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Cell mediated immune responses through TLR4 prevents DMBA-induced mammary carcinogenesis in mice

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Abstract

Toll like receptors (TLRs) activate signals that are critically involved in the initiation of adaptive immune responses and many tumorigenic chemicals have been associated with activation of those pathways. To determine the role of TLR4 in mammary carcinogenesis, we subjected TLR4 deficient and wild type (WT) mice to oral gavage with carcinogenic polyaromatic hydrocarbon 7, 12-dimethylbenz(a)anthracene (DMBA). TLR4 deficient mice developed more tumors relative to the WT mice. T cells of TLR4 deficient mice produced elevated levels of IL-17 and lower levels of IFN-γ relative to WT mice. IL-12 secreted by CD11c+ cells was higher in WT mice whereas greater amounts of IL-23 were produced by CD11c+ cells from TLR4 deficient mice. Moreover, there was higher incidence of regulatory T cells in TLR4 deficient mice than WT mice. Similarly, various markers of angiogenesis (MMP-2 and MMP-9, CD31, and VEGF) were highly expressed in tumors from TLR4 deficient mice than WT mice. The results of this study indicate that TLR4 plays an important role in the prevention of DMBA induced mouse mammary tumorigenesis and efforts to divert the cell-mediated immune response may therefore prove to be beneficial in the prevention of mammary tumors.

Keywords

Toll-like receptor 4 (TLR4); Breast cancer; 7,12-dimethylbenz(a)anthracene (DMBA); chemical carcinogenesis

Introduction

Breast cancer ranks second only to lung cancer among cancer deaths in women. An estimated 192,370 new cases of invasive breast cancer were expected to occur among women in the US during 2009 [1]. These cancers start in the lobules or ducts of the breast, and then invade the duct or glandular walls and spread to the surrounding tissues. The seriousness of invasive breast cancer is strongly influenced by the extent or spread of the cancer when it is first diagnosed [2]. Breast cancer remains a destructive disease despite the advent of new and aggressive therapeutics. Global differences in breast cancer incidence and

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studies of migrating populations suggest that both genetic and environmental factors play an important role in the pathogenesis of the disease [3].

Polyaromatic hydrocarbons (PAH) have been implicated as etiologic agents in breast cancer. Exposure occurs primarily through the smoking of tobacco, inhalation of polluted air, and ingestion of char-broiled foods [4]. One example of a carcinogenic polyaromatic hydrocarbon is 7, 12-dimethylbenz(a)anthracene (DMBA) which in animal models, induces mammary adenocarcinoma by producing carcinogen specific adducts with DNA. The dose dependence is well-established, and tumors develop without any systemic toxicity. DMBA requires metabolic activation; thus, the model is well suited to studying initiation and promotion or for evaluating effects of agents that may affect parameters of carcinogen metabolism and activation [5].

Toll-like receptors (TLRs) play a major role in innate immune responses by recognizing invading pathogens and possibly leading to inflammatory responses, which in turn lead to the activation of adaptive immune responses [6]. Analogous to other infectious and non-infectious diseases, the direct relation between TLR4 and cancer development is obscure. Some reports have provided evidence that TLR4 signaling inhibits tumor progression [7, 8], while others suggest that TLR4 facilitates tumor progression and angiogenesis [9]. The results of our previous study have shown that TLR4 plays an important role in the prevention of DMBA skin tumorigenesis [10].

In this study we examined whether differences in TLR4 signaling might influence the carcinogenic activities of DMBA in the breast, and, if so, whether biomarkers associated with a lack of host protective immune responses and the invasive phenotype were present in TLR4 deficient animals.

Materials and Methods

Animals and Reagents

Female C3H/HeN mice were purchased from Charles River Laboratories (Boston, MA). Female C3H/HeJ mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). Mice used for experiments were 6–8 weeks of age. All animal procedures were performed according to NIH guidelines under protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Alabama at Birmingham.

T-cells, T cell subsets (CD4+, CD8+, and CD4+CD25+), and CD11c+ cells were purified using magnetic beads from Miltenyi Biotec (Auburn, CA). Antibodies for MMP-2, MMP-9, VEGF and PCNA were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), CD31 antibody was from BD Pharmingen (San Diego, CA). 7,12-dimethylbenz(a)anthracene (DMBA) was purchased from Sigma Chemical Co. (St. Louis, MO). All primers were custom made from Invitrogen (Carlsbad, CA).

Mouse Tumorigenesis protocol

35 female C3H/HeN mice and 40 C3H/HeJ mice were gavaged with DMBA as described earlier with some modifications [11]. Briefly, each mouse was given a dose of DMBA (30 mg/kg body weight in 0.1ml of corn oil) by oral gavage weekly for 5 weeks, beginning at 6–8 weeks of age. Mice were monitored twice weekly and sacrificed when tumors developed. Mice bearing tumors >0.5 cm in diameter were euthanized by CO_2 inhalation and necropsied. Mammary tumors were excised and portions of the tissues were prepared for histology.

Histology

Upon necropsy, tumors were removed and immediately fixed overnight in 10% (v/v) phosphate-buffered formalin. The tissues were then processed, embedded in paraffin, and sectioned at 7 μm. The sections were mounted on glass slides and stained with hematoxylin and eosin using routine laboratory procedures. The tumors were reviewed in a blinded manner by a board certified pathologist.

RNA extraction and RT-PCR

Total RNA was extracted from the tumor samples using Trizol reagent (Invitrogen, Carlsbad, CA) following the protocol recommended by the manufacturer. The concentration of total RNA was determined by measuring the absorbance at 260 nm using BioRad spectrophotometer. Purity of isolated RNA was determined with the ratio of absorbance 260nm/280nm >1.9. cDNA was synthesized from 1 μg RNA using a Reverse transcriptase kit (Promega) according to the manufacturer's instructions. Single stranded DNA was amplified using gene specific primers and PCR Master Mix (Promega). The primers for IL-17, IFN-γ, TGF-β, perforin, granzyme, MMP-2, MMP-9 and GAPDH have been described elsewhere [12–17]. The standard PCR conditions were set according to the annealing temperatures of each primer. The expression levels of genes were normalized to the expression level of the GAPDH mRNA in each sample.

Quantitative Real time PCR—The mRNA expression of IL-17, IFN-γ, VEGF, PCNA, MMP-2 and MMP-9 from tumor samples was also determined using real-time PCR. For mRNA quantification, cDNA was synthesized as described above. Using iQ™ SYBR Green Master Mix (Bio-Rad), cDNA was amplified using real-time PCR with a Bio-Rad MyiQ thermocycler and SYBR Green detection system (Bio-Rad). Samples were run in triplicate to ensure amplification integrity. The primers used for IL-17, IFN-γ, GAPDH were as described above. The primers for Foxp3, TGF-β, PCNA, VEGF, MMP-2, MMP-9, IL-12p40, IL-12p35, and IL-23p19 have been described earlier [18–25]. The primers for CD31 were Forward 5'-TCCCTGGGAGGTCGTCCAT-3' and Reverse 5'-GAACAAGGCAGCGGGGTTTA-3' (accession no. M32599) and CD25 Forward 5'-ACTCCCATGACAAATCGAGAAAG-3' and reverse 5'-TCTCTTGGTGCATAGACTGTGT-3' (accession no. NM_008367). The standard PCR conditions were 95°C for 10 min and then 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The expression levels of genes were normalized to the expression level of the GAPDH mRNA in each sample. For mRNA analysis, the calculations for determining the relative level of gene expression were made using the cycle threshold (C_t) method. The mean C_t values from duplicate measurements were used to calculate the expression of the target gene with normalization to a housekeeping gene used as internal control and using the formulae $2^{-\Delta\Delta CT}$.

Cell isolation and co-culture—Spleens from C3H/HeN and C3H/HeJ mice were obtained 6 days after oral gavage with 30mg/kg DMBA per mouse. Single cell suspensions were made from spleen and the lysates were passed through cell strainers. After lysis of erythrocytes, cells were washed twice with HBSS. $CD11c^+$, $CD90^+$, $CD4^+$ and $CD8^+$ cells were isolated using MACS cell isolation magnetic beads (Miltenyi Biotech) according to manufactures' instructions. For *in vitro* cytokine production $CD11c^+$ cells from naïve or DMBA treated C3H/HeN or C3H/HeJ mice were stimulated for 30 min with DMBA. CD11c+ cells were added in a ratio of 1:10 with T cells from DMBA treated C3H/HeN or C3H/HeJ mice and cultured for 72h. The supernatants were collected and cytokines were determined using ELISA. In some cases after 48h cells were again purified using specific MACS beads and RNA was isolated as described above for QReal-time PCR analysis.

Suppressive activity of regulatory T cells

Regulatory T cells ($CD4^+CD25^+$) and effector ($CD4^+CD25^-$) T cells were isolated using regulatory T-cell isolation kit (Miltenyi Biotech) according to the manufacturer's protocol. In order to assess the suppressive activity of $CD4^+CD25^+$ T cells, $CD8^+$ T cells were incubated in presence of plate bound anti-CD3*e* and anti-CD28 antibody (25μg/ml; 30μl/well) with $CD4^+CD25^+$ cells in a ratio of 1:0, 1:1 and 1:2. After 48h cells were purified and RNA isolated to determine the suppressive activity of $CD4^+CD25^+$ cells on CD8+ cells through the production of perforin, granzyme and IFN-γ.

ELISA—Cytokines IL-17, IFN-γ, IL-23p19, IL-12p70 and IL-12p40 were measured by ELISA as described earlier (10) using antibodies from BD Pharmingen (San Diego, CA). TGF-β cytokine was measured using ELISA kit from Invitrogen (Carlsbad, CA) according to manufacturer's instructions.

Immunohistochemical analysis

Paraffin-embedded sections were deparaffinized and stained with the specific primary antibody followed by 3,3-diaminobenzidine (DAB) staining as described earlier [26]. Sections were subjected to antigen retrieval and blocking of endogenous peroxidase activity. For immunostaining, sections were incubated with biotin labeled anti-CD31 antibody (BD Pharmingen, San Diego, CA), or rabbit polyclonal anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA). They were then incubated with the appropriate biotinylated secondary antibody followed by conjugated horseradish peroxidase (HRP)-streptavidin (Abcam, Cambridge, MA) followed by incubation with 3,3'-diaminobenzidine (BD Biosciences, San Diego, CA) working solution at room temperature, sections were counterstained with diluted Harris hematoxylin (Sigma Chemicals, St. Louis, MO). In all immunohistochemical staining, negative staining controls were used to rule out any nonspecific staining.

Statistical analysis—The differences between experimental groups were analyzed using the Student's t-test. The Fisher exact test was employed for analysis of the tumors per mice and for percent tumor free mice. In all cases, a $p<0.05$ was considered significant.

Results

TLR4 deficient mice are more susceptible to mouse mammary tumor development induced by DMBA

The role of TLR4 in DMBA induced mammary carcinogenesis was investigated by comparing C3H/HeN and C3H/HeJ mice for tumor development after feeding the mice by oral gavage with DMBA for 5 weeks. C3H/HeN mice have normal TLR4 signaling; C3H/HeJ mice have a mutant TLR4 gene which renders them deficient in TLR4 signaling (27). The number of tumors per mouse was significantly greater in C3H/HeJ mice than in C3H/HeN mice [Fig. 1A]. Also, at the end of the experiment the percent tumor free mouse in the C3H/HeJ group was significantly lower than in the C3H/HeN group. By week 7, C3H/HeJ mice had developed tumors whereas in C3H/HeN mice tumors did not begin to develop until week 13; these differences were statistically significant [Fig. 1B]. When examined histologically, most of the tumors were squamous carcinomas and adenosquamous carcinomas. However, there was no difference in the proportions of the type of tumors in the two groups.

TLR4 deficient mice express higher levels of IL-17 and lower levels of IFN-γ in DMBA induced mammary carcinogenesis

In order to determine the type of T cells activated after DMBA treatment, we gavaged mice with DMBA and after 6 days spleens of those mice were harvested. T cells were purified and co-cultured with CD11c+ cells from cultures from naïve syngenic mice. C3H/HeJ mice produced higher levels of IL-17 than those from C3H/HeN mice[Fig. 2A (i)]. Further purification showed that more IL-17 was produced by CD4+ T cells, although in C3H/HeJ mice CD8+ T cells also produced significant amounts of this cytokine [Fig. 2A (ii)]. Steps were then taken to determine the composition of the inflammatory infiltrate in the two strains of mice. Tumor samples were collected from C3H/HeJ and C3H/HeN mice after induction of mammary tumors and RNA was isolated. Tumor samples from C3H/HeJ mice showed a significant increase in IL-17 mRNA levels compared to the C3H/HeN mice [Fig. 2A (iii)].

When the T-cells were analyzed for IFN-γ, we found higher levels of IFN-γ in C3H/HeN mice than C3H/HeJ mice [Fig. 2B (i)]. Both CD4+ and CD8+ cells produced IFN-γ but higher levels of IFN-γ than CD4+ cells [Fig. 2B (ii)]. When the tumors were analyzed for IFN-γ, higher levels were seen in C3H/HeN mice compared to C3H/HeJ mice as observed by real time and RT-PCR data of IFN-γ [Fig. 2B (iii)].

TLR4 deficient mice express higher levels of IL-23 and lower levels of IL-12 after DMBA induced mammary carcinogenesis

Since TLR4 deficient C3H/HeJ mice had higher levels of IL-17 in their tumors and C3H/HeN mice had greater amounts of IFN-γ, we were interested in determining the levels of IL-12 and IL-23 in the tumors. We found higher levels of IL-23p19 in the tumors of C3H/HeJ mice than in C3H/HeN mice. In contrast, there were greater levels of IL-12p35 and IL-12p40 in tumors of C3H/HeN mice than C3H/HeJ mice (Fig. 3A (i–iii)]. Additional experiments were performed in which CD11c+ cells from these mice were treated *in vitro* and found higher IL-12p40 and IL-23p19 levels in C3H/HeJ than C3H/HeN mice and IL-12p70 was found higher in C3H.HeN mice further confirming our results (Data not shown). Furthermore, when bone marrow derived dendritic cells were treated with DMBA, C3H/HeJ mice secreted smaller amounts of IL-12 ($p<0.05$) and more IL-23 ($p<0.05$) than C3H/HeN mice (Data not shown). Addition of anti-IL-12p70 antibody in co-culture of T cells and CD11c+ cells significantly inhibited the production of IFN-γ [Fig. 3B (i)], whereas addition of anti-IL-23p19 antibody significantly inhibited the production of IL-17 in culture [Fig. 3B (ii)].

TLR4 deficiency leads to increase in suppressive activity by regulatory T cells

IL-17-producing cells (T_H-17 cells) represent a T helper lineage that requires transforming growth factor-β (TGF-β) and IL-6 for differentiation and IL-23 for maintenance. We were therefore also interested in determining whether there was enhanced expression of these cytokines in tumors of C3H/HeJ mice. IL-6 also inhibits TGF-β-driven Foxp3 expression and together with TGF-β induces T_H-17 cells [28, 29]. We found that T-cells from C3H/HeJ mice produced higher levels of TGF-β than C3H/HeN mice. Further IL-6 levels were higher in T-cells from C3H/HeJ mice compared to C3H/HeN mice (Data not shown). We also observed that C3H/HeJ mice had higher levels of TGF-β and IL-6 in their tumors as compared to C3H/HeN mice (Data not shown) consistent with the hypothesis that the differential expression of IL-17 producing cells might be due to alterations in TGF-β expression.

Since TGF-β was higher in C3H/HeJ mice we investigated whether there is higher activity of regulatory T-cells as well. IL-10 and TGF- β levels were higher in CD4+CD25+ T cells

of C3H/HeJ mice as compared to C3H/HeN mice [Fig. 4 A&B]. To further estimate the levels of regulatory cells produced, we analyzed the levels of Foxp3 and CD25 in CD4+CD25+ cells and found higher levels of these two markers of regulatory T cells in C3H/HeJ mice [Fig. 4C]. Moreover the levels were also high in tumors of C3H/HeJ mice as compared to C3H/HeN mice [Fig. 4D]. Next, we investigated whether these regulatory cells were potent in their suppression, so we co-cultured these with CD8+ cells in increasing concentration. These cells were then purified and analyzed for IFN-γ, granzyme and perforin. We found that T regulatory cells from C3H/HeJ mice significantly inhibited CD8+ cells [Fig. 4E].

TLR4 deficiency promotes tumor angiogenesis and proliferation

IL-17 production has been linked to tumor angiogenesis and enhanced tumor growth [10]. Because TLR4 deficient C3H/HeJ mice had more tumors and significantly greater amounts of IL-17 mRNA, experiments were conducted to determine whether there were differences with respect to angiogenesis in these strains. CD31 is an important marker for angiogenesis and is known to contribute to the formation of new vasculature [30]. Angiogenic factors, such as matrix metalloproteinases (MMPs) and vascular endothelial growth factors (VEGFs), are important regulators of tumor growth, both at the primary tumor site and at sites of distant metastasis [31]. We found higher levels of MMP-2 and MMP-9 in tumors of C3H/HeJ mice compared to C3H/HeN mice. We further stained tumor sections for MMP-2 and MMP-9 and observed higher staining of MMP-2 in C3H/HeJ mice than in C3H/HeN mice. Similar results were seen when tumors were stained with MMP-9 [Fig. 5A]. Next, we stained the sections for angiogenesis markers CD31, VEGF and proliferation marker PCNA. The intensity of these markers was higher in mammary tumors from C3H/HeJ mice compared to C3H/HeN mice [Fig 5B]. Real-time PCR data also showed higher levels of mRNA for CD31, VEGF and PCNA in C3H/HeJ mice tumors compared to tumors from C3H/HeN mice [Fig. 5B]. These results indicate that TLR4 deficiency promotes tumor angiogenesis and proliferation in this model.

Discussion

Toll-like receptors (TLRs) have emerged as a key component of the innate immune system. They contribute to host defenses by identifying invading pathogens and activating potentially beneficial antimicrobial inflammatory and adaptive immune responses [32]. TLR4 was initially investigated because it recognizes lipopolysaccharide present in the cell wall of gram negative bacteria. Recent studies have shown that a variety of exogenous nonbacterial agonists such as taxol [33, 34] and fibronectin [35, 36] and some endogenous proteins, including heat shock proteins 27 and 60 [37] also initiate TLR4 signaling, suggesting that TLR4 is important for non-infectious diseases as well. TLR4 is also associated with signaling by many environmental chemicals, many of which are tumorigenic [38]. In this study, we evaluated the role of TLR4 in DMBA-induced mammary tumorigenesis. We compared C3H/HeN mice that have normal TLR4 function with C3H/HeJ mice that have a mutation in TLR4 which renders the receptor functionally deficient. This is the only known difference between C3H/HeN and C3H/HeJ mice, and, as a result, the two strains have been used extensively to investigate the biological effects of TLR4 [39–42]. We found that mice with deficient TLR4 function were more susceptible to DMBA-induced mammary tumor development than mice in which TLR4 function was normal.

The role that TLR4 plays in cancer has been examined in other systems. These results indicate that increased susceptibility to the carcinogenic effects of DMBA in TLR4 deficient animals is not restricted to one organ system. Similar observations have been made in a two-stage chemical lung carcinogenesis model as well. Bauer et al. reported that the presence of a functional TLR4 signaling pathway inhibited the occurrence of lung cancer in mice [43].

However, the complexity of the role of TLR4 is illustrated by its effect in other tumor systems, in which TLR4 activation has been shown to be a risk factor for tumors. There are some studies which evaluate the role of cellular immunity against different cancers. In a model of lung cancer, Ehrlich et al. have reported that the cytostatic ability of splenocytes from mice bearing DMBA-induced tumors was significantly elevated. The spleens of mice bearing these tumors also contained cells able to suppress NK activity or to compete against target cells for NK cells [44]. In another model of rat pancreatic, and colon cancer, Stevens et al. have used anti-tumor cell-mediated immunity as a measure of exposure to chemical carcinogens. Their findings suggested that the anti-tumor immunity is specific to carcinogens which interact with entodermally derived tissue [45].

The difference in mammary tumorigenesis in the two strains was associated with differences in their inflammatory infiltrates. In C3H/HeN mice, T-cells that produced IFN-γ were more common, whereas in C3H/HeJ mice, cells that produced IL-17 were the predominant infiltrating T-cell type. IL-17 has been shown to increase the growth of several different types of tumors [46–49]. It has been proposed that this is due at least in part to its pro-angiogenic effects [49].

In mice, it has been reported that both TGF-β and IL-6 are required for the generation of IL-17 producing T-cells [50, 51]. We observed that in DMBA-induced mammary tumors greater amounts of TGF-β and IL-6 were produced in C3H/HeJ mice which had greater numbers of IL-17 producing cells. It is noteworthy that Nam et al. found using a transplantable model of breast carcinoma that IL-17 producing $CD8^+$ T-cells promoted breast tumor development through a TGF-β dependent mechanism. Thus, our observations provide additional support for the finding that one mechanism by which TGF-β and IL-6 augment mammary tumor development is through their capacity to influence the type of T-cell T_H1 vs T_H17 that develops. We have previously demonstrated in a model of DMBA induced skin cancer that DMBA induces DMBA specific CD8 T effector cells [52]. Apart from this $CD4^+CD25^+$ regulatory T cells are known negative regulators of T cell immune responses *in vitro* and *in vivo* (53). T regulatory cells can suppress the proliferation of both $CD4^+$ and $CD8^+$ cells in co-culture experiments (53). In this study, we found higher levels of CD4+CD25+Foxp3+ cells in C3H/HeJ mice than in C3H/HeN mice and these cells produced higher levels of TGF-β and IL-10 upon DMBA treatment. We also found they were potent suppressors of CD8+ T-cells to produce IFN-γ, perforin or granzyme.

One of the mechanisms involved in regulatory T-cell suppressive activity is through production of immunosuppressive cytokines TGF-β and IL-10. The significance of these observations can be used in vaccination procedures by stimulating TLR4 dependant pathways as an adjuvant in order to enhance the polarity of T-cells to CD8+ cells and for the production of higher levels of IFN-γ and hence can be able to prevent carcinogen-induced tumor development.

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Abbreviations

DMBA	7–12-dimethylbenz(a)anthracene
TLR4	toll-like receptor-4
TGF-β	transforming growth factor-beta

VEGF	vascular endothelial growth factor
MMP	matrix metalloproteinases

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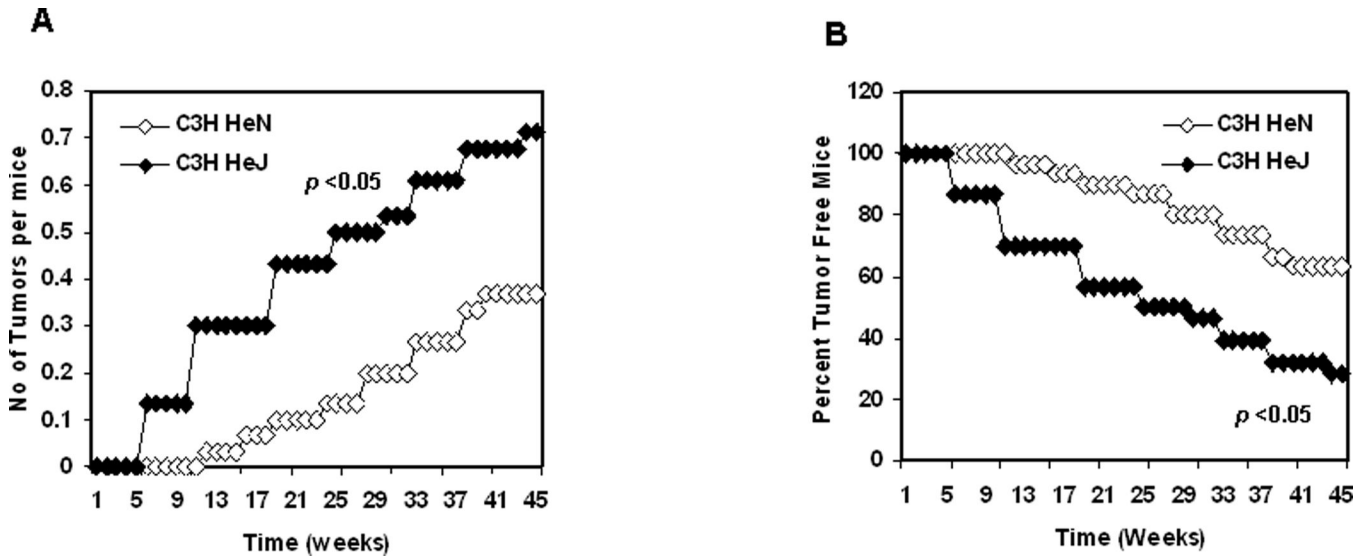
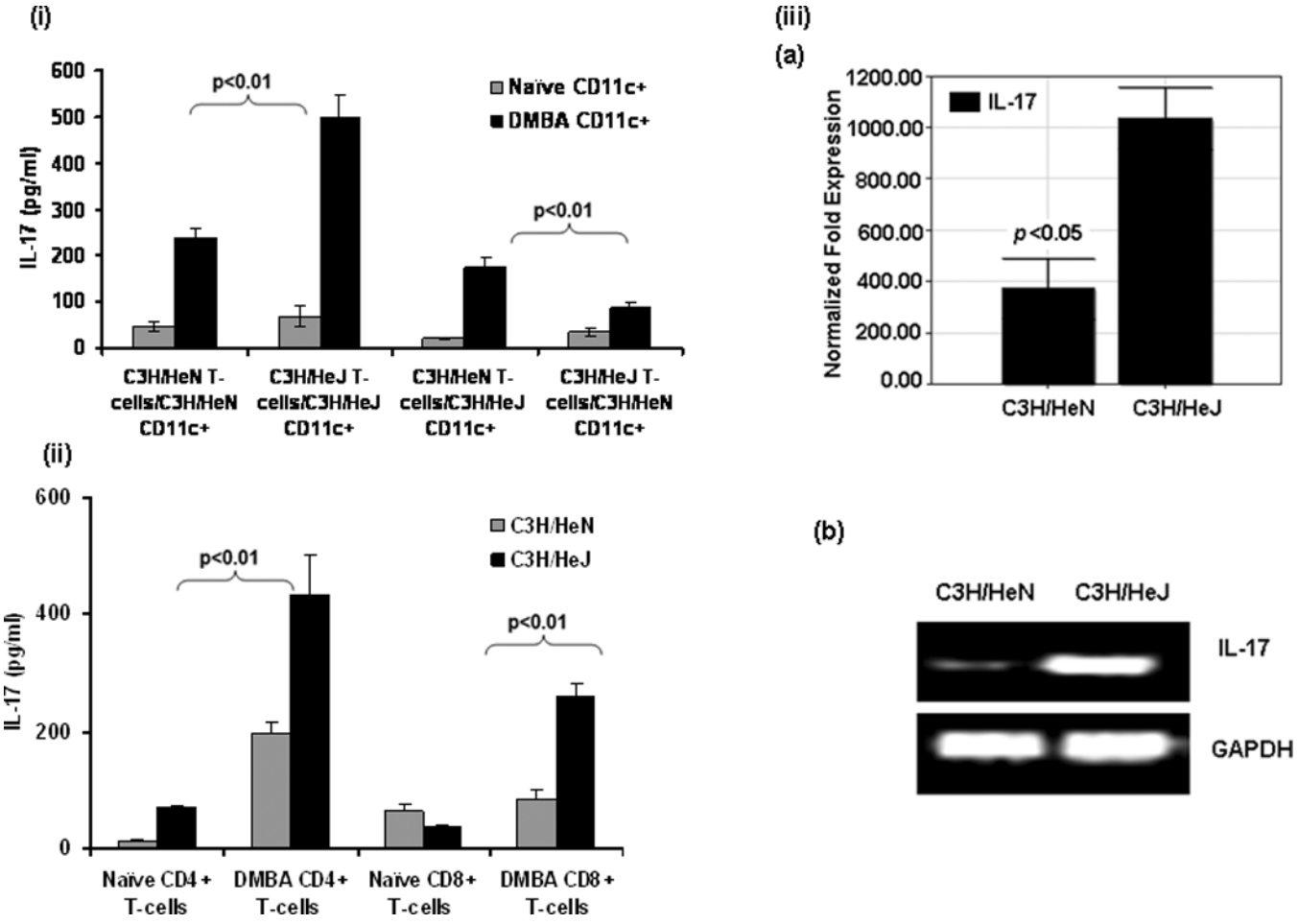


Figure 1.

TLR4 deficient mice are more susceptible to DMBA induced mammary carcinogenesis. TLR4 deficient C3H/HeJ and TLR4 competent C3H/HeN mice were subjected to a mammary carcinogenesis protocol using DMBA as described in the Methods section. (A) The number of tumors per mouse was plotted as a function of the number of weeks on the test. There were significantly fewer tumors in C3H/HeN mice ($p<0.05$) than in C3H/HeJ mice. (B) The percentage of tumor free mice was plotted as a function of the number of weeks of the test. There were significantly more tumor free mice in C3H/HeN group ($p<0.01$) than in C3H/HeJ group.



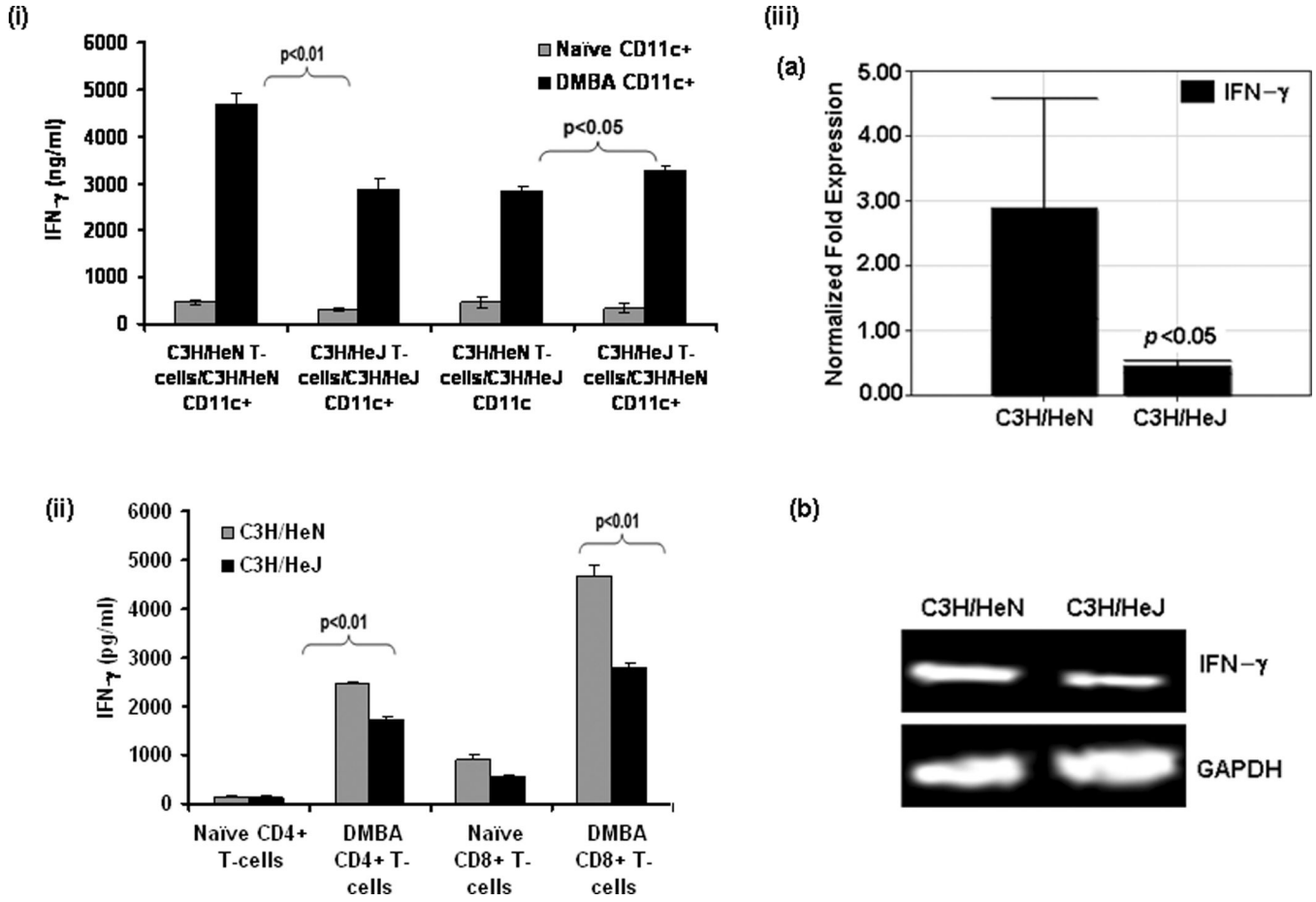


Figure 2.

(A) TLR4 competent C3H/HeN mice produce lower levels of IL-17 than TLR4 deficient C3H/HeJ mice. Mice were fed once with DMBA by oral gavage, and on day 6 mice were sacrificed and spleens were removed. T cells, T-cell subsets (CD4+ or CD8+ T cells), or CD11c+ cells were purified from spleen as described in the Methods section. T cells/T cell subsets were incubated with CD11c+ cells for 72h and IL-17 levels were determined in the supernatant by ELISA. (i) T cells and CD11c+ cells were isolated from spleen cells as described in the Methods section. (ii) CD4+ T cells produced more IL-17 (p<0.01) than CD8+ T cells. CD4+ as well as CD8+ T cells from C3H/HeJ mice produced more IL-17 (p<0.01) than CD4+ and CD8+ T cells from C3H/HeN mice. (iii) C3H/HeJ mice showed elevated levels of IL-17(p<0.05) in their tumors. IL-17 levels were measured in tumors excised from the mice of both groups using (a) Quantitative Real-time PCR and (b) RT-PCR.

(B) TLR4 competent C3H/HeN mice produce higher levels of IFN-γ than TLR4 deficient C3H/HeJ mice. Mice were fed once with DMBA by oral gavage, and on day 6 mice were sacrificed and spleens were removed. T cells, T-cell subsets (CD4+ or CD8+ T cells), or CD11c+ cells were purified from spleen as described in the Methods section. T cells/T cell subsets were incubated with CD11c+ cells for 72h and IFN-γ levels were determined in the supernatant by ELISA. (i) T cells and CD11c+ cells were isolated from spleen cells. (ii) CD4+ T cells produced less IFN-γ (p<0.01) than CD8+ T cells. CD4+ as well as CD8+ T cells produced from C3H/HeJ mice produced less IFN-γ (p<0.01) than CD4+ and CD8+ T cells from C3H/HeN mice. (iii) C3H/HeN mice showed elevated levels of IFN-γ (p<0.05) in

their tumors. IFN-γ levels were measured in tumors excised from the mice of both groups using (a) Quantitative Real-time PCR and (b) RT-PCR.

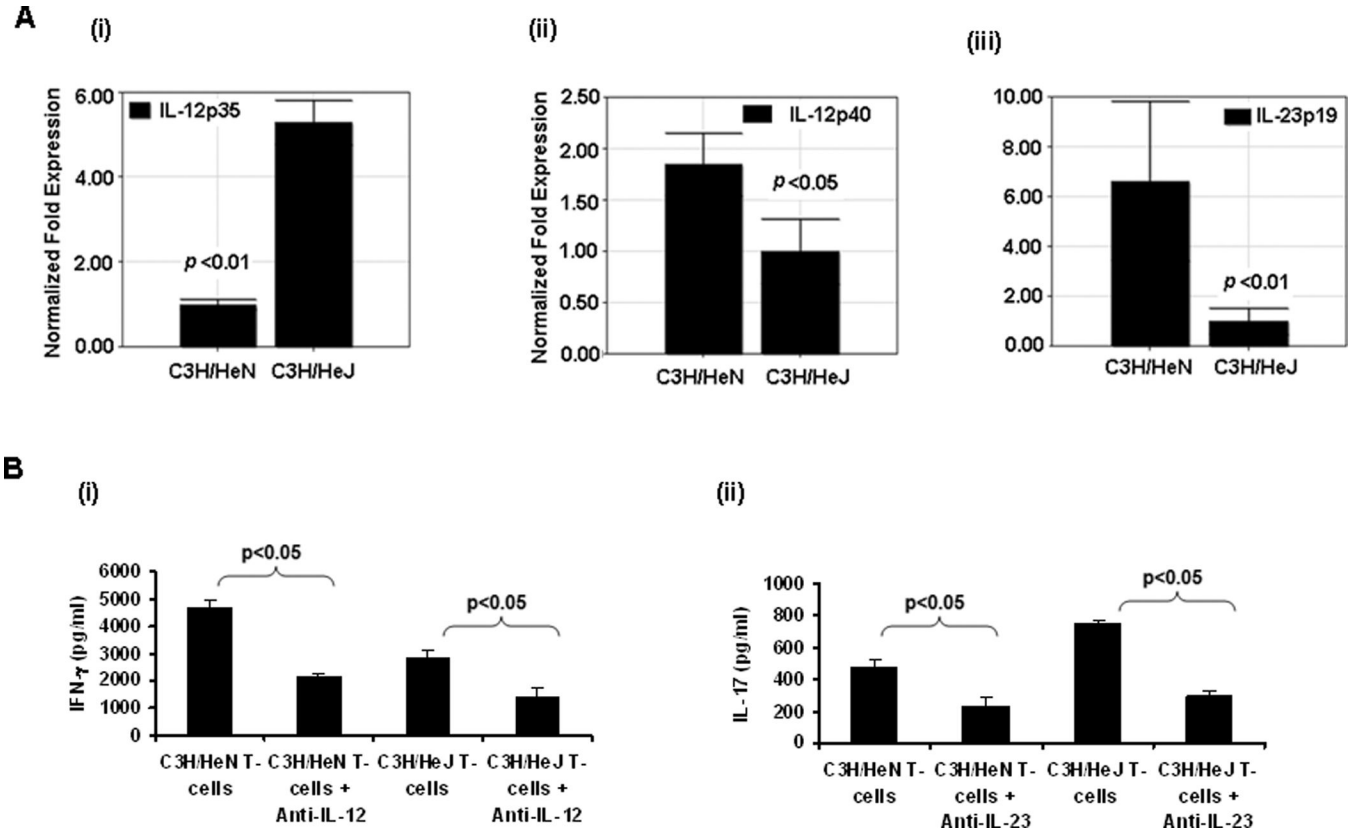


Figure 3.

(A) TLR4 deficient mice C3H/HeJ mice express lower levels of (i) IL-12p35 (p<0.01), and higher levels of (ii) IL-12p40 (p<0.05) as well as (iii) IL-23p19 (p<0.01) in their tumors compared to C3H HeN mice as determined by quantitative real-time PCR. (B) Mice were fed once with DMBA by oral gavage, and on day 6 mice were sacrificed and spleens were removed. T cells, or CD11c+ cells were purified from spleen as described in the Methods section. T cells were incubated with CD11c+ cells for 72h and IFN-γ and IL-17 levels were determined in the supernatant by ELISA. (i) Addition of anti-IL-12p70 antibody to cultures significantly inhibits the production of IFN-γ (p<0.05), and (ii) addition of anti-Il-23p19 antibody to cultures significantly inhibits the production of IL-17 (p<0.05).

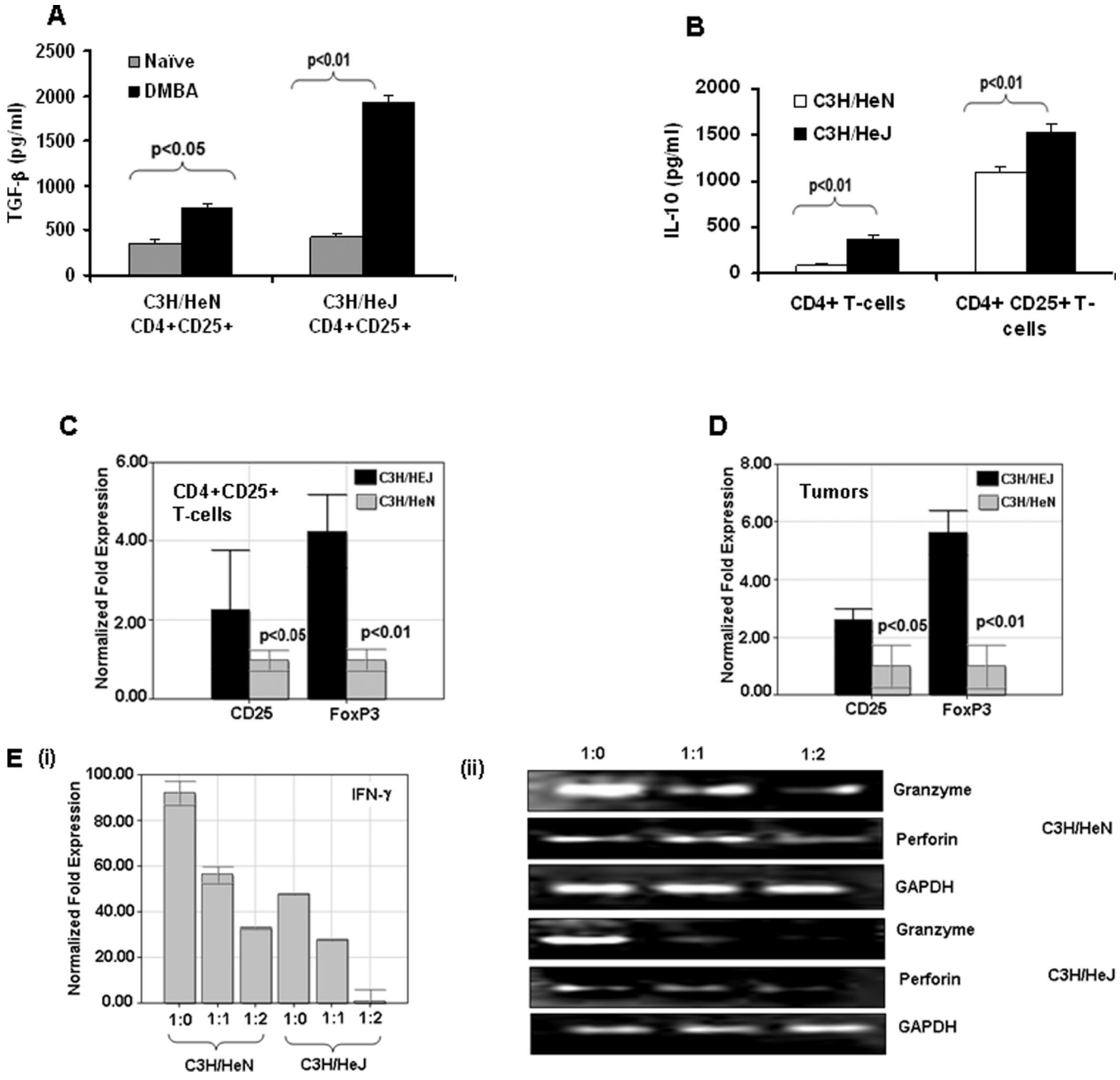
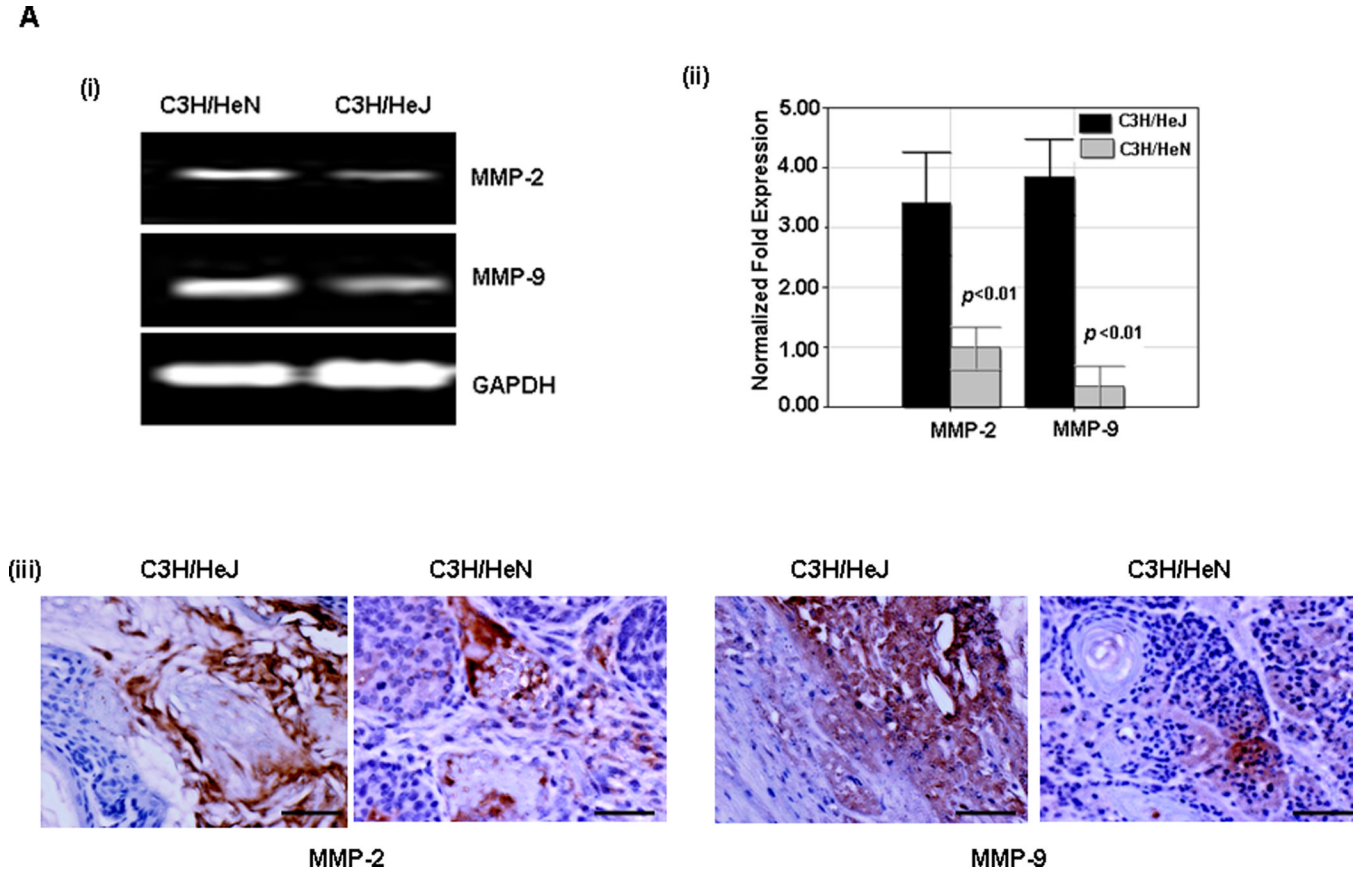


Figure 4.

(A) CD4+CD25+ T-regulatory cells from TLR4 deficient C3H/HeJ mice produce more TGF-β ($p<0.01$) and (B) IL-10 ($p<0.01$) compared to T-regulatory cells from TLR4 competent C3H/HeN mice. Mice were fed once with DMBA by oral gavage. On day 6, mice were sacrificed and spleens were removed. CD4+ or CD4+CD25+ T cells, were purified from spleen and stimulated with anti-CD3/anti-CD28 antibody as described in the Methods section. (C) CD4+ T-cells of TLR4 deficient C3H/HeJ mice expressed higher levels of Foxp3 ($p<0.01$) and CD25 ($p<0.05$) compared to TLR4 competent C3H/HeN mice. CD4+CD25+ T cells were purified after gavage with DMBA as described in the Methods section and incubated for 48h. RNA was isolated from cells as described in the Methods section. (D) Tumors of TLR4 deficient C3H/HeJ mice express higher levels of Foxp3

($p<0.01$) and CD25 ($p<0.05$) compared to tumors of TLR4 competent C3H/HeN mice. (E) T-regulatory cells from TLR4 deficient C3H/HeJ mice showed an increase suppressive activity compared to TLR4 competent C3H/HeN mice. CD8+ T cells were purified and incubated with CD4+CD25+ T cells in the ratio of 1:0, 1:1 and 1:2. After 48h cells CD8+ cells were purified and RNA was isolated. (i) IFN-γ levels were estimated by quantitative real-time PCR and (ii) granzyme and perforin levels were assessed using RT-PCR.



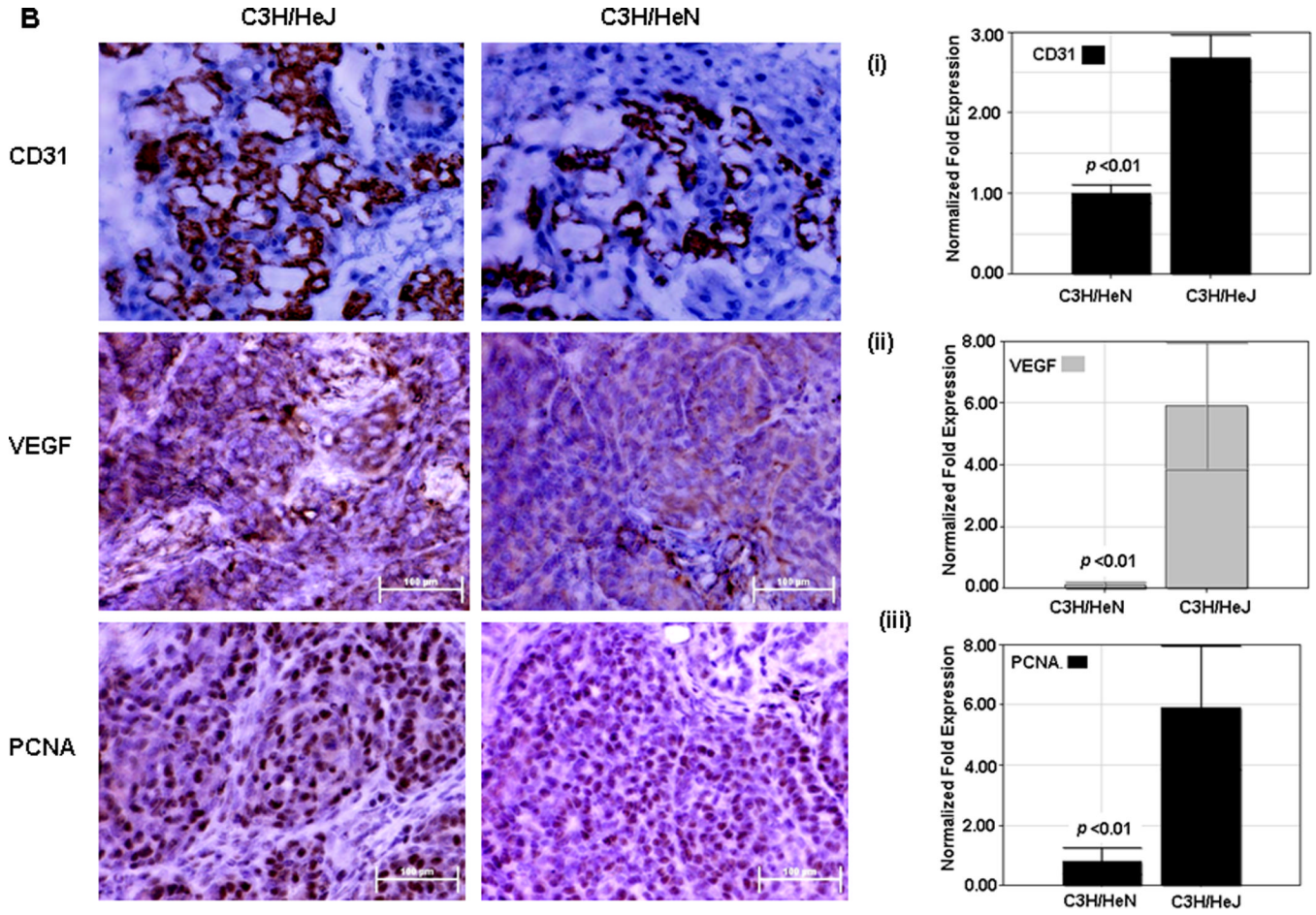


Figure 5.

TLR4 deficient C3H/HeJ mice are more prone to angiogenesis compared to TLR4 competent C3H/HeN mice. (A) MMP-2 and MMP-9 levels were higher in tumors of C3H/HeJ mice compared to C3H/HeN mice as determined by (i) RT- PCR and (ii) Quantitative Real-time PCR (p<0.01). (iii) Similar results were obtained when the tumor sections were stained for MMP-2 and MMP-9 by DAB. The staining for MMP-2 and MMP-9 was of higher intensity in C3H/HeJ mice than in C3H/HeN mice. Bar=100μM. (B) The tumors were also stained for angiogenic factors VEGF, and CD31, and the proliferation marker PCNA by immunostaining as described in Methods section. C3H/HeJ mice showed higher levels of VEGF, CD31 and PCNA in their tumors compared to C3H/HeN mice. Representative examples of micrographs of staining from C3H/HeJ and C3H/HeN mice from experiments conducted in tumor samples from at least three mice showed identical patterns. Bar=100μM. (E) Quantitative Real-time PCR also showed higher levels of mRNA expression of CD31 (p<0.01) (i), VEGF (p<0.01) (ii), and PCNA (p<0.01) (iii) in tumors of C3H/HeJ mice as compared to C3H/HeN mice.