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Modulation of tumor-specific CD8+ T-cell responses

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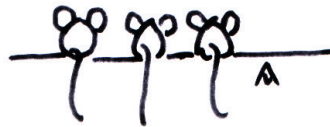
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Chapter 7

CD4⁺ T-cells are able to promote tumor growth
through inhibition of tumor-specific CD8⁺ T-cell
responses in tumor-bearing hosts

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Modulation of the immune response by established tumors may contribute to the limited success of therapeutic vaccination for the treatment of cancer compared to vaccination in a preventive setting. We analyzed the contribution of the CD4⁺ T cell population to the induction or suppression of tumor-specific CD8⁺ T cells in a tumor model in which eradication of tumors crucially depends on CD8⁺ T cell-mediated immunity. Vaccine mediated induction of protective anti-tumor immunity in the preventive setting was CD4 T cell dependent, since depletion of this T cell subset prevented CD8⁺ T cell induction. In contrast, depletion of CD4⁺ cells in mice bearing established E1A⁺-tumors empowered the mice to raise strong CD8⁺ T cell immunity capable of tumor eradication without the need for tumor-specific vaccination. Spontaneous eradication of tumors, that had initially grown out, was similarly observed in MHC class II deficient mice, supporting the notion that the tumor-bearing mice harbor a class II MHC-restricted CD4 T cells subset capable of suppressing tumor-specific CD8⁺ T cell immune response. The deleterious effects of the presence of CD4⁺ T cells in tumor-bearing hosts could be overcome by CD40-triggering or injection of CpG. Together these results show that CD4⁺ T cells with a suppressive activity are rapidly induced following tumor development and that their suppressive effect can be overcome by agents that activate professional antigen presenting cells. These observations are important for the development of immune interventions aiming at treatment of cancer.

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Introduction

In general, protective anti-tumor immunity in tumor-free hosts can be installed more easily than therapeutic immunity following vaccination of tumor-bearing recipients. One of the possible reasons for this difference is the generation of an immunosuppressive environment by the growing tumor. Since most cancer vaccine strategies aim at therapy of existing tumors rather than prophylaxis, it is crucially important to investigate the factors contributing to the altered immune response in tumor-bearing hosts. Furthermore, overcoming this detrimental influence is a major challenge for successful immunotherapy of established tumors.

Tumor-specific CD8⁺ T cell responses are often crucial to tumor eradication (1-4). Although CD4⁺ T helper cells are usually intimately involved in the induction of CD8⁺ cells, relatively little is known about the interplay between CD4⁺ and CD8⁺ T cells in tumor-bearing animals. Help provided by CD4⁺ T cells can be crucial for the induction of effective anti-tumor immunity (5-8). However, recent evidence suggests that CD4⁺ cells can also contribute to tumor growth. For example, CD4⁺ T cells were shown to enhance tumor incidence in HPV16 transgenic mice by increased recruitment of neutrophils, which provide MMP-9 necessary for angiogenesis and neoplastic cell proliferation (9). CD4⁺ NKT cells can interfere with immune surveillance resulting in incomplete elimination and recurrence of tumors. Depletion of these cells within the first 10 days after tumor cell inoculation resulted in enhanced survival (10). CD4⁺CD25⁺ T cells, which have been implicated in control of autoimmune responses, have also been shown to play a role in suppression of anti-tumor responses (11-13). These cells most likely suppress responses against self-antigens on the tumor cells, but could potentially also be directed against neo-antigens expressed by the tumor. Recently, tumor-antigen specific regulatory T cells were cloned from tumor-infiltrating lymphocytes of a melanoma patient (14). Together these observations indicate that CD4⁺ T cells can play an important and diverse role in the development of tumors.

To gain more insight in the contribution of CD4⁺ T cells to the development of tumor-specific CTL immunity in tumor-bearing hosts, we wished to analyze the evolution of the naturally occurring anti-tumor CTL response. To this end, we made use of a tumor model in which eradication of the tumor crucially depends on CD8⁺ cell-mediated immunity against a neo-antigen, the adenovirus type 5 derived E1A protein (15). In a prophylactic setting, induction of the E1A-specific CD8 response is dependent on CD4⁺ cells. However, in tumor-bearing mice depletion of CD4⁺ cells results in a remarkable increase in the number of tumor-specific CD8⁺ T cells as well as in tumor eradication and enhanced survival. Because CD4⁺ T cells are required for proper CTL induction before the emergence of established tumors, these findings indicate that tumor-growth can modulate the CD4⁺ T cell responses from one supporting CTL activation to a response that inhibits development of tumor-specific CTL immunity. These observations are important to the design of immunotherapeutic strategies for the treatment of cancer.

Materials and Methods

Cell cultures

All in vitro cultures were performed in IMDM (Life Technologies, Gaithersburg, MD) supplemented with 8% FCS, 50 μ M 2-ME, glutamine, and penicillin.

Mice

C57BL/6 (H-2^b) mice were purchased from IFFA Credo (Paris, France). C57BL/6 Kh (H-2^b) and class II-/- (H-2^b) mice were bred at TNO-PG (Leiden, The Netherlands).

Tumor challenge

In tumor challenge experiments, C57BL/6 mouse embryo cells, which express Ad5E1A and EJras (AR6) were collected and washed in PBS. 10^6 - 10^7 cells were injected s.c. into C57BL/6 mice. Tumor volumes were measured with a caliper. Mice were sacrificed when the tumors grew larger than 1000 mm³.

In vivo depletion of cell types

CD4⁺, CD8⁺ and NK⁺ cells were depleted by injection of 50 µg of respectively anti-CD4 (GK1.5), anti-CD8 (2.43) and anti-NK (NK1.1) antibodies in PBS intraperitoneally (i.p.) at day 0, 1, 3, 6 and 9. CD25 cells were depleted by injecting 300 µg of anti-CD25 antibody (PC61) once i.p.. Depletion of specific cell types was started at least 22 days after tumor cell injection.

Treatment of tumors with CpG

The CpG1826 ODN, which is a 20-mer containing two CpG-motives (TTCATCAGTTCCT-GACGTT), was provided by Coley Pharmaceutical (Langenfeld, Germany) and used at their suggested optimal working concentration of 50 µg/injection, intratumorally in 40 µl of PBS at day 22 and 25 after tumor cell injection.

Flow cytometry analysis

Tumors were removed, cut into small pieces, and treated with collagenase (400 units/ml) for 15 min at 37°C. Living cells were subsequently isolated by performing a Ficoll-gradient. Single-cell suspensions of spleen and lymph nodes were prepared by mechanical disruption. Blood samples were depleted of erythrocytes by ammonium chloride treatment for 5 min at room temperature.

Cells were stained with directly allophycocyanin (APC)-conjugated monoclonal antibody against CD8 (PharMingen) and phycoerythrin (PE)-conjugated E1A₂₃₄₋₂₄₃-loaded H-2 D^b tetramers. Data acquisition and analysis was done on a Becton Dickinson FACScan with CELLQUEST software.

Results

Role of CD4⁺ T cells in naive and tumor-bearing mice

Ad5E1A-transformed tumor cells express the E1A protein containing highly immunogenic CD4 and CD8 epitopes. Despite the presence of this immunogenic protein, Ad5E1A-transformed tumor cells form progressively growing tumors in immunocompetent mice. Eradication of these tumors crucially depends on E1A-specific CD8⁺ T cells (15). We have previously shown that the E1A-peptide, recognized by CD8⁺ T cells, is presented in the tumor draining lymph nodes and that E1A-specific CD8⁺ T cells are detected in tumor draining lymph nodes in 30% of tumor-bearing mice (16). However, the tumors grow progressively, indicating that the CD8⁺ T cell response is incapable of eradicating the tumor. To obtain more mechanistic insight in the ineffective anti-tumor immune response, we studied the role of CD4⁺, CD8⁺ and NK1.1⁺ cells by depletion of each specific cell type in mice bearing

established tumors three weeks after tumor cell injection. As described before (17), depletion of CD4⁺ T cells before vaccination of non-tumor-bearing animals completely abrogates the induction of E1-specific CD8⁺ T cell immunity, demonstrating the crucial role of CD4⁺ T cells in the induction of E1-specific T cell-mediated immune responses (data not shown). CD8 depletion in tumor-bearing mice resulted in enhanced tumor outgrowth (Fig. 1), indicating some anti-tumor capacity of the “spontaneously” induced anti-tumor CD8⁺ T cells. NK1.1 depletion did not have any effect on the outgrowth of the tumor (Fig. 2), indicating that NK1.1⁺ cells do not play an important role in anti-tumor immunity in these tumor-bearing mice. Surprisingly, depletion of CD4⁺ cells in tumor-bearing mice resulted in tumor eradication and prolonged survival of mice that had received an otherwise lethal tumor challenge (Fig. 1, 2). Tumor-rejection occurred without the need for tumor-specific vaccination, suggesting that the depletion of CD4⁺ T cells in these mice unleashed potent anti-tumor immunity.

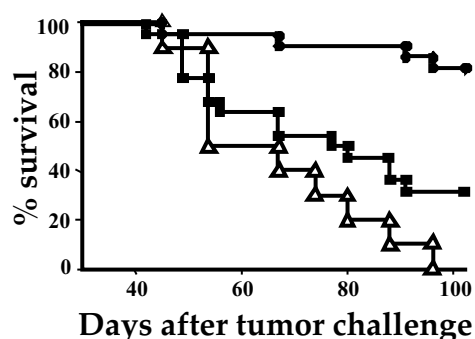


Figure 1: The role of CD4⁺ and CD8⁺ cells in spontaneous anti-tumor immunity.

C57BL/6 mice were injected with 1×10^6 live Ad5E1/ras cells in the flank s.c. and left untreated (closed squares, $n=22$) or depletion was started 24 days later, at a time mice had developed established tumors, with GK1.5, anti-CD4, (closed circles, $n=22$) or 2.43, anti-CD8 antibody (open triangles, $n=10$). Data from three individual experiment are pooled and the percent of surviving mice is shown. Untreated versus CD4-depleted, $P < 0.001$ (log-rank test).

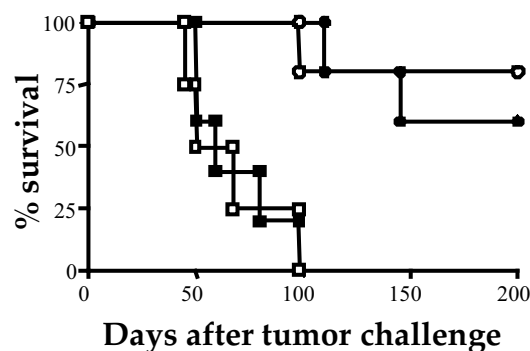


Figure 2: The role of CD4⁺ and NK⁺ cells in spontaneous anti-tumor immunity.

C57BL/6 mice were injected with 1×10^6 live Ad5E1/ras cells in the flank s.c. and left untreated (open squares, $n=4$) or depletion was started 24 days later, at a time mice had developed established tumors, with GK1.5, anti-CD4, (open circles, $n=5$) or NK1.1, anti-NK1.1 antibody (closed squares, $n=5$) or both (closed circles, $n=5$). The percent of surviving mice is shown. Untreated versus CD4-depleted, $P < 0.01$; untreated versus CD4- and NK-depleted, $P = 0.0027$ (log-rank test).

CD4⁺ T cells suppress expansion of anti-tumor CD8⁺ T cells

Because E1A-specific CD8⁺ T cells are crucially involved in the eradication of E1A-transformed tumors (15), we determined whether depletion of CD4⁺ cells affected the number of E1A-specific CD8⁺ T cells. A remarkable increase in the number of E1A-specific CD8⁺ T cells in peripheral blood was observed after CD4 depletion (Fig. 3). Furthermore, whereas E1A-specific CD8⁺ T cells in untreated tumor-bearing mice are only detected in tumor-draining lymph nodes in 30% of the animals, the E1A-response emerging after CD4 depletion was readily detected in other lymphoid organs and the tumor (Fig. 4). These results indicate that the CD4⁺ cells suppress the initial induction or expansion of the tumor-specific CD8⁺ T cells

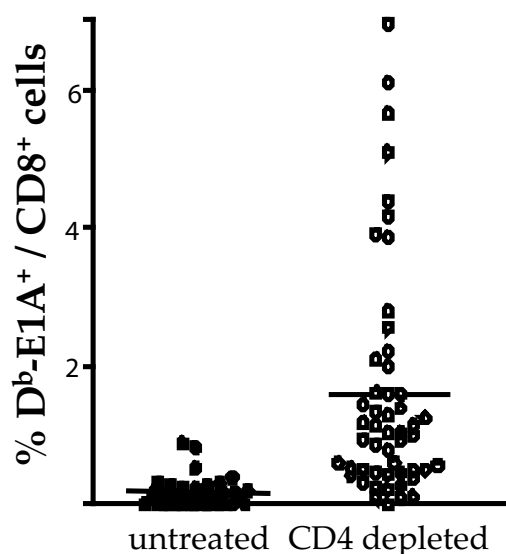
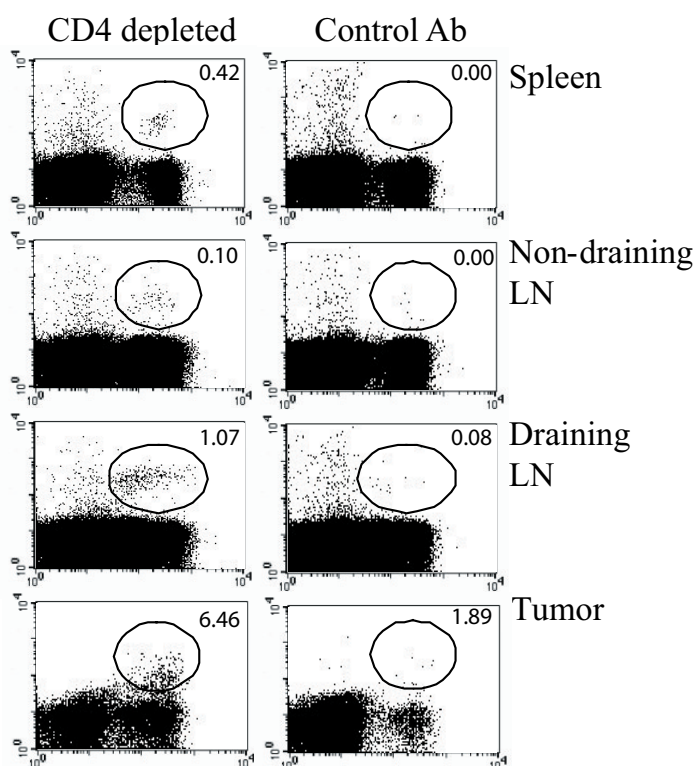


Figure 3: CD4 depletion leads to increased numbers of tumor-specific CD8⁺ T cells.

C57BL/6 mice were injected with 1×10^6 live Ad5E1/ras cells in the flank s.c. and at least 21 days later, at a time mice had developed established tumors, depletion of CD4⁺ cells was started ($n=52$), or mice were left untreated ($n=35$). Ten to thirteen days after the start of the depletion the percentage of CD8⁺ T cells capable of interacting with H-2 D^b-E1A₂₃₄₋₂₄₃ in peripheral blood was determined by FACS analysis. Combined data from 6 independent experiments are shown. $P < 0.0001$ (Mann Whitney test).

Figure 4: CD4 depletion leads to increased numbers of tumor-specific CD8⁺ T cells.

C57BL/6 mice were injected with 1×10^7 live Ad5E1/ras cells in the flank s.c. and injected 21 days later, at a time mice had developed established tumors, with GK1.5, anti-CD4, or control antibody (6E9). Nineteen days after the start of the depletion the percentage of CD8⁺ T cells capable of interacting with H-2 D^b-E1A₂₃₄₋₂₄₃ was determined by FACS analysis in spleens, lymph nodes and tumors. Numbers represent the percent of tetramer-positive cells in the CD8-positive population. Representative dot plots are shown. Tumors from 2 control-treated mice and 4 CD4-depleted mice were pooled before analysis.



in tumor-bearing mice. When CD4 depletion was combined with CD8⁺ cell depletion, the beneficial effect of CD4 depletion alone was completely abrogated, confirming that tumor rejection mice depleted for CD4⁺ cells requires CD8⁺ T cells (Fig. 5).

CD4 is not only expressed on a subset of T cells, but also on subsets of NKT cells and DCs. Therefore, any of these cell types may theoretically be involved in the observed effects of CD4 depletion. To investigate whether the tumor-suppressive CD4⁺ cells were T cells, class II^{-/-} mice, which lack functional peripheral CD4⁺ T cells, but contain CD4⁺ NKT cells (18) and DCs, were challenged with tumor cells. Tumors were initially detected in all mice, but spontaneously regressed after 10 days (Fig. 6). Anti-tumor E1A-specific CD8⁺ T cells were

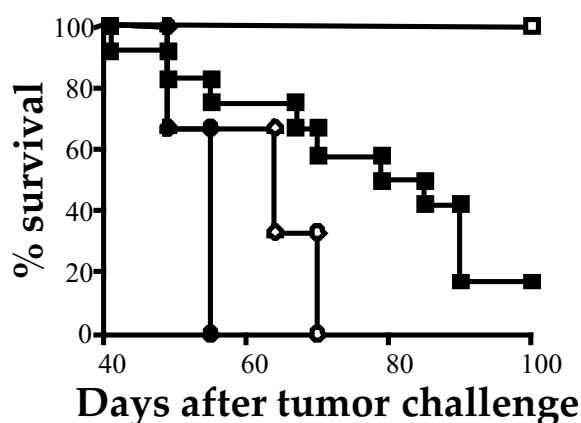


Figure 5: The role of CD8⁺ cells in CD4 depleted mice.

C57BL/6 mice were injected with 1×10^7 live Ad5E1/ras cells in the flank s.c. and left untreated (closed squares) or injected 22 days later, at a time mice had developed established tumors, with GK1.5, anti-CD4, (open squares) or 2.43, anti-CD8, antibody (closed circles) or both (open circles). The percent of surviving mice is shown. Untreated versus CD4-depleted, $P = 0.0061$; untreated versus CD4- and CD8-depleted, $P = 0.092$; CD4- versus CD4- and CD8-depleted, $P = 0.0042$ (log-rank test).

induced in all mice injected with tumor cells (data not shown), indicating that no suppression occurred in these mice. Together these results indicate that CD4⁺T cells, but not other CD4 cells, suppress the anti-tumor CD8⁺ T cell response in wild-type tumor-bearing mice. Furthermore, the combined observations indicate that rapidly (i.e. within 3 weeks) after outgrowth of an E1A-expressing tumor, a CD4⁺T cell population emerges that prevents the generation of effective CD8⁺ T cell immunity, as the presence of CD4⁺ T cells was required for E1A-specific CTL immunity in the prophylactic setting, but prevented such immunity in tumor-bearing hosts.

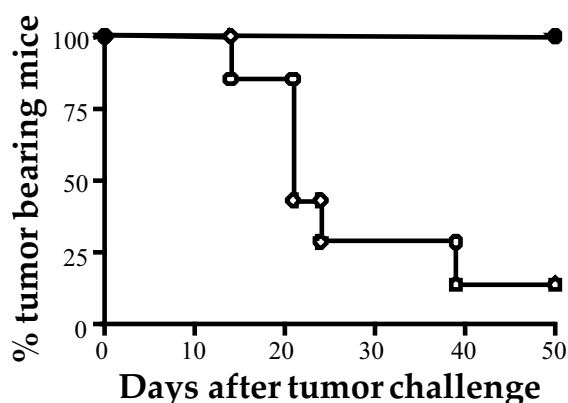


Figure 6: Tumors are spontaneously eradicated in class II-/- mice.

C57BL/6 (closed circles) and class II-/- (open circles) mice were injected with 1×10^7 live Ad5E1/ras cells in the flank s.c.. Tumor outgrowth was measured. The percent of tumor-bearing mice is shown. The experiment was repeated with similar results. $P < 0.01$ (log-rank test).

CD4⁺CD25⁺ T cells are not involved in suppression

To study whether “conventional” CD4⁺CD25⁺ regulatory cells are involved in suppression of tumor-specific CD8⁺ T cell mediated immunity, we depleted mice for CD25⁺ cells. In several other models CD25 depletion shortly before tumor challenge increased anti-tumor immunity and tumor eradication (11-13, 19). Therefore, depletion was started three days before tumor challenge. CD4⁺CD25⁺ cells were reduced in numbers by 90-95% as determined by flow cytometric analysis (data not shown). CD25⁺ cell depletion did, however, not influence tumor-growth (data not shown).

To study whether the tumor induces a CD4⁺CD25⁺ suppressor population during development, CD25⁺ cells were also depleted three weeks after tumor challenge. No influence of this depletion on tumor outgrowth was observed (Fig. 7). Since CD25 is expressed on all activated T cells, depletion of CD25⁺ cells may reduce the number of

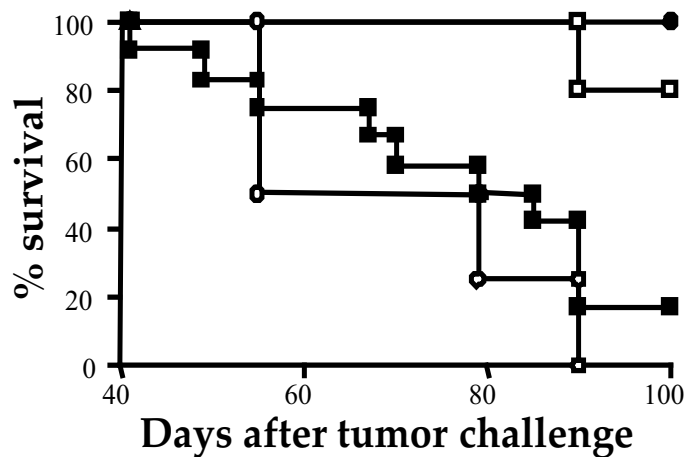


Figure 7: CD25 depletion does not affect tumor-specific CD8 T cells or tumor outgrowth.

C57BL/6 mice were injected with 1×10^7 live Ad5E1/ras cells in the flank s.c. and left untreated (closed squares) or injected 22 days later, at a time mice had developed established tumors, with GK1.5, anti-CD4 antibody (closed circles) or PC61, anti-CD25 antibody (open circles), or both (open squares). The percent of surviving mice is shown. Untreated versus CD4-depleted, $P < 0.01$; untreated versus CD25-depleted, $P = 0.47$; untreated versus CD4- and CD25-depleted, $P = 0.018$ (log-rank test).

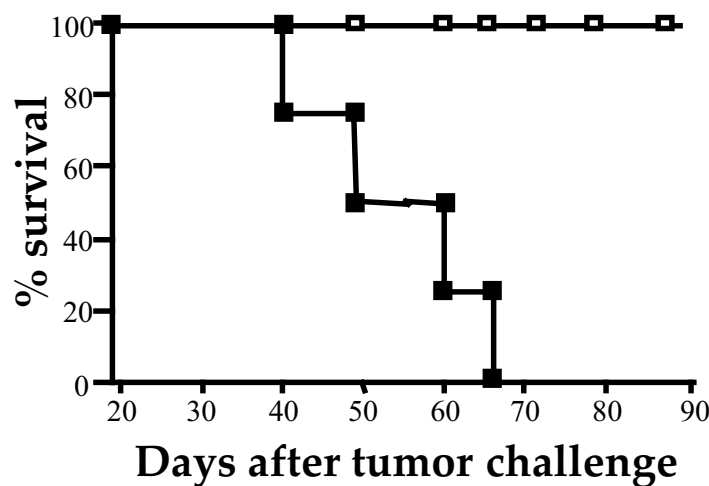


Figure 8: Injection of CpG overcomes suppression of anti-tumor CD8⁺ T cell responses and increases survival of tumor-bearing animals.

C57BL/6 mice were injected with 1×10^6 live Ad5E1/ras cells in the flank s.c. and left untreated (closed squares) or injected 22 and 25 days later, at a time mice had developed established tumors, peritumorally with CpG (open squares). The percent of surviving mice is shown. $P < 0.01$ (log-rank test)

effector CD8⁺ T cells as well. However, mice depleted for both CD4⁺ and CD25⁺ cells still eradicated the tumor (Fig. 7). E1A-specific CTL emerged in these mice (data not shown), indicating that effector CD8⁺ T cells remain functional and are not depleted by the anti-CD25 antibody. Together, these results indicate that CD4⁺CD25⁺ cells are not crucially involved in suppression of the E1A-specific CD8⁺ T cell mediated response in tumor-bearing mice.

APC activation overcomes CD4⁺ T cell suppression

Since CD4⁺ T cells suppress the initiation phase of spontaneous CD8⁺ T cell immunity, we hypothesized that therapeutic intervention schemes supporting the induction of CD8⁺ T cells may overcome this immunosuppression. Provision of proper costimulation by activated APC plays a major role in the induction of CD8⁺ T cells. Furthermore, activation of APC is an important determinant in the balance between tolerisation and immunization (20). Therefore, we investigated whether APC activation can overcome CD4⁺ T cell mediated suppression in tumor-bearing mice. Injection of CpG, or anti-CD40 antibodies, induced APC activation as demonstrated by an increased number of CD11c cells expressing high levels of B7.2 in the lymphoid organs (data not shown). Furthermore, a strong E1A-specific CD8⁺ T cell response was generated after injection of CpG (Fig. 8), or anti-CD40 antibodies (16), data not shown, which was capable of complete eradication of tumor cells resulting

in survival of the mice. Thus, treatment with agents that cause proper APC activation can overcome suppressive CD4⁺ T cells in tumor-bearing mice.

Discussion

In this study, we show that CD4⁺ T cells in tumor-bearing mice suppress anti-tumor CD8⁺ T cell responses as depletion of CD4⁺ cells in mice bearing established tumors results in the rapid, systemic expansion of effective tumor-specific CD8⁺ T cell immunity without the need for tumor-specific vaccination. Remarkably, the suppressive CD4⁺ cells appear to be induced rapidly after tumor-growth, because the same CD8⁺ T cell response that is suppressed by CD4⁺ T cells in tumor-bearing mice, is helper cell dependent in vaccinated tumor-free mice. Thus, our data indicate that rapidly after tumor outgrowth (i.e. within 3 weeks) CD4⁺ T cells emerge that instead of providing help for CD8⁺ cell induction, hamper the spontaneous development of effective tumor-specific CD8⁺ cell responses. Suppression in tumor-bearing animals can be overcome via injection of anti-CD40 antibodies or CpG, indicating that the suppressive effect mediated by CD4⁺ T cells can be by-passed through the activation of CD40-, respectively TLR9-positive cells, most likely DCs.

Therapeutic vaccination for cancer treatment has proven more difficult than prophylactic vaccination. Although the reasons for this observation are not known, various aspects are likely to contribute to the ineffectiveness of therapeutic anti-tumor vaccination. Here we show that one of these aspects relates to the induction of suppressive CD4⁺ T cells rapidly after tumor cell inoculation and growth. CD4⁺ T cells play a central role in the orchestration of various immune functions such as macrophage activation, B cell maturation and isotype-switching. Likewise, CD4⁺ T cells are important in the induction of anti-tumor CD8⁺ T cell responses after vaccination, as immunization in the absence of CD4⁺ T cells often leads to the abrogation of tumor-specific CD8⁺ T cell response (17, 21) due to the limited secondary expansion of these CD8⁺ T cells upon re-encounter of antigen (22). It is conceivable that given the central role of CD4⁺ T cells in immune regulation, several effector pathways will be affected by the generation of the wrong type of CD4⁺ T cell immunity. Our results indicate that the prevention of effective CTL-mediated anti-tumor immunity by suppressor CD4⁺ T cells in the context of a growing tumor expressing highly immunogenic T cell epitopes represents a potent mechanism that contributes to uncontrolled tumor growth. This suppressive environment potentially impedes effective anti-tumor immunotherapy.

An important distinction explaining the discrepancy between prophylactic and therapeutic vaccination might relate to the presence or absence of 'danger' signals which alarm the immune system to generate a response. In general, prophylactic vaccination is performed with for example irradiated tumor cells or tumor antigens in adjuvant. Such vaccines harbor the intrinsic ability to alert the immune system through the release of compounds like heat-shock-proteins (23) and uric acid (24) that trigger DC-activating receptors such as TLRs. This will lead to the activation of DCs which in turn will communicate the stress inflicted by the vaccine to T cells and activate them. In contrast, outgrowth of tumors is not accompanied by strong danger signals. Therefore, tumor antigens will be shunted into the cross-presentation pathways that normally prevent unwanted activation of T cells (25) to avoid tissue destruction in the absence of stimulatory signals. We anticipate that even when the tumor antigens are immunogenic (neo-)antigens, like the E1A-protein, they are not presented in a pro-inflammatory context and therefore will not provoke an effective immune response. It is tempting to speculate that in this setting T cells are induced that in

hibit potentially destructive immune responses. Such T cells are crucial to maintain normal tissue homeostasis in case the immune system is confronted with new proteins during for example pregnancy, lactation or inhalation. However, in tumor-bearing hosts such T cells may be detrimental to the emergence of effective anti-tumor immunity and may hamper effective anti-tumor immunotherapy.

In mice bearing E1A-expressing-tumors a small number of E1A-specific CD8⁺ T cells is present in the tumor-draining lymph nodes without any additional intervention (16). Induction of these CD8⁺ T cells is either independent of CD4⁺ helper T cells, or initially a population of helper CD4⁺ T cells is present, for example induced by tumor cell inoculation, which is later overruled by the suppressor CD4⁺ T cells. The presence of an E1A-specific CD8⁺ T cell population in tumor-bearing MHC class II ^{-/-} mice which lack functional peripheral CD4⁺ T cells, indicates that induction of tumor-specific CD8⁺ T cells can occur without CD4⁺ T cell help. Likewise, in transgenic mice bearing spontaneously developed pancreatic tumors, adoptive transfer of high numbers of naïve tumor-specific T-cell-receptor-transgenic CD8⁺ T cells generates effector CD8⁺ T cells that display pronounced proliferative potential and increased IFN-gamma production after CD4⁺ cell depletion (26).

Recently, suppression by CD4⁺CD25⁺ T cells, in vitro or in vivo, in an environment of systemic antigen presentation, was shown to be reverted by TLR signals (27, 28). Likewise, we show that CpG or CD40 triggering can overcome tumor-induced CD4⁺ T cell mediated suppression. In vitro, IL-6 produced by APC upon TLR triggering with LPS or CpG was crucial to overcome suppression from regulatory T cells (27). It is conceivable that these and other beneficial mechanisms contribute to the effects observed after injection of anti-CD40 and CpG.

In conclusion, our results are important for improvement of immunotherapeutic interventions in cancer patients. In the design of therapeutic immuno-interventions, ways to overcome a suppressive environment need to be taken into account to successfully induce powerful anti-tumor immunity. Intervention regimens, like treatment with non-depleting blocking anti-CD4 antibodies (29, 30), could prove to be effective, but also interventions aiming at bypassing suppressive effects installed by the CD4⁺ T cells could be successful. In this respect, we have shown that CpG injection or CD40 ligation support a strong tumor-specific CD8⁺ T cell response in a suppressive setting, resulting in eradication of tumor cells and (prolonged) survival. This indicates that a change in the environment in which tumor antigens are presented can shift the balance from suppression to strong induction of anti-tumor immunity. Interestingly, both depletion of the suppressive cell population or treatment with APC activating agents in tumor-bearing mice, resulted in tumor rejection without the need for tumor-specific vaccination.

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