation and IL-10 receptor blockade, but not when mice receive sublethal irradation.

Others also performed two vaccination studies in spontaneous tumor models [12,13]. In accordance with our results, vaccination of tumor-bearing mice resulted in a reduced outgrowth of tumors. However, tumor burden was reduced dramatically by combining the vaccination with a celecoxib-supplemented diet that inhibits the cyclooxygenase-2 (COX-2) enzyme. Immune responses induced by this treatment were not defined, but in vivo experiments showed that the CEA-specific effect was relatively small compared to the much greater impact of the COX-2 inhibitor on tumor development. These data also offer a plausible explanation for the fact that this effective, but not CEA-specific, treatment did not result in the induction of colitis.

It can be concluded from these studies in mouse models that tolerance of CEA is a major barrier for immunotherapy targeting this antigen and that the CEA-specific repertoire can only suffice when strong non-specific stimulation is given to the immune system. How do these data relate to the outcome of clinical trials performed with CEA-specific vaccinations? So far, at least 9 clinical trials targeting CEA have been conducted using various vaccines, and most of these studies have been performed with viral vectors (Table II introduction). Only modest CEA-specific responses were observed in treated patients and no striking effects on tumor growth and metastasis in cancer patients have been described [14]. In accordance with the lack of objective cancer regression, the responding patients in these clinical trials also did not show any signs of auto-immunity in CEA-expressing tissues.

Implications for immunotherapy of colorectal cancer

Together these data from mouse studies and clinical trials with currently available CEA-targeting cancer vaccines raise the question whether CEA should still be considered as a useful and practical target antigen. I showed in this thesis that tolerance of CEA is induced in the thymus and in the periphery and is very difficult to overcome. Notably, CEA-specific anti-tumor efficacy could be induced when immune modulation was combined with adoptive transfer of CEA-specific T cells. CEA specific TCR gene transfer into autologous lymphocytes would be a possible approach to gain this effect in cancer patients. However, our data also indicate that this treatment regimen increases the risk of autoimmune pathology. Initial experiments with CEA-specific CD4 TCR gene transfer in mouse lymphocytes showed that this resulted in a potent CEA-specific CD4+ T-cell response (Fig. 1). Notably, adoptive transfer of these CEA-specific CD4+ T cells into CEA-tg mice treated with myeloablative irradiation and BMT failed to control tumor growth, but did cause severe colitis. These findings are in line with our previous results showing an important role for both CD4+ and CD8+ T cells in clearance of MC38-CEA and a pivotal role for CD4+ T cells in inducing experimental colitis. We are currently investigating selective application of CEA-specific CD8+ T-cell responses, which might result in anti-tumor efficacy without the induction of autoimmune pathology, as we have previously shown that CEA-specific CD8+ T cells do not induce colitis.

Thus, very powerful treatment regimens in combination with suppression of immune regulation are necessary to circumvent tolerance in CEA-tg mice, but it can be concluded that CEA-specific immune responses can play an important role in the eradication of CEA-positive tumors, also when these treatments do not lead to autoimmune reactions. However, when CEA-specific immunity becomes the major effector mechanism, especially when high CD4+ T-cell immunity is induced, this may lead to severe autoimmune pathology of CEA-expressing tissues. Mild side effects might be acceptable when cancer treatment is effective, but may also be very dangerous because CEA is expressed on vital tissues. In view of these issues, I believe immunotherapy targeting CEA should be very carefully performed and possibly other target antigens, that might be used in combination with CEA, should be considered. We are currently testing a combination of p53 and CEA specific immunotherapy. The advantage of p53 is that this antigen is overexpressed by multiple tumors, while p53 expression is low in normal tissues and no autoimmunity

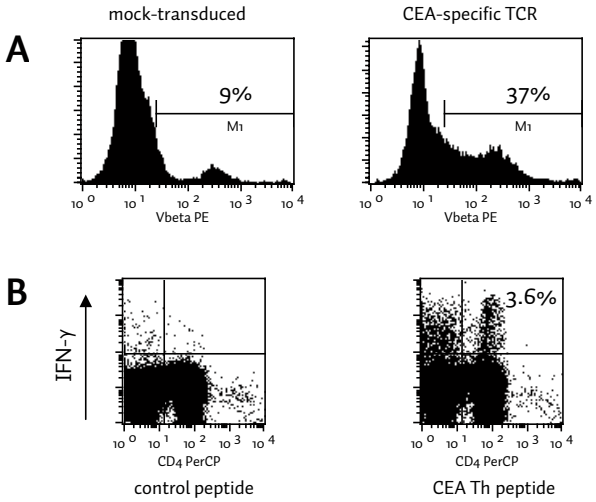

FIGURE 1. Genetic transfer of TCR chains resulted in expression of functional TCRs. A. Flow cytometric analysis of total splenocytes 3 days after retroviral transduction with CEA CD4+ T-cell receptor α and β chains or mock-transduction. Cells were stained with anti-Vβ3-PE. **B**. Intracellular IFN-γ staining of CEA TCR-transduced splenocytes. Cells were incubated in the presence of a CEA Th peptide (right panel) or control peptide (left panel) and stained with anti-CD4-PerCP and anti-IFN-γ-APC.

has been observed so far [15]. Other self-antigens that are overexpressed on coloncancer, like Ep-CAM and gastrin have mainly the same disadvantages as CEA, in that it is also expressed on healthy tissues and tolerance limits the specific T-cell repertoire. In principle, tumor antigens corresponding to fetal gene products will have triggered little or no tolerance making them good tumor rejection antigens. The human 5T4 oncofetal antigen could be an attractive target showing only low expression in normal tissues but is frequently expressed by carcinomas of diverse origin [16]. With DNA microarray techniques, a very detailed analysis can be performed of expression levels of molecules in tumor cells compared to healthy tissues. These studies might reveal new interesting target antigens that can be used in the development of new treatment strategies. In addition, improved adjuvants have been described, like toll-like receptor agonists [17], to activate the innate immune system. Molecules and cytokines that are able to stimulate, like anti-4-1BB and OX-40 [18,19], or even can rescue tolerant CD8+ T cells, like PD-1 and IL-15 [20,21] are under investigation. Another promising approach, which we have also used in the CEA-tg mouse model, is elimination of immune regulatory mechanisms. This can be accomplished by blockade of immunoregulatory cytokines, such as IL-10 and TGF-β or blockade of CTLA-4, an important regulatory mechanism and/or by depleting regulatory T cells [22-25]. These interventions should all be well considered and only be used when the immune response is clearly identified. As we have shown for CEA-specific immunity, depleting regulatory mechanisms can result in antigen-specific autoimmune pathology. However, when the immune response is not specifically targeted, these interventions might lead to systemic autoimmune reactions as shown by CTLA-4 blockade in melanoma patients [26]. In addition, immune modulation will only suffice when an effective T-cell repertoire is present. Ex-vivo expansion and TCR

gene transfer of autologous lymphocytes are two possibilities to obtain sufficient effector T cells and these techniques are now intensively studied in both mouse models and clinical trials [27-30].

Conclusion

Considering all research performed concerning immunotherapy of cancer that started about 15 years ago in mouse models, it can be concluded that we have made extraordinary progress in the elucidation of interactions between the immune system and tumor cells. Progress in clinical effects of immunotherapy has mainly been shown for virus-induced cancers and melanomas, but so far, no objective cancer regression has been described in patients with colorectal cancer. To improve clinical effects and optimize treatment strategies, all studies should comprise detailed analysis of induced immune responses and the subsequent role of this response in anti-tumor immunity.

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