

Antagonistic Roles of CD4⁺ and CD8⁺ T-Cells in 7,12-Dimethylbenz(a)anthracene Cutaneous Carcinogenesis

Nabiha Yusuf,^{1,2} Tahseen H. Nasti,¹ Santosh K. Katiyar,¹ Michael K. Jacobs,¹ Megan D. Seibert,¹ Alexis C. Ginsburg,¹ Laura Timares,^{1,2} Hui Xu,^{1,2} and Craig A. Elms^{1,2}

¹Department of Dermatology and Skin Diseases Research Center, University of Alabama at Birmingham and

²Birmingham VA Medical Center, Birmingham, Alabama

Abstract

The role that cell-mediated immune responses play during cutaneous carcinogenesis has received little attention. In this study, we evaluated the contribution of CD4⁺ and CD8⁺ T cells in C3H/HeN mice that were subjected to a two-stage 7,12-dimethylbenz(a)anthracene (DMBA) initiation, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) promotion skin carcinogenesis protocol. In CD8 knockout (CD8^{-/-}) mice, allergic contact hypersensitivity to DMBA was reduced compared with wild-type (WT) C3H/HeN mice. On the other hand, CD4 knockout (CD4^{-/-}) mice developed an exaggerated contact hypersensitivity response. CD4⁺ T cells from DMBA contact-sensitized mice preferentially produced interleukin 4 (IL-4), IL-10, and IL-17; CD8⁺ T cells, on the other hand, secreted IFN- γ . When CD4^{-/-}, CD8^{-/-}, and WT mice were subjected to a standard two-stage DMBA/TPA cutaneous carcinogenesis protocol, the percentage of mice with tumors was much greater ($P < 0.001$) in CD8^{-/-} mice than in WT mice. In contrast, the percentage of tumors was significantly less ($P < 0.001$) in CD4^{-/-} mice than in WT mice. Similar results were obtained when the data were evaluated as the number of tumors per mouse. These findings indicate that (a) CD8⁺ T cells are the predominant effector cells in allergic contact hypersensitivity to DMBA and that CD4⁺ T cells have an inhibitory role and (b) the development of CD8⁺ T cells plays a protective role in skin tumor development whereas CD4⁺ T cells have the opposite effect. Manipulation of T-cell subpopulations that are induced by carcinogenic chemicals, like DMBA, could be a means of preventing skin cancers caused by these agents. [Cancer Res 2008;68(10):3924–30]

Introduction

Murine models of polycyclic aromatic hydrocarbon (PAH) skin carcinogenesis have been used extensively to investigate the mechanisms by which chemicals cause cancer (1, 2). As a consequence of these studies, it is now known that epidermal keratinocytes undergo an orderly sequence of molecular and biochemical alterations that eventuate in invasive squamous cell carcinomas. During the initiation stage, specific mutations in the H-ras oncogene occur when carcinogenic PAHs, such as 7,12-dimethylbenz(a)anthracene (DMBA), benzo(a)pyrene [B(a)P], or

3-methylcholanthrene, are applied to the skin (3–6). With repeated exposure to such tumor-promoting agents as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), croton oil, or benzoyl peroxide during the promotion stage, epigenetic changes in these mutant cells result in the appearance of premalignant papillomas (7). Continued exposure to tumor promoters results in further genetic alterations that facilitate the progression of some of the premalignant papillomas into fully invasive squamous cell carcinomas.

PAH-induced skin tumors vary in their immunogenicity depending on the specific PAH that is initially applied to the skin. T lymphocytes identify antigenic moieties on the surface of these cells that cause the regression of existing tumors and their metastases (8). This has led to efforts to bolster the immune response to a variety of solid tumors as a form of immunotherapy (8, 9).

There is now growing evidence that, in addition to its effect on tumors that already exist, T cells have an influence on earlier stages in the carcinogenesis pathway. For example, in FVB mice, $\gamma\delta$ T cells inhibit the development of epithelial malignancies (10, 11). When subjected to a DMBA initiation/TPA promotion protocol, tumors are more likely to arise in mice that lack $\gamma\delta$ T cells, whereas tumor incidence is reduced in mice that lack $\alpha\beta$ T cells (11). In other studies, it has been shown that infiltrating CD4⁺ T cells are present in both premalignant and malignant lesions in a transgenic model of multistage squamous carcinogenesis induced by human papillomavirus (HPV) oncogenes (12). HPV16 mice deficient in CD4⁺ T cells were found to have delayed neoplastic progression and a lower incidence of tumors than their wild-type (WT) counterparts.

We have previously shown that topical application of a variety of different mutagenic and carcinogenic PAHs to C3H/HeN mice results in the development of an allergic contact hypersensitivity response mediated by CD8⁺ T cells (13, 14). The development of that response is controlled by genes at the MHC class I and Ah receptor loci (15). Moreover, allergic contact hypersensitivity was associated with inhibition of tumor development, giving rise to the hypothesis that induction of the T cell-mediated immune response to DMBA protects an individual from the carcinogenic effects of that agent.

The purpose of this study was to define the contribution of CD4⁺ and CD8⁺ T cells to tumor development in mice subjected to a two-stage DMBA initiation/TPA promotion skin tumorigenesis protocol. We observed that skin carcinogenesis was retarded in mice deficient in CD4⁺ T cells compared with WT mice, whereas significantly greater numbers of tumors occurred in mice deficient in CD8⁺ T cells. These results indicate that, in addition to their effect on fully developed tumors, T cells participate during tumor development as well. CD4⁺ T cells promote tumor development, whereas CD8⁺ T cells have the opposite effect.

Requests for reprints: Nabiha Yusuf, Department of Dermatology, University of Alabama at Birmingham, 1670 University Boulevard, VH 566A, P.O. Box 202, Birmingham, AL 35294-0019. Phone: 205-934-7432; Fax: 205-934-5745; E-mail: nabihay@uab.edu.

©2008 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-07-3059

Materials and Methods

Animals and reagents. C3H/HeN mice were purchased from Charles River Laboratories. Female mice 6 to 8 wk of age were used in the experiments. CD4 knockout (CD4^{-/-}) and CD8 knockout (CD8^{-/-}) mice on a C57BL/6 background were purchased from The Jackson Laboratory and were backcrossed onto a C3H/HeN background. The CD4 deletion was backcrossed for 12 generations via traditional methods. These mice had >99% C3H/HeN background genes. The CD8 deletion was placed on the C3H/HeN background via rapid backcross methodology (16, 17). Briefly, mice at each backcross generation were genotyped with two sets of polymorphic microsatellite repeat markers (Research Genetics), making it possible to select progeny which retained the desired C3H genome and had lost a larger portion of the undesired C57BL/6 DNA. By selective breeding of these mice, it was possible to eliminate the majority of the C57BL/6 genome and replace it with C3H DNA after four generations of backcrossing; subsequently, the mice were backcrossed by traditional methods. The mice that were used for the experiments had >99% C3H/HeN background genes. All animal procedures were performed according to NIH guidelines under protocols approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. DMBA, TPA, and acetone were purchased from Sigma Chemical Co.

Assessment of contact hypersensitivity responses. The induction and elicitation of contact hypersensitivity responses in mice were carried out as described previously (14). Briefly, mice were sensitized with 100 μ L DMBA (0.1% w/v in acetone). The sensitized mice were challenged 5 d later by application of 20 μ L DMBA (0.1% w/v in acetone) to the ear. The contact hypersensitivity response was assessed by measuring ears before elicitation and daily for 5 d after elicitation with a dial thickness gauge micrometer (Mitutoyo). The maximum increment in ear thickness compared with the baseline preelicitation level was used to quantify the magnitude of the response. Naive mice, which were not sensitized but were ear challenged, served as negative controls.

Purification of T-cell subpopulations. *In vitro* purification of DMBA primed CD4⁺ or CD8⁺ T cells after contact sensitization and tumorigenesis experiments was conducted using MACS system according to the manufacturer's instruction (Miltenyi Biotech, Inc.). The efficiency of depletion and purity of T-cell subpopulations were determined by flow cytometric analysis using specific antibodies to target cells. The efficiency of purification was 98%.

Skin tumorigenesis. A two-stage skin carcinogenesis protocol was used to study the effect of CD4⁺ and CD8⁺ T cells, in which DMBA was the initiating agent and TPA was the promoting agent, using methods that have been described previously (18). Briefly, the dorsal skin of mice (10 mice per panel) was painted with 100 μ L of DMBA (0.1% w/v in acetone). Beginning 1 wk later, TPA (40 nmol in acetone) was applied biweekly to the site that had been treated previously with DMBA. Mice were evaluated biweekly for tumors. Only tumors that had attained a size of ≥ 1 mm and were present for ≥ 2 wk were counted.

Measurement of cytokine production by T cells. Spleens were harvested from tumor-bearing C3H/HeN mice, and T-cell subpopulations were purified with magnetic beads using MACS system according to the manufacturers' instruction (Miltenyi Biotech, Inc.). The cells (2×10^5 in 200 μ L) were cultured in anti-CD3 antibody-coated 96-well plates. The plates were incubated with anti-CD3 antibody (25 μ g/mL; 30 μ L/well) at 37°C for 90 min and then washed with PBS before adding the cell suspensions. Supernatants were harvested 48 h later for quantification of cytokines by ELISA kits from Invitrogen using standard protocols.

TPA-induced ear swelling. Baseline mouse ear thickness measurements were made with an engineer's micrometer. To study the effect of CD4⁺ and CD8⁺ T cells on the ear swelling response to TPA, the ears of CD4^{-/-}, CD8^{-/-}, and WT mice were painted with TPA (40 nmol in acetone). The ear thickness was measured at 24-h intervals until the ear swelling receded. The increment in ear thickness compared with baseline levels was used to quantify the inflammatory response caused by TPA. There were five mice in each panel tested.

***In vitro* stimulation of T cells by bone marrow-derived dendritic cells and measurement of cytokine production.** Mice were sensitized with 100 μ L DMBA (0.1% w/v in acetone). The sensitized mice were sacrificed after 5 d, and T-cell subpopulations were purified from draining lymph nodes with magnetic beads using MACS system according to the manufacturer's instruction (Miltenyi Biotech, Inc.). Bone marrow-derived dendritic cells (BMDC) were prepared and used for *in vitro* stimulation of primed T cells as described (19, 20). Purified naive and DMBA-primed CD4⁺ and CD8⁺ T cells (2×10^6 /mL), which were prepared as described above, were stimulated with DMBA-labeled BMDC (2×10^5 /mL). To determine the specificity of the T-cell response, BMDCs were treated with DMBA or B(a)P and stimulated *in vitro* with DMBA primed T cells. Cytokine concentrations in culture supernatants were measured 48 h later by cytokine-specific ELISA.

Statistical analysis. The differences between experimental groups for contact hypersensitivity and cytokine ELISAs were analyzed using the Student's *t* test. The Fisher exact test was used for analysis of the tumors per mouse and the percentage of mice with tumors. In all cases, a *P* value of <0.05 was considered significant.

Results

Differential role of CD4⁺ and CD8⁺ T cells in the contact hypersensitivity response to DMBA. In previous studies, we had shown that topical application of DMBA results in the development of hapten-specific contact hypersensitivity (13, 14). Initial experiments were performed to determine the role of CD4⁺ and CD8⁺ T cells in this response. Panels of CD4 knockout (CD4^{-/-}) and CD8 knockout (CD8^{-/-}) mice and their WT counterparts were contact-sensitized with DMBA and ear challenged 5 days after that. CD8 knockout mice showed total inhibition of contact hypersensitivity compared with untreated controls (Fig. 1), confirming our previous results (13). Conversely, CD4^{-/-} mice augmented contact hypersensitivity. Similar results were observed when mice were depleted of CD4⁺ and CD8⁺ T cells by administration of anti-CD4 and anti-CD8 antibodies (data not shown). These results indicate that CD8⁺ T cells are effector cells for contact hypersensitivity to DMBA and CD4⁺ T cells have an inhibitory effect on the response.

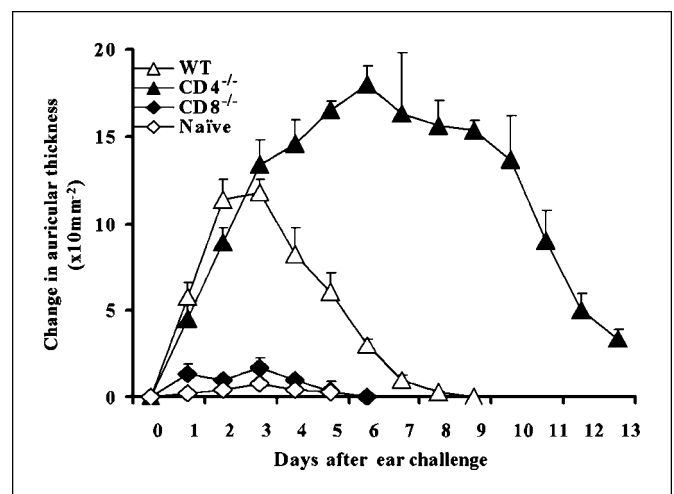


Figure 1. DMBA contact hypersensitivity in CD4^{-/-}, CD8^{-/-}, and WT C3H/HeN mice. Mice were sensitized to DMBA and ear challenged 5 d later. CD4^{-/-} mice showed a significant increase in the contact hypersensitivity response compared with CD8^{-/-} and WT mice. Points, mean with five mice per group; bars, SE. The experiment was repeated thrice.

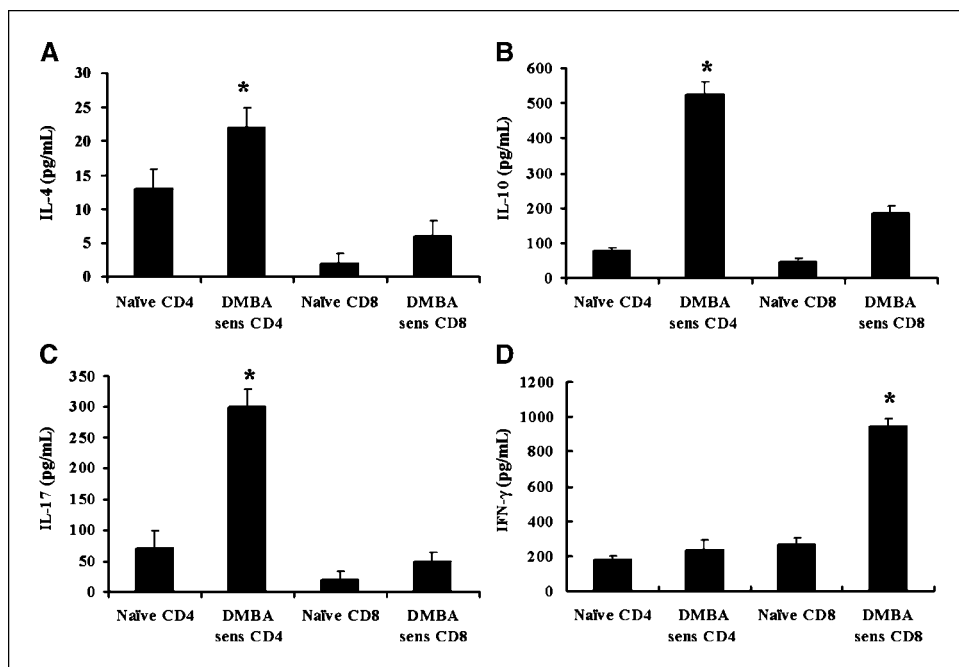


Figure 2. Cytokine profiles of DMBA (0.1%)–sensitized T cells stimulated with BMDCs from C3H/HeN mice. Mice were sensitized to DMBA and CD4⁺ and CD8⁺ T cells were purified as described in Materials and Methods. Purified T cells were then stimulated with DMBA labeled BMDCs. Cytokine levels were measured in supernatants by ELISA as described in Materials and Methods. CD4⁺ T cells from DMBA-sensitized mice produced significantly greater amounts of IL-4, IL-17, and IL-10 (*, $P < 0.05$; A, B, and C), whereas CD8⁺ T cells from CD4^{−/−} mice produced significantly greater (*, $P < 0.05$) amounts of IFN-γ (D). Columns, mean with three mice per group; bars, SD. Each experiment was repeated twice.

Cytokine production by T cells in response to DMBA. Effector T-cells for dinitrofluorobenzene contact hypersensitivity secrete the cytokines IFN-γ and interleukin 17 (IL-17), and regulatory T cells that inhibit the response produce IL-4 and IL-10 (19, 21). The types of cytokines produced by DMBA-responsive T cells were next examined. DMBA was applied to the skin, and 5 days later, the draining lymph nodes were removed. Purified CD4⁺ and CD8⁺ T-cell suspensions were prepared and restimulated *in vitro* for 48 hours with DMBA-treated BMDCs. The supernatants were then removed and examined by ELISA for production of IFN-γ, IL-4, IL-10, and IL-17 (Fig. 2). There was a significant increase in IL-4,

IL-10, and IL-17 ($P < 0.05$) concentrations in supernatants of CD4⁺ T cells from DMBA-sensitized mice compared with those from naive mice, but there was not a statistically significant increase in IFN-γ production. In contrast, there was a much more modest increase in IL-4, IL-10, and IL-17 concentrations and a much greater increase in IFN-γ from DMBA-sensitized mice compared with naive CD8⁺ T cells. These results indicated that hapten primed CD4⁺ T cells produced significant levels of IL-4, IL-10, and IL-17 ($P < 0.05$) and CD8⁺ T cells produced significant levels ($P < 0.05$) of IFN-γ (Fig. 2). To determine whether these cytokine-producing T cells from hapten-sensitized mice were specific for DMBA, we

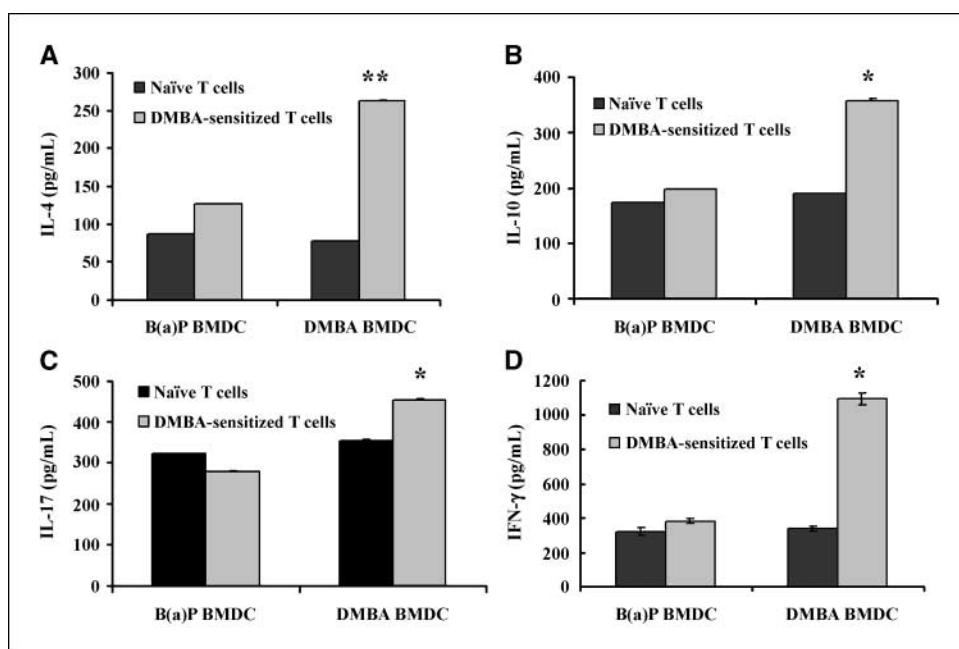


Figure 3. Cytokine profiles of DMBA (0.1%)–sensitized T cells stimulated with BMDCs from C3H/HeN mice. Mice were sensitized to DMBA, and T cells were purified as described in Materials and Methods. Purified T cells were then stimulated with DMBA-labeled or B(a)P-labeled BMDCs. Cytokine levels were measured in supernatants by ELISA, as described in Materials and Methods. T cells from DMBA-sensitized mice produced significantly greater amounts of IL-4 (**, $P < 0.001$), IL-17, IL-10, and IFN-γ (*, $P < 0.05$; A–D) than naive T cells with DMBA-labeled BMDCs. However, there was no significant difference in cytokine production on stimulation of DMBA-sensitized T cells with B(a)P-labeled BMDCs. Columns, mean with three mice per group; bars, SD. Each experiment was repeated twice.

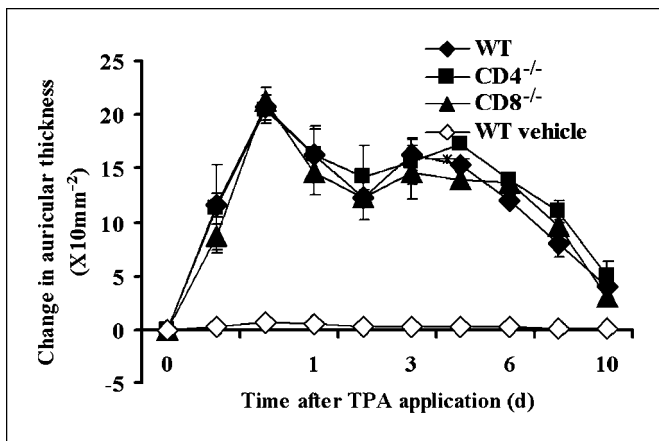


Figure 4. Comparison of the ear swelling response to TPA in CD4^{-/-}, CD8^{-/-}, and WT C3H/HeN mice. TPA (40 nmol) was applied to the ear of CD4^{-/-}, CD8^{-/-} and WT C3H/HeN mice. Points, mean of ear swelling response; bars, SE. There is no significant ($P > 0.05$) difference in ear swelling response between the groups.

stimulated DMBA primed T cells with either DMBA-labeled or B(a)P-labeled BMDCs *in vitro*. Stimulation of DMBA-labeled T cells with DMBA-labeled BMDCs showed significantly higher levels of cytokines IL-4, IL-10, IL-17, and IFN- γ ($P < 0.05$) compared with T cells stimulated with B(a)P-labeled BMDCs (Fig. 3).

TPA ear swelling response. TPA produces a brisk inflammatory response when applied to the skin, which is non-antigen specific in nature. We wished to examine the contribution of CD4⁺ and CD8⁺ T cells to the TPA response (Fig. 4). Panels of CD4^{-/-} and CD8^{-/-} mice and their WT counterparts were treated with 40 nmol of TPA on their ear, and the increase in ear swelling was measured until it returned to baseline levels. There was no significant difference in the ear swelling response after TPA treatment in any of the groups, indicating that neither CD4⁺ nor CD8⁺ T cells are major participants in the inflammatory response to TPA.

DMBA-induced skin tumorigenesis. The contribution of CD4⁺ and CD8⁺ T cells to DMBA skin tumorigenesis was next examined. Panels of CD4^{-/-} and CD8^{-/-} mice and their WT counterparts

were subjected to a two-stage cutaneous chemical carcinogenesis protocol, in which DMBA was the initiating agent and TPA was the promoter. Because C57BL/6 mice do not develop an immune response to DMBA and because the CD4^{-/-} and CD8^{-/-} mice that are commercially available are on a C57BL/6 background, we first backcrossed the CD4 and CD8 mutations onto the C3H/HeN background. As shown in Fig. 5A, the percentage of CD4^{-/-} mice that developed tumors was significantly less than WT mice and the percentage of CD8^{-/-} mice that developed tumors was significantly greater than WT mice. For example, after 10 weeks on the protocol, 100% of CD8^{-/-} mice had developed tumors. This compared with tumors in 50% of WT mice. At that time, tumors were present in only 20% of the CD4^{-/-} mice. These differences were highly significant ($P < 0.001$).

The number of tumors per mouse was also significantly greater ($P < 0.001$) in CD8^{-/-} mice compared with WT mice; CD4^{-/-} mice developed significantly fewer tumors than their WT counterparts (Fig. 5B). Similar results were obtained when the data were evaluated as the cumulative number of tumors per group (data not shown). It is important to note, however, that the tumor latency was the same (~4 weeks) in all three groups. Whereas the tumors in the CD8^{-/-} and WT group persisted and grew progressively, the tumors in CD4^{-/-} regressed, and after 11 weeks, only 10% of the CD4^{-/-} mice had tumors (Fig. 5A). These differences were also highly significant ($P < 0.001$). We thus conclude that CD4^{-/-} mice are resistant to DMBA skin tumorigenesis and CD8^{-/-} mice are more susceptible to it. Tumors were resected, stained, and evaluated histologically. All the tumors were squamous cell carcinomas and papillomas; there were no fibrosarcomas. No significant differences in the proportions of papillomas and carcinomas were observed. Hence, absence in T-cell populations did not induce a shift in the tumor type.

Characterization of T-cell subpopulations during chemical carcinogenesis. To further characterize T-cell subsets that produce IL-4, IL-10, IFN- γ , and IL-17 during chemical carcinogenesis, T cells were restimulated with anti-CD3 antibody *in vitro* and cytokine profiles of CD4⁺ and CD8⁺ T cells were analyzed by ELISA as described earlier. The results showed that CD4⁺ T cells from tumor-bearing mice produced IL-4, IL-10, and IL-17 and CD8⁺ T cells produced IFN- γ (Fig. 6).

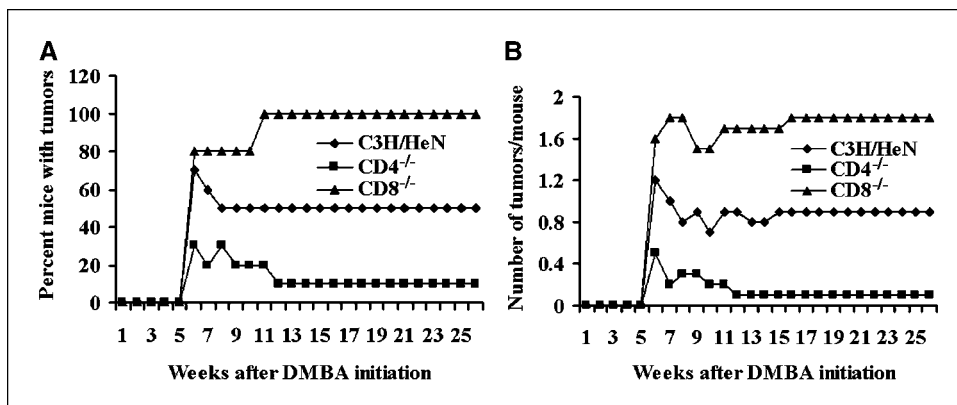


Figure 5. Comparison of the percentage of mice with tumors in CD4^{-/-}, CD8^{-/-}, and WT C3H/HeN mice. The mice were subjected to the DMBA initiation/TPA promotion cutaneous carcinogenesis protocol described in Materials and Methods. A, the percentage of mice with tumors was plotted as a function of the number of weeks of the test. There was a significantly higher percentage of CD8^{-/-} mice with tumors compared with WT mice (**, $P < 0.001$) and a significantly lower percentage of CD4^{-/-} mice with tumors than WT mice (**, $P < 0.001$). B, the number of tumors per mouse was also plotted as a function of the number of weeks on the test. There were significantly more tumors in CD8^{-/-} mice (**, $P < 0.001$) than in WT mice. There were significantly less tumors (**, $P < 0.001$) in CD4^{-/-} mice compared with WT mice.

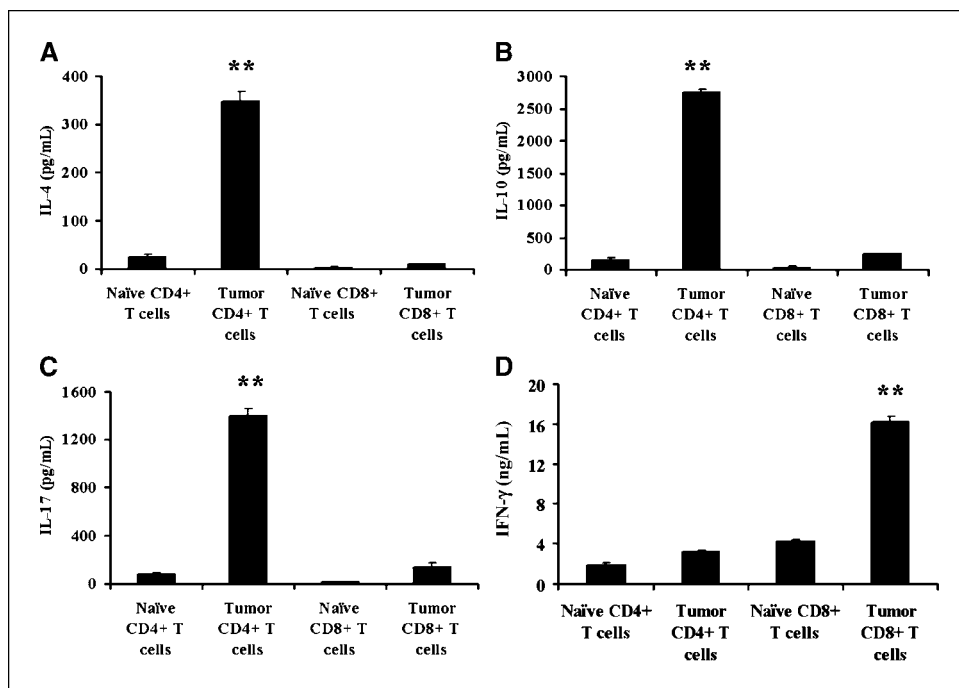


Figure 6. Cytokine profiles of T cells from C3H/HeN mice after 25 wk of cutaneous chemical carcinogenesis. The mice were subjected to the same DMBA initiation/TPA promotion cutaneous carcinogenesis protocol as is described in Materials and Methods. Purified T cells were then stimulated with anti-CD3 antibody. Cytokine levels were measured in supernatants by ELISA, as described in Materials and Methods. CD4⁺ T cells from DMBA-sensitized mice produced significantly greater amounts of IL-4, IL-17, and IL-10 (**, $P < 0.001$; A, B, and C) than CD8⁺ T cells. CD8⁺ T cells from CD4^{-/-} mice produced significantly greater (* $P < 0.05$) amounts of IFN- γ (D) than naïve CD4⁺ or CD8⁺ T cells. Columns, mean with three mice per group; bars, SD. Each experiment was repeated twice.

Discussion

PAHs are ubiquitous environmental pollutants that are by-products of the incomplete combustion of fossil fuels (2). They are present in cigarette smoke, automobile emissions, and charcoal-broiled food. These agents are mutagens and carcinogens, and those properties have been exploited to study the mechanisms by which chemicals cause cancer. In previous studies, we have shown that carcinogenic PAHs also elicit a T cell-mediated immune response in certain strains of mice (13–15). As a consequence, we have developed the hypothesis that the development of such an immune response has a protective effect, retarding the development of skin cancers by these agents. The results presented here support that hypothesis. CD4 knockout mice had an exaggerated contact hypersensitivity response to DMBA compared with WT mice, and those mice developed fewer tumors than WT mice. In contrast, a reduced contact hypersensitivity response was observed in CD8^{-/-} mice; greater numbers of tumors arose in those mice. The results indicate that CD8⁺ T cells prevent and CD4⁺ T cells augment tumor development.

It has recently been proposed that the relationship between the immune system and developing tumor cells can be divided into three stages, termed elimination, equilibrium and escape (22, 23). In elimination, innate and adaptive immune responses act to destroy malignant cells. If malignant cells persist, then the immune mechanisms can operate for an extended period of time, during which the growth of tumor cells is controlled so that a clinically apparent tumor is not present. When the growth of tumor cells is no longer restrained by the immune system and clinically apparent tumors arise, tumors then enter the escape stage. In the studies described here, the development of CD8⁺ T-cell response could be eliminating mutant cells so they cannot advance into clinically apparent tumors. Alternatively, the presence of an immune response induced by the chemical carcinogen could shift the balance of the T cell-mediated immune response to one in which

an equilibrium between the immune system and mutant cells exists, such that the mutant cells are present but the overall tumor number is held in check by antitumor immune mechanisms.

The capacity of CD4⁺ T cells to augment the development of tumors is not without precedent. Daniel et al. have used a transgenic model of viral skin carcinogenesis, in which the viral oncogene HPV16 is targeted to the epidermis (12). They have shown that CD4⁺ T cells are present in the infiltrate of developing skin cancers that promote tumorigenesis through the production of the enzyme matrix metalloproteinase-9 (MMP-9). In other studies, they have observed that MMP-9 stimulates proliferation of the mutant keratinocytes and promotes their invasion into connective tissue. An unexpected finding was that the CD4⁺ T cells in their system were directed at Staphylococcal antigens on bacteria present within the emergent tumor, rather than on antigens expressed by the tumor itself. Thus, in their system, the CD4⁺ T cells were acting during the promotion stage of carcinogenesis and stimulated tumor development through bystander interactions with the tumor. The role of CD4⁺ T cells during the initiation stage could not be addressed in the system used by Daniel et al., because in their transgenic animal model, mutant cells were already present at the time of birth.

Girardi et al. have also examined the contribution of T cells during skin tumor development in FVB mice using the same DMBA initiation/TPA promotion protocol that we used in our study (10, 11). They discovered that T cells that express the $\gamma\delta$ T-cell receptor have a suppressive effect on tumor development and that $\alpha\beta$ T-cells augment the induction of tumors. The CD4⁺ and CD8⁺ T-cell subpopulations were not examined individually in those studies. Moreover, FVB mice do not develop a contact hypersensitivity response when DMBA is applied to their skin.³ Taken

³ N. Yusuf and C.A. Elmetts, unpublished data.

together, the results of these two studies, as well as ours, highlight the complex interactions that occur between T lymphocytes and malignant cells during tumor development. Some of these are favorable to the host, whereas others are clearly disadvantageous. It is also likely that different T-cell subpopulations have different effects at different stages of tumor development.

The T-cell cytokine profile in DMBA contact hypersensitivity is similar to that of other contact sensitizers (19, 21). In dinitrofluorobenzene, oxazolone, and FITC contact hypersensitivity, CD4⁺ T cells produce IL-4 but do not produce IFN- γ and CD8⁺ T cells produce IFN- γ but do not produce IL-4. With respect to DMBA, we found that at least two populations of DMBA specific T cells were generated. One of these, the CD4⁺ T cell, inhibited the DMBA contact hypersensitivity response, had a Th2 cytokine profile, and accelerated the development of DMBA-induced skin tumors. In contrast, the CD8⁺ T cell that was generated functioned as an effector cell for DMBA contact hypersensitivity, produced Th1 cytokines, and prevented the development of DMBA-induced skin tumors.

Of particular interest with respect to the cytokine profile of the CD4⁺ T cells was the observation that they produce IL-17. The IL-17 family are inflammatory cytokines that can be produced by both CD4⁺ and CD8⁺ T cells (24, 25). IL-17 producing T cells, so-called Th_{IL-17} cells, have been implicated in the pathogenesis of a variety of autoimmune and allergic diseases (20, 23–29). The effects of IL-17 in cancer have been conflicting. IL-17 has been shown to increase the growth of several different types of tumors (30–34). It has been proposed that this is at least in part due to its proangiogenic effects (20, 30, 32). However, it has also been observed that Th_{IL-17} may actually have an inhibitory effect on some malignancies (31, 33). For example, in an *in vitro* system, IL-17 rendered U-2 OS osteosarcoma cells more vulnerable to natural killer cell lysis (31). In other studies, it was observed that the growth of IL-17-transfected murine immunogenic tumors was reduced when implanted into immunocompetent mice, but not

when injected into athymic nude mice (33). Most studies examining the relationship between IL-17 and tumors have evaluated its effect on tumor growth. Our studies suggest that IL-17 producing T cells also have an effect on tumor development. Further studies will need to be performed to analyze how Th_{IL-17} cells influence carcinogen-induced tumor development in this system.

The significance of our observations relates to the immunoprevention of carcinogen-induced tumor development. If it is true that IFN- γ -producing T cells are important effector cells for DMBA contact hypersensitivity and those cells are involved in reducing the development of DMBA-induced tumors, then it may be possible to use this information to develop methods that will augment the induction of CD8⁺ T cells and to diminish the generation of CD4⁺ T cells that have a permissive role of chemical carcinogenesis. In so doing, it may be possible to devise vaccination procedures that bolster the immune response to DMBA and ultimately to prevent DMBA-induced tumors in susceptible individuals. For example, IL-12 stimulates the production of CD8⁺ T cells that produce IFN- γ . Immunization procedures with noncarcinogenic agents that stimulate an anti-DMBA cell-mediated immunity coupled with cytokines or adjuvants that enhance the generation of subpopulations of CD8⁺ effector T cells may be an effective immunopreventive strategy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 8/8/2007; revised 1/30/2008; accepted 3/13/2008.

Grant support: NIH grants P30 AR050948 and P30 CA13148 and Veterans Administration Merit Review award 18-103-02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

- Mukhtar H, Mercurio M, Agarwal R. Murine skin carcinogenesis: relevance to humans. In: Mukhtar H, editor. Skin cancer: mechanisms and human relevance. Boca Raton: CRC Press; 1995. p. 3–8.
- DiGiovanni J. Multistage carcinogenesis in mouse skin. *Pharmacol Ther* 1992;54:63–128.
- Quintanilla M, Brown K, Ramsden M, Balmain A. Carcinogen-specific mutation and amplification of Ha-ras during mouse skin carcinogenesis. *Nature* 1986;322:78–80.
- Brown K, Buchmann A, Balmain A. Carcinogen-induced mutations in the mouse c-Ha-ras gene provide evidence of multiple pathways for tumor progression. *Proc Natl Acad Sci U S A* 1990;87:538–42.
- Bizub D, Wood AW, Skalka AM. Mutagenesis of the Ha-ras oncogene in mouse skin tumors induced by polycyclic aromatic hydrocarbons. *Proc Natl Acad Sci U S A* 1986;83:6048–52.
- Pelling JC, Fischer SM, Neades R, Strawhecker J, Schweickert L. Elevated expression and point mutation of the Ha-ras proto-oncogene in mouse skin tumors promoted by benzoyl peroxide and other promoting agents. *Carcinogenesis* 1987;8:1481–4.
- DiGiovanni J. Genetic factors controlling responsiveness to skin tumor promotion in mice. *Prog Clin Biol Res* 1995;391:195–212.
- Mandelboim O, Vadai E, Fridkin M, et al. Regression of established murine carcinoma metastases following vaccination with tumour-associated antigen peptides. *Nat Med* 1995;1:1179–83.
- Morgan RA, Dudley ME, Wunderlich JR, et al. Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 2006;314:126–9.
- Girardi M, Oppenheim DE, Steele CR, et al. Regulation of cutaneous malignancy by $\gamma\delta$ T cells. *Science* 2001;294:605–9.
- Girardi M, Glusac E, Filler RB, et al. The distinct contributions of murine T cell receptor (TCR) $\gamma\delta$ and TCR $\alpha\beta$ T cells to different stages of chemically induced skin cancer. *J Exp Med* 2003;198:747–55.
- Daniel D, Meyer-Morse N, Bergsland EK, Dehne K, Coussens LM, Hanahan D. Immune enhancement of skin carcinogenesis by CD4⁺ T cells. *J Exp Med* 2003;197:1017–28.
- Anderson C, Hehr A, Robbins R, et al. Metabolic requirements for induction of contact hypersensitivity to immunotoxic polyaromatic hydrocarbons. *J Immunol* 1995;155:3530–7.
- Klemme J, Mukhtar H, Elmets C. Induction of contact hypersensitivity to dimethylbenz[a]anthracene and benzo[a]pyrene in C3H/HeN mice. *Cancer Res* 1987;47:6074–8.
- Elmets C, Athar M, Tubesing K, Rothaupt D, Xu H, Mukhtar H. Susceptibility to the biological effects of polyaromatic hydrocarbons is influenced by genes of the major histocompatibility complex. *Proc Natl Acad Sci U S A* 1998;95:14915–9.
- Gould K, Dietrich W, Borenstein N, Lander E, Dove W. Mom1 is a semi-dominant modifier of intestinal adenoma size and multiplicity in Min/+ mice. *Genetics* 1996;144:1769–76.
- Marcela P, Su P, Ebeling C, et al. Theoretical and empirical issues for marker-assisted breeding of congenic mouse strains. *Nat Genet* 1997;17:280–4.
- Elmets CA, Zaidi SI, Bickers DR, Mukhtar H. Immunogenetic influences on the initiation stage of the cutaneous chemical carcinogenesis pathway. *Cancer Res* 1992;52:6106–9.
- Xu H, Dilulio N, Fairchild R. T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production: interferon γ -producing (Tc1) effector CD8⁺ T cells and interleukin (IL) 4/IL-10-producing (Th2) negative regulatory CD4⁺ T cells. *J Exp Med* 1996;183:1001–12.
- Yusuf N, Nasti TH, Long JA, et al. Protective Role of Toll-like Receptor 4 during the initiation stage of cutaneous chemical carcinogenesis. *Cancer Res* 2008;68:615–22.
- He D, Wu L, Kim HK, Li H, Elmets CA, Xu H. CD8⁺ IL-17-producing T cells are important in effector functions for the elicitation of contact hypersensitivity responses. *J Immunol* 2006;177:6852–8.
- Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* 2004;21:137–48.

23. Dunn GP, Old LJ, Schreiber RD. The Three Es of cancer immunoediting. *Annu Rev Immunol* 2004;22:329–60.
24. Aggarwal S, Gurney AL. IL-17: prototype member of an emerging cytokine family. *J Leukoc Biol* 2002;71:1–8.
25. Kolls JK, Linden A. Interleukin-17 family members and inflammation. *Immunity* 2004;21:467–76.
26. Mangan PR, Harrington LE, O'Quinn DB, et al. Transforming growth factor- β induces development of the TH17 lineage. *Nature* 2006;441:231–4.
27. Yao Z, Fanslow WC, Seldin MF, et al. Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. *Immunity* 1995;3:811–21.
28. Langrish CL, Chen Y, Blumenschein WM, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 2005;201:233–40.
29. Komiyama Y, Nakae S, Matsuki T, et al. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol* 2006;177:566–73.
30. Numasaki M, Watanabe M, Suzuki T, et al. IL-17 enhances the net angiogenic activity and *in vivo* growth of human non-small cell lung cancer in SCID mice through promoting CXCR-2-dependent angiogenesis. *J Immunol* 2005;175:6177–89.
31. Honorati MC, Neri S, Cattini L, Facchini A. IL-17 enhances the susceptibility of U-2 OS osteosarcoma cells to NK cell lysis. *Clin Exp Immunol* 2003;133:344–9.
32. Numasaki M, Fukushi J, Ono M, et al. Interleukin-17 promotes angiogenesis and tumor growth. *Blood* 2003;101:2620–7.
33. Benchetrit F, Ciree A, Vives V, et al. Interleukin-17 inhibits tumor cell growth by means of a T-cell-dependent mechanism. *Blood* 2002;99:2114–21.
34. Tartour E, Fossiez F, Joyeux I, et al. Interleukin 17, a T-cell-derived cytokine, promotes tumorigenicity of human cervical tumors in nude mice. *Cancer Res* 1999;59:3698–704.