封面格式

行政院及所屬各機關出國報告

（出國類別： 研 究 ）

分子胎兒醫學

服務機關：成大醫學院 婦產科

出國人職稱：主治醫師

姓名：許耿福

出國地區：美國

出國期間：88 年 6 月 30 號至 89 年 6 月 22 號

報告日期：89 年 11 月 12 號

系統識別號:C08903504

公 務 出 國 報 告 提 要

頁數: 10　含附件: 否

報告名稱:

分子胎兒醫學

主辦機關:

國立成功大學醫學院附設醫院

聯絡人／電話:

陳秀梅／06-2353535轉2049

出國人員:

許耿福　國立成功大學醫學院附設醫院　主治醫師

出國類別:　研究

出國地區:　美國

出國期間:　民國 88 年 06 月 30 日 民國 89 年 06 月 22 日

報告日期:　民國 90 年 2 月 1 日

分類號/目:　J2／西醫　J2／西醫

關鍵詞:　FLT3-Ligand gene, Vaccine, naked DNA

內容摘要:

DNA 疫苗爲目前最新型的抗癌疫苗. 利用基因槍的方式可以把子宮頸癌有關的人類乳突病毒(HPV)E7 的蛋白質直接送入人體內. 在動物實驗方面, 把 HPV E7 接連 Flt3 這種 cytokine 的 DNA 後 送入老鼠體內. 不僅可以增加動物體內特殊抗 E7 T 細胞的量, 同時可以達到抑制攜帶 E7 的抗腫瘤細胞的擴散. 因此, 可以考慮將來以此種疫苗作人類的第一階段臨床試驗.

目次

内容 頁數

封面……………………………………………………………………0

書名頁…………………………………………………………………1

摘要……………………………………………………………………2

目次……………………………………………………………………3

正文……………………………………………………………………4

附件一(行政院所屬各機關出國報告審核表)

附件二(藉由連結抗原基因到 Flt3-Ligand 基因上以提高 DNA 疫苗的效力, I)

附件三(藉由連結抗原基因到 Flt3-Ligand 基因上以提高 DNA 疫苗的效力, II)

附件四(Enhancement of DNA Vaccine Potency by Linkage Gene to a Gene Encoding the Extracellular Domain of Flt3-Ligand)

封底

使用連接抗原基因到 Flt3-Ligand 基因上進而提高 DNA 疫苗的效力

目的:

核酸疫苗由於其穩定性和簡便, 在產生特殊抗原免疫中扮演著引人注目的角色, 但仍然需要提高其 DNA 疫苗的效力. 近年來, 在特定的抗原呈現細胞中, 尤其是在樹狀突細胞裡, Flt3-ligand 已經被鑒定出是一種重要的組織介素. 在一重組分子連接 Flt3-ligand (FL) 到抗原的模型上時, 可能其目標是將抗原連結到樹狀突細胞上. 使用 HPV-16E7 爲一抗原模型, 利用基因槍的方式把子宮頸癌有關的人類乳突病毒 (HPV-E7) 的蛋白質直接送入人體內. 在動物實驗方面, 把 HPV-16E7 接連 Flt3 這種組織介素的 DNA 後, 送入老鼠體內, 不僅可以增加動物體內特殊抗 E-7 T 細胞的量, 同時可以達到抑制攜帶 E7 的腫瘤細胞的擴散. 因此可以考慮將來以此種疫苗做人類第一階段臨床試驗.

過程: 學習分子醫學的技術及動物實驗的方法, 藉以了解腫瘤細胞的特性及 DNA 疫苗的潛力,以為將來於臨床治療病人

心得:

特殊抗原癌細胞之免疫治療上最近出現了新治療方法來控制癌細胞因爲它能夠在不破壞正常細胞的情況下發展出特殊的抗腫瘤細胞. 許多的証明顯示, 建議 professional antigen presenting cells (APCs), , 尤其是在樹狀突細胞 (DCs), 是腫瘤細胞免疫治療中期的主要中間角色. 一有效的疫苗大部分可能需要抗原目標到 professional APCs 使特殊抗原 T 細胞活化.

近年來, DNA 疫苗已經成爲特殊抗原之免疫治療上所引人注目之方法. Naked DNA 是安全的, 它具低免疫性, 而且可以被重複執行. 另外, DNA 疫苗容易被大量製備, 花費低但製備出來的純度極高, 而且比蛋白質來得穩定 [Robinson, 1995 #2917; Pardoll, 1995 #2830; Donnelly, 1997 #2242]. 我們所關心之一的, 是 DNA 疫苗的發展潛力. 由於它們沒有其故有的能力像病毒疫苗在 vivo 中擴展. 我們推論, DNA 疫苗爲一抗原融合暗碼, 可以直接被誘導至其免疫位置上, 就像樹狀突細胞 (DCs)可能提高疫苗的潛力 (Boyle et al., 1998). GM-CSF 爲其中之一種分子在 DNA 疫苗具發展潛力. 例如, 融合 GM-CSF 和 DNA 疫苗裡的抗原可被引導增加免疫反應, 保護對抗 HIV [Lee, 1999#11] 和 C 型肝炎 [Lee, 1998#22]. 雖然這種特殊的作用過程對增加 DNA 疫苗潛力並不清楚, 但它被相信 chimeric GM-CSF/ antigen 也許扮演著在 DCs 上的一個 immunostimulatory signal. 自從 DCs 和它們的前導細胞解釋了 GM-CSF 的高層接受者 (GM-CSFR), chimeric GM-CSF/ antigen 爲目標並專注 DCs 的抗原連結和更多促進 DNA 疫苗潛力. 另一種重要的分子在 DCs 上也具有成長-刺激的影響, 並在 vivo 中能夠產生大量的 DCs 是 Flt3-Ligand [Maraskovsky, 1996 #45; Shurin, 1997#230]. Flt3-Ligand 一直出現, 就像一個重要的分子給發展中的腫瘤疫苗, 增加了在 vivo 中 DCs 的功能和數量. Flt3 (fms-like tyrosine kinase3), 是一 murine tyrosine kinase 的接受者, 在 1991 年首先被描述出來 [Matthews, 1991 #237; Rosnet, 1991 #238] 並且被發現和 c-kit 和 c-fms 接受者爲同一族之成員, 是爲第三型 receptor

kinase family (for review see [Lyman, 1998#319]). 在造血組織中, flt3 視爲被受限制的至 CD34- positive progenitors 上. flt3 已經被使用在確認和後來 corresponding ligand 的無性繁殖, Flt3-L [Lyman, 1993 #240; Hannum, 1994 #239]. Flt3 卓越的型式被合成像是 transmembrane 蛋白質. 這些蛋白質功能藉由繫連到催化 tyrosine kinase receptors. flt3 接受者的解釋是初級受限制, 在造血細胞之間, 到大部分自然的原始細胞, 包括樹狀突前導細胞. 至今, flt3-ligand 還未被使用在 chimeric DNA 疫苗中.

我們選擇 HPV-16E7 爲一疫苗發展的抗原模型, 因爲 HPVs, 尤其是 HPV-16, 和大部分子宮頸癌有關連. HPV oncogenic 蛋白質, E6 和 E7 在大部分 HPV 子宮頸癌中是重要的誘導, 和持續細胞的轉移. 疫苗或 immunotherapies targeting E7 和/ 或 E6 蛋白質可提供機會以避免及處理 HPV 有關之子宮頸惡性腫瘤. 在我們近年來的研究, 我們調查是否基因 E7 全長連結到 flt3-ligand 可以提高疫苗的潛力. 我們比較 DNA 疫苗包含 wild-type HPV-16E7 with DNA 疫苗包含 E7 全長融合到 flt3-ligand 以提供它們免疫反應的發生和保護動物對抗 HPV-16E7-expressing murine tumors [Lin, 1996 #37]的能力

建議:

無

Enhancement of DNA Vaccine Potency By Linkage of Antigen Gene to an FLT3-ligand Gene

*Chien-Fu Hung[1], *Keng-Fu Hsu[1,5], Wen-Fang Cheng[1,6] Chee-Yin Chai[1,7], Liang-May He[1], Drew M. Pardoll[2] and T.-C. Wu[1,2,3,4]

Departments of Pathology[1], Oncology[2], Obstetrics and Gynecology[3], and Molecular Microbiology and Immunology[4], The Johns Hopkins Medical Institutions, Baltimore, Maryland 21287, USA; Department of Obstetrics and Gynecology[5], National Cheng Kung University Hospital, Tainan, Taiwan; Department of Obstetrics and Gynecology[6], National Taiwan University Hospital, National Taiwan University, Taipei, Taiwan; Department of Pathology[7], School of medicine, Kaohsiung Medical University, Kaohsiung, Taiwan.

Address correspondence and reprint requests to: Dr. T.-C. Wu, Department of Pathology, the Johns Hopkins Hospital, 600 North Wolfe Street, Baltimore, Maryland 21205. Tel: (410) 614-3899. Fax: (410) 614-3548. Email: wutc@welchlink.welch.jhu.edu

* Equal contribution to this article

Running title: Tumor immunotherapy with FLT3-ligand DNA vaccine

Introduction:

The discovery of distinct antigenic feature of neoplastic cells has suggested that it may be possible to create antitumor vaccine. [Rosenberg, 1999 #20] Recently, DNA vaccines have become an attractive approach for generating antigen-specific immunotherapy. The naked plasmid DNA is safe, has low immunogenicity, and can be repeatedly administered. Also, DNA vaccines can be easily prepared in large scale with high purity and are highly stable relative to proteins and reduced cost [Robinson, 1995 #2917; Pardoll, 1995 #2830; Donnelly, 1997 #2242].

The flt3 ligand (FL) belongs to the small family of hematopoietic cytokins, including also stem factor(SCF) and macrophage colony-stimulating factor (M-CSF), that are specific for tyrosine kinase receptors.[Hannum, 1994 #349; Lyman, 1993 #355] It also increase the yield of dendritic cells generated from hematopoietic precursor. [Maraskovsky, 1996 #273; Strobl, 1997 #231] , and effectivly enhances antitumor immune responses in mice[Lynch, 1997 #243] .However, so far, all studies were focus on the Flt3–ligand administration by protein format not in the DNA format. Here ,

we show that by fusing the Flt3-ligand to E7 deliveried by gen gun, it can produce a strong antitumor effect in murine tumor model.

We chose HPV-16 E7 as a model antigen for vaccine development because HPVs, particularly HPV-16, are associated with most cervical cancers. HPV oncogenic proteins, E6 and E7, are co-expressed in most HPV-containing cervical cancers and are important in the induction and maintenance of cellular transformation. Therefore, vaccines or immunotherapies targeting E7 and/or E6 proteins may provide an opportunity to prevent and treat HPV-associated cervical malignancies. HPV-16 E7 is a well-characterized cytoplasmic/nuclear protein that is more conserved than E6 in HPV-associated cancer cells [Wu, 1994 #7]. In our current study, we investigated whether genes linking full-length E7 to Flt3-ligand can enhance the potency of DNA vaccines. We compared DNA vaccines containing wild type HPV-16 E7 with DNA vaccines containing full-length E7 fused to Flt3-ligand for their immune response generation and their ability to protect animals against the HPV-16 E7-expressing murine tumors. We show that linkage of E7 to Flt3-ligand dramatically increases expansion and activation of E7-specific CD8+ T cells, completely bypassing the CD4 arm. In addition, FL-E7 generated robust CTL activity by both direct and cross priming in vitro. This enhanced CD8 response results in potent antitumor immunity against established tumors.

Summary

Using HPV-16 E7 as a model antigen, we evaluated the effect of linkage to Flt3-ligand on the potency of antigen-specific immunity generated by naked DNA vaccines. We found that vaccines containing Flt3-ligand fusion genes

increased the frequency of E7-specific CD8+ T cells relative to vaccines containing the wild type E7 gene. More importantly, this fusion converted a less effective vaccine into one with significant potency against established E7-expressing tumors. Surprisingly, Flt3-ligand fusion vaccines exclusively targeted CD8+ T cells; immunologic and antitumor effects were completely CD4 independent. FL-E7 generated robust CTL activity by both direct and cross priming in vitro. These results indicate that fusion of Flt-ligand to an antigen gene may greatly enhance the potency of DNA vaccines via direct and cross priming CD8 dependent pathways.

RESULTS

Vaccination with FL-E7 Fusion DNA Enhances E7-Specific CD8^{+} T Cell-Mediated Immune Responses

CD8$^+$ T lymphocytes are one of the most crucial components among antitumor effectors[Melief, 1995 #21]. To determine the E7-specific CD8$^+$ T cell precursor frequencies generated by FL-E7 DNA vaccines intracellular cytokine stains were used. Intracellular cytokine staining is sensitive functional assays used to measure IFN-γ production at the single-cell level, which can thus be applied to quantify antigen-specific CD8$^+$ T cells[Murali-Krishna, 1998 #22]. As shown in **Figure 1A** the quantity of E7(49-57)-specific CD8$^+$ T cell precursors can be determined by flow cytometry analysis using double staining for CD8 and intracellular IFN-□. As shown in **Figure 1B,** mice vaccinated with FL-E7 DNA generated the highest number of E7-specific IFN-□$^+$ CD8$^+$ T cell precursors (around 94/3x10^5 splenocytes) by flow cytometry analysis, whereas mice vaccinated with E7 DNA showed no signal above the vector-vaccinated controls. we have demonstrated that FL-E7 DNA immunization led to a significant increase in the E7-specific CD8$^+$ T cell precursor frequencies.

Vaccination with FL-E7 Fusion DNA does not Enhance E7-Specific CD4$^+$ T Cell-Mediated Immune Responses

To determine E7-specific CD4$^+$ T precursor cells and the cytokine profiles generated by FL-E7 DNA vaccines, we performed double staining for CD4 surface marker and intracellular IFN-□ or IL-4 on splenocytes from immunized mice, followed by flow cytometry analysis. The splenocytes from immunized mice were cultured *in vitro* with E7 peptide (aa 30-67) overnight and stained for both CD4 and intracellular IFN-□. The E7 peptide (aa 30-67) contains a major T helper epitope in the E7 open reading frame

protein of HPV-16[Tindle, 1991 #23]. The percentage of IFN-□ secreting $CD4^+$ T cells was analyzed using flow cytometry. As shown in **Figure 2** mice vaccinated with Fl-E7 DNA generated a similar number of $CD4^+$ IFN-□ $^+$ double positive cells compared to mice vaccinated with wild-type E7 DNA or naïve control.

As a positive control for the ability of CD4 vs. intracellular IFN-□ staining to detect E7-specific CD4+ T cells, analysis of mice vaccinated with a DNA vaccine expressing Sig/E7/LAMP-1, which targets E7 to the MHC class II compartment [32], demonstrated a 3 fold increase in the $CD4^+$ IFN-□ $^+$ double positive cells relative to mice vaccinated with E7 DNA or control plasmid (data not shown).

We then analyzed IL-4 secreting E7-specific $CD4^+$ T cell in mice vaccinated with various DNA vaccines. No significant $CD4^+$ $IL\text{-}4^+$ double-positive cells could be identified in the mice that received FL-E7 DNA, E7 DNA mixed with Flt3-ligand DNA, wild type E7 DNA, empty plasmid DNA, or no vaccination as in **Figure 2.** MICK-2 IL-4 secreting cells (Pharmingen, San Diego, CA) were used as positive controls to ensure the success of intracytoplasmic IL-4 staining for this study .

Vaccination with FL-E7 Fusion DNA dose not Generate E7-Specific Antibodies

The quantity of anti-HPV 16 E7 antibodies in the sera of the vaccinated mice was determined by a direct e nzyme-linked immunoabsorbent assay (ELISA) 2 weeks after the last vaccination. No anti-E7 antibodies could be detected in the sera of mice of any vaccinated group (**Figure 4**). The commercial anti-E7 monoclonal antibody (Zymed, San Francisco, CA) and sera from mice vaccinated with vaccinia virus containing the

Sig/E7/LAMP-1 chimera [Wu, 1995 #42] were used as positive controls to ensure the success of anti-E7 ELISA for this study. This result is consistent with the complete absence of apparent E7-specific CD4 stimulation by or FL-E7 DNA vaccines.

Vaccination with FL-E7 Fusion DNA Enhances Protection of Mice Against the Growth of TC-1 Tumors

To determine whether vaccination with the FL-E7 DNA construct protects mice against E7-expressing tumors, *in vivo* tumor protection experiments was performed. Mice were vaccinated with 2 □g naked DNA/mouse via gene gun and boosted with the same dose one week later.. The mice were then challenged with $1x10^4$ TC-1/mouse subcutaneously in the right leg 7 days after the last vaccination. 100% of those receiving FL-E7 DNA vaccination remained tumor-free 60 days after TC-1 challenge, while only 20% of mice receiving E7 DNA vaccination remained tumor-free. In contrast, all of the unvaccinated mice and mice receiving FL DNA or no vaccination developed tumor growth after tumor challenge **[Fig 5]**In summary, these results indicated that FL- E7 fusion DNA could significantly enhance the antitumor immunity against the growth of TC-1 tumors.

Therapeutic Vaccination with FL-E7 Fusion DNA Cures Mice with Established E7-Expressing Tumors

To test the efficacy of DNA vaccines in eradicating established TC-1 tumors, *in vivo* tumor treatment experiments were performed.

The C57BL/6 mice were intravenoualy challenged with 1×10^4 cells/mouse TC-1 tumor cells from tail vein. Three days after challenge with TC-1 tumor cells, mice were given 2 μg FL DNA, E7 DNA, FL-E7 DNA, E7+FL DNA

via gene gun. One week later, these mice were boosted with the same regimen as the first vaccination. Mice were sacrificed 25 days after TC-1 tumor cell challenge and lung tumor nodules were counted and lung were weight. There were multiple grossly tumor growth in control, E7, FL vaccinted mice[Fig. 6A] In the mice vaccinated with FL-E7 have the least lung nodules number, and the lightest weights[Fig. 6B] , while the mice vaccinated with wild type E7 , Flt3-ligand or FL mixed E7 DNA showed the similar number of lung nodules and lung weigh.

CD8$^+$ T Cells But Not CD4+ T cells is Essential for the Antitumor Effect Generated by DNA Vaccine with E7 Fused to Flt3-ligand

To determine the subset of lymphocytes that are important for the rejection of E7-positive tumor cells, we performed in vivo antibody depletion experiments. The antibody depletion was started one week before the tumor challenge and terminated on day 40 after tumor challenge. As shown in **Figure 7** , all naïve mice and all of the mice depleted of CD8$^+$ T cells grew tumors within 14 days after tumor challenge. In contrast, all of the nondepleted mice and all of the mice depleted of CD4$^+$ T cells remained tumor-free 60 days after tumor challenge. NK1.1 depletd mice have 60% tumor free after 60 days TC-1 challenge. These results suggest that CD8$^+$ T cells that are activated in a CD4-independent fashion are essential for the antitumor immunity generated by FL-E7 vaccine and NK cells may play some role in antitumor effect.

FL-E7 DNA Vaccine could Enhance the Cell-Mediated Immune Responses via both Direct and Cross Priming Effects

To evaluate if FL-E7 could induce a cell-mediated immune response via direct and/or cross priming effects, 293 cells were utilized. For direct priming effect, 293 K^bD^b cells were transfected with various DNA vaccines firstly. Transfected 293 K^bD^b cells were as target cells and E7 specific $CD8^+$ T cells were served as effector cells. CTL assays with various E/T ratios were performed. The FL-E7 DNA generated significantly higher percentages of specific lyses on the 9:1 (20.5±1.0% versus 10.43±0.9%, $P<0.001$) and 27:1 (47.1±5.5% versus 15.1±3.0%, $P<0.001$) E/T ratios as compared with mice vaccinated with wild-type E7 DNA vaccine (Figure 9). For cross priming effect, 293 cells were transfected with various DNA vaccines and lysates of trasnfected 293 cells were got from cycles of freeze-thaw. The different dilutions of lysates of transfected 293 cells with various DNA vaccines were pulsed to bone marrow-derived DCs. These DCs were used as target cells while E7-specific $CD8^+$ T cells served as effector cells. CTL assays were performed with fixed E/T ratio (9/1). The FL-E7 DNA generated significantly higher percentages of specific lyses on the 1/10 dilution (74.9±5.7% versus 25.4±4.2%, $P<0.001$) and 1/50 dilution (17.9±3.7% versus 5.3±2.4%, $P<0.001$) as compared with mice vaccinated with wild-type E7 DNA vaccine (Figure 8). These results revealed that FL-E7 DNA could enhance cell-mediated immune response via both direct and cross priming effects to generate the antigen-specific anti-tumor effect.

Vaccination with FL, FL-E7 Fusion DNA does not Increase DC and NK cell in Spleen

C57BL/7 mice were immunized intradermerally with gene gun with FL-E7, E7, FL. Splenocytes were analyzed 14 days from last vaccination Percentage of CD11c positive DC cells in splenocyes were measured [Fig.

9A] . Gates for the population were based on the control bone marrow dendritric cells. No significantly difference in the control(non-vaccinated) and E7, FL, FL+E7,and FL-E7 DNA vaccinated mice.

For $CD3^{-}KN1.1^{+}$ cell determination in spleen, cells were stained simultaneously with FITC-conjugated anti-CD-3 and PE-conjugated anti-NK1.1 mAbs and analyzed by flow cytometry[Fig. 9B} Two-color contour plot and percentage cells are presented. No
significant increase shown in the control and vaccinated mice.

Discussion:

In T cells , Intracelluar FL colocalization with giantin and ERGIC-53, indicating that the protein resident in endoplasmic reticulum and Golgi apparatus.[Chklovskaia, 1999 #80]

To produce a vaccine against cancer, antigens must be found that are preferentially expressed by tumour cells and can induce an immune response against the tumour.[Tao, 1993 #18] Here we show that by fusing a tumour-derived idiotype to GM-CSF, it can be converted into a strong immunogen capable of inducing idiotype-specific antibodies without other carrier proteins or adjuvants and of protecting recipient animals from challenge with an otherwise lethal dose of tumour cells. This approach may be applicable to the design of vaccines for a variety of other diseases.

Immunization with DNA constructs encoding the idiotype (ld) of a murine B-cell lymphoma induced specific anti-ld antibody responses and protected mice against tumor challenge. Use of DNA encoding an ld/GM-CSF (idiotype/granulocyte-macrophage colony- stimulating factor) fusion protein improved vaccine efficacy, and xenogeneic immunoglobulin constant region determinants were required for immunogenicity.[Syrengelas, 1996 #19]

MATERIALS AND METHODS

Plasmid DNA Constructs and Preparation

For the generation of HPV-16 E7-expressing plasmid (pcDNA3-E7), E7 fragment ,cut from TrcA-E7 by BamHI and HindIII ,was cloned into pcDNA3 vector (Invitrogen) cut BamHI and HindIII. For the generation of HSP-expressing plasmid (pcDNA3-HSP), the signal peptide and extracellular portion of mouse flt3 ligand (FL) was polymerase chain

reaction-cloned into pcDNA3 vector by using primers:
gggtctagaatgacagtgctggcgccagc and gggggatcc ctgcctgggccgaggctctgg, with a mouse flt3 ligand (FL) DNA template (flt3 ligand was purchased from ATCC).
For the generation of FL-E7 chimera (pcDNA3-FL-E7:), E7 DNA ,cut fragment from pcDNA3-E7 by BamHI and HindIII was cloned into pcDNA3-FL cut by BamHI and HindIII.
The accuracy of these constructs was confirmed by DNA sequencing.
Plasmid DNA with FL, E7 or FL-E7 gene insert and the "empty" plasmid vector were transfected into subcloning efficient DH5™ cells (Life Technologies, USA). The DNA was then amplified and purified using double CsCl purification (BioServe Biotechnologies, Laurel, Maryland).
The integrity of plasmid DNA and the absence of Escherichia coli DNA or RNA were checked in each preparation using 1% agarose gel electrophoresis.
DNA concentration was determined by the optical density measured at 260 nm. The presence of the inserted E7 fragment was confirmed by restriction enzyme digestion and gel electrophoresis.

DNA Vaccination

Gene gun particle-mediated DNA vaccination was performed using a helium-driven gene gun (Bio-rad, Hercules, CA) according to the protocol provided by the manufacturer. Briefly, DNA coated gold particles were prepared by combining 25 mg of 1.6 μm gold microcarriers (Bio-rad, Hercules, CA) and 100 μl of 0.05 M spermidine (Sigma, St, Louis, MO). Plasmid DNA (50 μg) and 1.0 M $CaCl_2$ (100 μl) were added sequentially to the microcarriers while mixing by vortex. This mixture was allowed to precipitate at room temperature for 10 minutes. The microcarrier/DNA

suspension was then centrifuged (10,000 r.p.m. for 5 sec) and washed 3 times in fresh absolute ethanol before resuspending in 3 ml of polyvinylpyrrolidone (0.1 mg/ml) (Bio-rad, Hercules, CA) in absolute ethanol. The solution was then loaded into tubing and allowed to settle for 4 min. The ethanol was gently removed and the microcarrier/DNA suspension was evenly attached to the inside surface of the tubing by rotating the tube. The tube was then dried by 0.4 liters per minute of flowing nitrogen gas. The dried tubing coated with microcarrier/DNA was then cut to 0.5-inch cartridges and stored in a capped dry bottle at 4 ^{0}C. As a result, each cartridge contained 1 μg of plasmid DNA and 0.5 mg of gold. The DNA coated gold particles (1 μg DNA/bullet) were delivered to the shaved abdominal region of the mice using a helium-driven gene gun (Bio-rad, Hercules, CA) with a discharge pressure of 400 p.s.i..

Intracytoplasmic Cytokine Staining and Flow Cytometry Analysis

Splenocytes from naïve or vaccinated groups of mice were incubated either with the E7 peptide (aa 49-57) that contains MHC class I epitope or the E7 peptide (aa 30-67) that contains MHC class II peptide. The E7 peptide was added at a concentration of 2 μg/ml for 20 hours. To detect E7-specific $CD8^+$ T cell precursors and E7-specific $CD4^+$ T helper cell responses, $CD8^+$ CTL epitopes aa 49-57 and aa30-67 were used, respectively. Golgistop (Pharmigen, San Diego, CA) was added 6 hours before harvesting the cells from the culture. Cells were then washed once in FACSCAN buffer and stained with phycoerythrin (PE)-conjugated monoclonal rat anti-mouse CD8 or CD4 antibody (Pharmingen, San Diego, CA). Cells were subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit according to

the manufacturer's instructions (Pharmingen). FITC-conjugated anti-IFN-□ or anti-IL-4 antibodies and the immunoglobulin isotype control antibody (rat IgG1) were all purchased from Pharmingen. Analysis was done on a Becton-Dickenson FACScan with CELLQuest software (Becton Dickson Immunocytometry System, Mountain View, CA).

Western Blot

The 293 cells were trasnfected with various DNA vaccines and lysed into cell lysis buffer (??????). Equal amounst of total protein were subjected to SDS-PAGE on a 10% gel and transferred onto Nylon C membranes (Amersham). The membrane was incubated with a murine anti-HPV 16 E7 monoclonal antibody ((Zymed, San Francisco, CA) with 1:1000 dilution. Specific antibody bound to protein bands were detected after absorption with anti-mouse peroxidase conjugates by chemoluminescence (ECL) according to the manufacturer's protocol (Amersham).

Cytotoxicity Assays

Cytotoxicity assays were performed in 96-well round-bottom platesand was determined by quantitative measurements of lactate dehydrogenase (LDH). The effector and target cells were mixed with different ratios. After 5 h incubation at 37°C, 50 μl of the cultured media were collected to assess the amount of LDH in the cultured media according to the manufacturer's protocol of the CytoTox assay kits (Promega, Madison, WI). LDH were detected and percentages of lysis were evaluated as previously mentioned (24). The percentage of lysis was calculated from the following equation: 100x (A—B)/(C—D) [where A is the reading of experimental-effector

signal value, B is the effector spontaneous background signal value, C is maximum signal value from target cells, D is the target spontaneous background signal value].

In Vitro Direct Priming Assays

To evaluate the direct priming effect, $293K^bD^b$ cells were transfected by lipofectamine with various DNA constructs. $293K^bD^b$ cells were incubated for 40-44 h after transfection. Then these $293K^bD^b$ cells cells were as target cell and the E7-specific CD8+ T cells were as effector cells. The $293K^bD^b$ cells mixed with E7-specific CD8 T cells at various effector/target (E/T) ratios. Cytotoxicity assays were performed and was determined by quantitative measurements of LDH as previously described.

In Vitro Cross Priming Assays

To evaluate the cross priming effect, 293 cells were transfected with different DNA construct as mentioned. After 40-44 hr of transfection, the 293 cells were trypsinized and freeze and thaw for 3 times. The lysed solution of transfected 293 cells were pulsed with bone marrowed derived-dentritic cells (DCs) for 48 hours with serial dilution. The pulsed DCs were as target cells and the E7-specific CD8 T+ cells were as effector cells. The DCs cells mixed with E7-specific CD8 T cells at various E/T ratios. Cytotoxicity assays were performed and was determined by quantitative measurements of LDH as previously described.

Anti-E7 ELISA

The anti-HPV 16 E7 antibodies in the sera were determined by a direct ELISA as previously described. A 96 microwell plate was coated with 100 µl 10 µg/ml bacteria-derived HPV-16 E7 proteins and incubated at 4 ^{0}C overnight. The wells were then blocked with PBS containing 20% fetal bovine serum. Sera were prepared from the mice on day 14 post-immunization, serially diluted in PBS, added to the ELISA wells, and incubated on 37 ^{0}C for 2 hr. After washing with PBS containing 0.05% Tween-20, the plate was incubated with 1/2000 dilution of a peroxidase-conjugated rabbit anti-mouse IgG antibody (Zymed, San Francisco, CA) at room temperature for one hour. The plate was washed 6 times, developed with TMB (Pierce, Rockford, IL), and stopped with 1M H_2SO_4. The ELISA plate was read with a standard ELISA reader at 450 nm.

Murine Tumor Cell Line

The production and maintenance of TC-1 cells has been described previously. In brief, HPV-16 E6, E7 and *ras* oncogene were used to transform primary C57BL/6 mice lung epithelial cells. The cells were grown in RPMI 1640, supplemented with 10% (vol/vol) fetal bovine serum, 50 units/ml penicillin/ streptomycin, 2mM L-glutamine, 1mM sodium pyruvate, 2mM nonessential amino acids and 0.4 mg/ml G418 at 37°C with 5% CO_2. On the day of tumor challenge, TC-1 cells were harvested by trypsinization, washed twice with 1X Hanks buffered salt solution and finally resuspended in 1X Hanks buffered salt solution to the designated concentration for injection.

Culture of 293K^bD^b Cells

$293K^bD^b$ was gifted from Dr. Yang JC (National Cancer Institute, National Institutes of Health, Bethesda), and grown in DMEM medium containing 10% heat-inactivated fetal calf serum, 0.3% glutamine, 0.01 M Hepes (National Institutes of Health Media Unit), 100 U/ml penicillin, 100 μg/ml streptomycin and 400 μg/ml G418.

Mice

We purchased 6- to 8-week-old male C57BL/6 mice from the National Cancer Institute (Frederick, Maryland) and kept in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, Maryland). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

Generation of bone marrow-derived Dendritic Cell(BM-DC).

BM-DC were propagated from C57BALB/c mice BM progenitors for 7 days. Briefly, bone marrow was collected from tibias of mice. Erythrocytes were lysed, and the remaining cells were passed through a nylon mesh to remove small pieces of bone and debris. The cells were collected and $1x10^6$ cells/ml were placed in 24-well plates in RMPI 1640, supplemented with 5% FCS, 50 μM ß-ME, 1% nonessential amino acids, 100 u peniccilin and 100 μg/ml sptretomycin (Life Technologies, Rockville, MD), and 100 U/ml GM-CSF (PharMingen, San Diego, CA). Two-thirds of the medium was replaced every 2 days, and nonadherent cells were harvested on day 7.

Generation of E7-specific $CD8^+$ cell lines

The generation of E7-specific CD8+ cell were performed as previous report[Ji, 1999 #1] Briefly, female C57BL/6 ($H-2^b$) mice were immunized

by vaccina-Sig/E7/LAMP-1. Splenocytes were harvested later. For initial in vitro stimulation, $4x10^6$ splenocytes were pulsed with IL-2 at a concentration of 20 U/ml and 1 μM E7 peptide (amino acids 49-57) for 6 days. Propagation of the E7-specific CTL cell line was performed by mixing $1x10^6$ splenocytes containing E7-specific CTLs with $3x10^6$ irradiated splenocytes and pulsing them with IL-2 at a concentration of 20 U/ml and 1 μM E7 peptide (amino acids 49-57). This procedure was repeated every 6 days. The specificity of the E7 CTL line was characterized by the CTL assay. Flow cytometry was performed to demonstrate the expression of the CD8 marker.

In Vivo Tumor Protection Experiments

For the tumor protection experiment, mice (5 per group) were vaccinated via gene gun with 2 μg of FL DNA, E7 DNA, FL-E7 DNA, E7+FL DNA. One week later, the mice were boosted with the same regimen as the first vaccination. One week after the last vaccination, mice were subcutaneously challenged with 1×10^4 cells/mouse TC-1 tumor cells in the right leg and then monitored twice a week.

In Vivo Tumor Treatment Experiments

The tumor cells and the DNA vaccines were prepared as described above. The mice were intravenoualy challenged with 1×10^4 cells/mouse TC-1 tumor cells from tail vein. Three days after challenge with TC-1 tumor cells, mice were given 2 μg FL DNA, E7 DNA, FL-E7 DNA, E7+FL DNA via gene gun. One week later, these mice were boosted with the same regimen

as the first vaccination. Mice were sacrificed 25 days after TC-1 tumor cell challenge.

In Vivo Antibody Depletion Experiments

In vivo antibody depletions have been described previousl[Lin, 1996 #9]. Briefly, C57BL/6 mice were vaccinated with 2 μg FL-E7 DNA via gene gun, boosted one week later, and challenged with 1 x 10^4 cells/mouse TC-1 tumor cells. Depletions were started one week prior to tumor challenge. MAb GK1.5 [Dialynas, 1983 #12]was used for CD4 depletion, MAb 2.43[Sarmiento, 1980 #11] was used for CD8 depletion, and MAb PK136 [Koo, 1986 #10]was used for NK1.1 depletion. Flow cytometry analysis revealed that the >95% of the appropriate lymphocytes subset were depleted with normal level of other subsets.

Legends to figures:

Fig. 1 . E7-specific $CD8^+$ T cell precursors in C57BL/6 mice immunized with FL-E7 DNA vaccines. C57BL/6 mice were immunized with Flt3-ligand DNA (FL), E7 DNA (E7), or E7 DNA mixed with Flt3-ligand DNA (E7+FL) or Flt3-ligand-E7 DNA (FL-E7) via gene gun or received no vaccination. For vaccinated mice, 2 μg DNA /mouse was given twice. (A) Splenocytes were harvested 14 days after the last DNA vaccination. Splenocytes from vaccinated mice were cultured *in vitro* with E7 peptide (aa 49-57) overnight and were stained for both CD8 and intracellular IFN-γ. The number of IFN-γ secreting $CD8^+$ T cell precursors in mice immunized with various recombinant DNA vaccines was analyzed by flow cytometry. Mice vaccinated with FL-E7 DNA generated the highest IFN-□ $CD8^+$ double positive T cells. The numbers of $CD8^+$ IFN-γ^+ double positive T cell in $3x10^5$ splenocytes are indicated in the upper right corner. (B) The number of IFN-□ -producing E7-specific CD8 $^+$ T cells were determined by flow cytometry in the presence (filled) and absence (open) of E7 peptide (aa 49-57). Results shown here are from one representative experiment of three performed. Data are expressed as mean number of $CD8^+$ IFN-$□^+$ cells/$3x10^5$ splenocytes ± SD.

Fig. 2. Flow cytometry analysis of IFN-γ secreting E7-specific $CD4^+$ cells in mice vaccinated with various recombinant DNA vaccines. C57BL/6 mice were immunized as described in Fig. 1. (A) Splenocytes from vaccinated mice were cultured *in vitro* with E7 peptide (aa 30-67) overnight and were stained for both CD4 and intracellular IFN-γ. The number of IFN-γ secreting $CD4^+$ T cells was analyzed by flow cytometry. Mice vaccinated with FL-E7 DNA generated comparable $CD4^+$ IFN-γ^+ double positive cells when compared to mice vaccinated with wild-type E7 DNA. The numbers of $CD4^+$ IFN-γ^+ double positive T cell in $3x10^5$ splenocytes are indicated in the upper right corner. (B) The number of IFN-□ -producing E7-specific CD4 $^+$ T cells were determined by flow cytometry in the presence (filled) and absence (open) of E7 peptide (aa 49-57). The data of intracellular cytokine staining shown here are one representative experiment of two performed.

Fig. 3. Flow cytometry analysis of IL-4 secreting E7-specific $CD4^+$ cells in mice immunized with various recombinant DNA vaccines. (A) Top panel: The IL-4 secreting activated mouse splenocytes (MiCK-2) from PharMingen were used as positive controls to assure the success of intracytoplasmic IL-4 staining for this study. The specificity of the IL-4 staining was demonstrated by the absence of $CD4^+$ IL-4^+ T cells when the IL-4 antibody was omitted. Lower panel: Splenocytes from vaccinated mice were cultured *in vitro* with E7 peptide (aa 30-67) overnight and stained for both CD4 and intracellular IL-4. The percentage of IL-4 secreting $CD4^+$ T cells was analyzed by flow cytometry. (B) Splenocytes from vaccinated mice were cultured *in vitro* with (filled) and without (open) E7 peptide (aa 30-67) overnight and were stained for both CD4 and intracellular IL-4. The numbers of IL-4 producing $CD4^+$ T cells were determined using flow

cytometry. Results shown here are from one representative experiment of three performed. No significant difference in the frequency of IL-4 secreting E7-specific $CD4^+$ cells was observed in mice immunized with various recombinant DNA vaccines. Data are expressed as mean number of $CD4^+$ $IL4^+$ cells/$3x10^5$ splenocytes ± SD.

Fig. 4. E7-specific antibody responses in C57BL/6 mice immunized with various recombinant DNA vaccines.
C57BL/6 mice were immunized with control plasmid (no insert), wild-type E7, FL , or E7 DNA mixed with Flt3-ligand DNA (E7+FL) or FL-E7 DNA via gene gun. Serum samples were obtained from immunized mice 14 days after vaccination. The presence of the E7-specific antibody was detected by ELISA using serial dilution of sera. The results from the 1:100 dilution are presented, showing mean absorbance (O.D.) at 450 nm ± SE.

Fig. 5. Vaccination with FL-E7 DNA protects mice against the growth of TC-1 tumors. C57BL/6 mice were no vaccination or immunized Flt3-ligand DNA (FL), E7 DNA (E7), or E7 DNA mixed with Flt3-ligand DNA (E7+FL) or Flt3-ligand-E7 DNA (FL-E7) via gene gun. For vaccinated mice, 2ug DNA/mouse was given twice. One week after the last vaccination, mice were challenged with $1x10^4$ TC-1 cells/ mouse subcutaneously in the right leg. The mice were monitored for evidence of tumor growth by palpation and inspection twice a week.

Fig. 6. Vaccination with FL-E7 DNA eradicates pre-existing TC-1 tumor in the lungs. Each mouse (5 per group) was initially challenged with $1x10^4$ TC-1 cells intraveneously from tail vein followed by DNA vaccination 3 days later with Flt3-ligand DNA (FL), E7 DNA (E7), or E7

DNA mixed with Flt3-ligand DNA (E7+FL) or Flt3-ligand E7 DNA (FL-E7) via gene gun or no vaccination. The mice were sacrificed at 25 days after TC-1 challenge. (A) Representative gross picture pictures of the lung tumors in each vaccinated group. There are multiple grossly visible lung tumor in the control mice and mice vaccinated with E7, FL and FL+E7 DNA. (B) The pulmonary nodules number and lung weight were measured and shown here in mean ± SD.

Fig. 7. Effect of lymphocyte subset depletions on the potency of FL-E7 DNA vaccine. C57BL/6 mice were immunized with 2 μg Fl-E7 DNA via gene gun and boosted with 2 μg FL-E7 DNA one week later. Two weeks after the last vaccination, mice were challenged with $1x10^4$ TC-1 cells/ mouse subcutaneously in the right leg. Depletions were initiated one week prior to tumor challenge and lasted 40 days after tumor challenge. MAb GK1.5 was used for CD4 depletion, MAb 2.43 was used for CD8 depletion, and MAb PK136 was used for NK1.1 depletion. Flow cytometry analysis revealed that the >95% of the appropriate lymphocytes subset were depleted with normal level of other subsets. The mice were monitored for evidence of tumor growth by palpation and inspection twice a week.

Fig. 8. FL-E7 DNA vaccine could enhance anti-tumor effect by promoting the E7-specific CTL response via Cross Priming and Direct Priming Assays . (A)To evaluate the direct priming effect, $293K^bD^b$ cells were transfected by lipofectamine with various DNA constructs. $293K^bD^b$ cells were incubated for 40-44 h after transfection. Then these $293K^bD^b$ cells cells were as target cell and the E7-specific CD8+ T cells were as effector

cells. The 293K^bD^b cells mixed with E7-specific CD8 T cells at various effector/target (E/T) ratios. Cytotoxicity assays were performed and was determined by quantitative measurements of LDH as previously described. FL-E7 DNA generated significantly higher percentages of specific lyses on the 27:1 ($P<0.001$) E/T ratios as compared with mice vaccinated with other DNA vaccines. The data of CTL assays for direct priming shown here are one representative experiment of two performed.(B) To evaluate the cross priming effect, 293 cells were transfected as mentioned. After 40-44 hr of transfection, the 293 cells were trypsinized and freeze and thaw for 3 times. The lysed solution of 293 cells were pulsed with bone marrowed derived-dendritic cells (DCs) for 48 hours with serial dilution. The pulsed DCs were as target cells and the E7-specific CD8 T+ cells were as effector cells. CTL assays with fixed E/T ratio (9/1) were performed. The FL-E7 DNA generated significantly higher percentages of specific lyses on the 1/10 and 1/50 dilution (both $Ps<0.001$) as compared with mice vaccinated with other DNA vaccines. The data of CTL assays of cross priming shown here are one representative experiment of two performed.

Fig. 9. Dendritic cells and Nature killer cells in spleens immunized with various DNA vaccines. C57BL/6 mice were immunized with control plasmid (no insert), wild-type E7, FL , or E7 DNA mixed with Flt3-ligand DNA (E7+FL) or FL-E7 DNA via gene gun. Splenocytes were obtained from immunized mice 14 days after vaccination. (A) The portion of CD11c positive DC cells in splenocyes were shown in percentage Gates for the population were based on the control bone marrow dendritric cells. (B) The splenocytes cells were stained simultaneously with FITC-conjugated anti-

CD-3 and PE-conjugated anti-NK1.1 mAbs and analyzed by flow cytometry. Two-color contour plot and percentage cells are presented at left-upper corner with $CD3^{-}NK1.1^{+}$ cells. The data shown here are one representative experiment of two performed.

Enhancement of DNA Vaccine Potency By Linkage of Antigen Gene to a FLT3-Ligand Gene

*Keng-Fu Hsu

Department of Obstetrics and Gynecology[5], National Cheng Kung University Hospital, Tainan, Taiwan;

ABSTRACT

Nucleic acid vaccines represent an attractive approach for generating antigen-specific immunity because of their stability and simplicity of delivery. However, there is still a need to enhance the potency of DNA vaccines. Recently, Flt3-ligand has been identified as an important cytokine in the generation of professional antigen presenting cells, particularly dendritic cells. A recombinant molecule linking Flt3-ligand (FL) to a model antigen may potentially target the antigen to dendritic cells. Using HPV-16 E7 as a model antigen, we evaluated the effect of linkage to Flt3-ligand on the potency of antigen-specific immunity generated by naked DNA vaccines administered intradermally via gene gun. We found that vaccines containing chimeric FL/E7 fusion genes dramatically increased the frequency of E7-specific $CD8^+$ T cells relative to vaccines containing the wild-type E7 gene. *In vitro* studies indicated that cells transfected with FL/E7 DNA were capable of presenting E7 antigen through the MHC class I pathway in a more efficient manner than cells transfected with wild-type E7 DNA. Furthermore, bone marrow-derived dendritic cells pulsed with FL/E7 fusion protein are capable of presenting E7 antigen through the MHC class I pathway in a more efficient manner than dendritic cells pulsed with wild-type E7 protein. More importantly, this fusion converted

a less effective vaccine into one with significant potency against established E7-expressing metastatic tumors. Interestingly, FL-E7 fusion vaccines mainly targeted CD8+ T cells and antitumor effects were completely CD4-independent. These results indicate that fusion of Flt3-ligand gene to an antigen gene may greatly enhance the potency of DNA vaccines via CD8-dependent pathways.

INTRODUCTION

Antigen-specific cancer immunotherapy has recently emerged as a promising approach for controlling cancer because it is capable of developing specific immunity against neoplastic cells while not attacking normal cells. Increasing evidence suggests that professional antigen presenting cells (APCs), particularly dendritic cells (DCs), are the central players for mediating cancer immunotherapy. An effective vaccine most likely requires a strategy that targets antigen to professional APCs to activate antigen-specific T cells.

Recently, DNA vaccines have become an attractive approach for generating antigen-specific immunotherapy. Naked DNA is safe, has low immunogenicity, and can be repeatedly administered. Furthermore, DNA vaccines can be easily prepared in large scale at lower relative cost with high purity and stability compared to proteins [Robinson, 1995 #2917; Pardoll, 1995 #2830; Donnelly, 1997 #2242]. One of the concerns about DNA vaccines is their potency, since they do not have the intrinsic ability to amplify *in vivo* as viral vaccines do. We reason that a DNA vaccine encoding a fusion antigen that is can be directed to sites of immune induction, such as dendritic cells may enhance vaccine potency (Boyle *et al.*, 1998). One such molecule is GM-CSF. It has been demonstrated that the linkage of GM-CSF gene to antigen gene has enhanced the potency of DNA vaccines. For example, fusion of GM-CSF with antigen in a DNA vaccine can lead to enhancement of immune responses and protection against HIV [Lee, 1999 #11] and hepatitis C [Lee, 1998 #22]. Although the specific mechanism for such enhancement of DNA vaccine potency is not clear, it is believed that chimeric GM-CSF/antigen may act

as an immunostimulatory signal to DCs, inducing differentiation from an immature DC form to a mature form and mobilizing active dendritic cells at the onset of a cell-mediated immune response [Banchereau, 1997 #14][Steinman, 1988 #44]. Since DCs and their precursor cells express high levels of GM-CSF receptors (GM-CSFR), chimeric GM-CSF/antigen may target and concentrate the linked antigen to DCs and further improve DNA vaccine potency. Another important molecule that also possesses a growth-stimulatory effect on DCs and to be capable of generating large numbers of DCs *in vivo* is Flt3-ligand [Maraskovsky, 1996 #45; Shurin, 1997 #230]. Flt3-L has emerged as an important molecule for the development of tumor vaccines that augment the function and quantity of DCs *in vivo*. Flt3 (fms-like tyrosine kinase 3), a murine tyrosine kinase receptor, was first described in 1991 [Matthews, 1991 #237; Rosnet, 1991 #238] and found to be a member of the same family of receptors as c-kit and c-fms receptors - the type III receptor kinase family (for review see [Lyman, 1998 #319]). In hematopoietic tissues, the expression of flt3 is restricted to the CD34-positive progenitors. Flt3 has been used to identified and subsequently clone the corresponding ligand, Flt3-L [Lyman, 1993 #240; Hannum, 1994 #239]. The predominant form of Flt3-L is synthesized as a transmembrane protein from which the soluble form is generated, presumably by proteolytic cleavage [Lyman, 1995 #241]. These proteins function by binding to and activating unique tyrosine kinase receptors. Expression of the Flt3 receptor is primarily restricted, among hematopoietic cells, to the most primitive progenitor cells, including dendritic cell precursors. To date, flt3-ligand have not been used in the form of chimeric DNA vaccines.

We chose human HPV-16 E7 as a model antigen for vaccine development because HPVs, particularly HPV-16, are associated with most cervical cancers. The HPV oncogenic proteins, E6 and E7, are important in the induction and maintenance of cellular transformation and co-expressed in most HPV-containing cervical cancers. Vaccines or immunotherapies targeting E7 and/or E6 proteins may provide an opportunity to prevent and treat HPV-associated cervical malignancies. In our current study, we investigated whether genes linking full-length E7 to flt3-ligand can enhance the potency of DNA vaccines. We compared DNA vaccines containing wild type HPV-16 E7 with DNA vaccines containing full-length E7 fused to flt3-ligand for their immune response generation and their ability to protect animals against the HPV-16 E7-expressing murine tumors [Lin, 1996 #37]. We show that linkage of E7 to flt3-ligand dramatically increases expansion and activation of E7-specific CD8+ T cells, completely bypassing the CD4 arm. This enhanced CD8 response results in potent antitumor immunity against established tumors.

DISCUSSION

In this study we demonstrated that FL can significantly enhance the potency of HPV-16 E7-expressing DNA vaccines. DNA vaccines with FL fused to HPV-16 E7 elicited strong E7-specific cellular immunity and generated significant $CD8^+$ T cell-dependent preventive effects against HPV-16 E7-expressing murine tumors. Furthermore, chimeric FL/E7 DNA vaccine was capable of controlling lethal lung metastasis.

Our data demonstrated that FL can preferentially enhance $CD8^+$ T cell responses of E7 DNA vaccines. In contrast, $CD4^+$ T cell responses were not detectably enhanced by FL linkage. The most plausible mechanisms for the enhancement of $CD8^+$ T cell responses in vaccinated miceis not clear. However, our data also indicated that the linkage of FL to E7 has led to the enhanced MHC class I presentation of E7 compared to wild type E7 in transfected cells (Figure 9). Therefore, the linkage of FL to E7 may facilitate the precessing of E7 into the MHC class I presentation pathway. Ballistic DNA delivery can introduce DNA directly into dermal dendritic cell precursors. The FL/E7 expressing DCs thereby may present E7 through MHC class I pathway more efficiently than E7 expressing DCs.

Another important mechanism that may contribute to the enhanced E7-specific CD8+ T cell immune responses *in vivo* is related to so call "cross-priming" effects of FL/E7 complexes where the chiemeric FL/E7 can lead exogenous proteins to the MHC-I restricted antigen presentation pathway. Our data suggested that chimeric FL/E7 protein can be processed by bone marrow-derived dendritic cells and presented more efficiently through MHC class I pathway than wild type E7 protein (Figure 10). However, the "cross-primimg" of chimeric FL/E7 probably does not play a major role in the gene gun-mediated FL/E7 DNA vaccines. It has been shown that direct priming of CD8+ T cells by gene-transfected DCs is the key event in gene gun-mediated DNA immunization [Porgador, 1998 #147; Akbari, 1999 #198], while cross-priming of DCs is not an major mechanism for gene gun-mediated DNA vaccination [Porgador, 1998 #147; Akbari, 1999 #198]. However, we can not completely rule out the possibility of cross-priming, because FL/E7 might be released from other cell types, such as keratinocytes (which

were also transfected by gene gun vaccination), and then enter the DCs via the cross-priming mechanism.

In this study, we failed to detect significant E7-specific IFN-γ or IL 4-secreting $CD4^+$ T cells in FL/E7 vaccinated mice (Figure 3). The observed enhancement of E7-specific CD8+ T cell immune responses was most likely not facilitated by E7-specific $CD4^+$ T helper cells. However, if the FL/E7 DNA vaccine generates FL-specific CD4+ T cells, these cells may contribute to the generation and expansion of E7-specific $CD8^+$ T cells. These FL-reactive T helper cells can exert a strong helper effect by reacting to conjugated peptides [Del Giudice, 1994 #57]. This may also contribute to the increase in E7-specific $CD8^+$ T cell precursors observed in mice vaccinated with the FL/E7 DNA vaccine.

While FL/E7 generates potent CD8+ T cell responses through enhanced MHC class I presentation, other constructs that target antigen to MHC class II presentation pathways may provide enhanced CD4+ T cell responses. This realization raises the notion of co-administration of vaccines that directly enhance MHC class I and class II restricted pathways. We have previously developed a chimeric Sig/E7/LAMP-1 DNA vaccine that uses the LAMP-1 endosomal/lysosomal targeting signal for enhancing the MHC class II presentation pathway of E7 [Wu, 1995 #42]. The FL/E7 vaccine described here in conjunction with a MHC class II-targeting vaccine such as Sig/E7/LAMP-1 may activate multiple arms of the immune system in a synergistic fashion, leading to significantly enhanced CD4+ and CD8+ T cell responses and potent antitumor effects.

While the FL/E7 vaccine holds promise for mass immunization, three safety issues need to be resolved. First, the DNA may integrate into the host genome, resulting

in the inactivation of tumor suppresser genes or the activation of oncogenes. This may lead to malignant transformation of the host cell. Fortunately, it is estimated that the frequency of integration is much lower than that of spontaneous mutation and integration should not pose any real risk [Nichols, 1995 #112]. The second issue concerns potential risks associated with the presence of HPV-16 E7 protein in host cells. E7 is an oncoprotein that disrupts cell cycle regulation by binding to tumor suppressor pRB protein in nuclei [Lukas, 1994 #119]. Thus, the presence of E7 in host cells may lead to accumulation of genetic aberrations and eventual malignant transformation in the host cells. The oncogenicity of E7 can be eliminated by introducing mutations into E7 DNA so that the resulting E7 protein cannot bind with pRB [Heck, 1992 #122] but still maintains most of its antigenicity. The third issue is the concern over the generation of autoimmunity that may be caused when FL lead to excessive dendritic cells *in vivo*. In our study, we performed pathological examination of the vital organs in the FL/E7-vaccinated mice, including the intestines. We did not observe any significant pathology, indicating that FL/E7 is a potent vaccine with minimal malevolent side effects.

In summary, our results indicate that fusion of FL to HPV-16 E7 gene can generate stronger E7-specific CD8+ T cell-mediated immune responses and antitumor effects against HPV-16 E7-expressing murine tumors generated by E7 DNA vaccines. Our results indicate that fusion of FL to an antigen gene may greatly enhance the potency of DNA vaccines and can potentially be applied to other cancer systems with known tumor-specific antigens.

Enhancement of DNA Vaccine Potency by Linkage of Antigen Gene to a Gene Encoding the Extracellular Domain of Flt3-Ligand

*Chien-Fu Hung[1], *Keng-Fu Hsu[1,5], Wen-Fang Cheng[1,6] Chee-Yin Chai[1,7], Liangmei He[1], Morris Ling[1] and T.-C. Wu[1,2,3,4]

Departments of Pathology[1], Oncology[2], Obstetrics and Gynecology[3], and Molecular Microbiology and Immunology[4], The Johns Hopkins Medical Institutions, Baltimore, Maryland 21287, USA; Department of Obstetrics and Gynecology[5], National Cheng Kung University Hospital, Tainan, Taiwan; Department of Obstetrics and Gynecology[6], National Taiwan University Hospital, National Taiwan University, Taipei, Taiwan; Department of Pathology[7], School of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan.

Source of support: This work was supported by NIH 5 PO1 34582-01, U19 CA72108-02, RO1 CA72631-01, the Richard W. TeLinde fund and the Alexander and Margaret Stewart Trust grant.

Running title: Tumor immunotherapy with a chimeric Flt3-ligand DNA vaccine

Keywords: Flt3-ligand, human papillomavirus, HPV, E7, DNA vaccines, immunotherapy.

* Chien-Fu Hung and Keng-Fu Hsu contributed equally to this paper.

Address correspondence and reprint requests to: Dr. T.-C. Wu, Department of Pathology, the Johns Hopkins Hospital, 600 North Wolfe Street, Baltimore, Maryland 21205. Tel: (410) 614-3899. Fax: (410) 614-3548. Email: wutc@jhmi.edu

ABSTRACT

Recently, Flt3 (fms-like tyrosine kinase 3)-ligand has been identified as an important cytokine in the generation of professional antigen presenting cells, particularly dendritic cells. A recombinant chimera of the extracellular domain of Flt3-ligand (FL) linked a model antigen may potentially target the antigen to dendritic cells and their precursor cells. Using HPV-16 E7 as a model antigen, we evaluated the effect of linkage to the extracellular domain of Flt3-ligand on the potency of antigen-specific immunity generated by naked DNA vaccines administered intradermally via gene gun. We found that vaccines containing chimeric FL-E7 fusion genes dramatically increased the frequency of E7-specific $CD8^+$ T cells relative to vaccines containing the wild-type E7 gene. *In vitro* studies indicated that cells transfected with FL-E7 DNA presented E7 antigen through the MHC class I pathway more efficiently than wild-type E7 DNA. Furthermore, bone marrow-derived dendritic cells pulsed with cell lysates containing FL-E7 fusion protein are capable of presenting E7 antigen through the MHC class I pathway more efficiently than dendritic cells pulsed with cell lysates containing wild-type E7 protein. More importantly, this fusion converted a less effective vaccine into one with significant potency against established E7-expressing metastatic tumors. The FL-E7 fusion vaccines mainly targeted CD8+ T cells and antitumor effects were completely CD4-independent. These results indicate that fusion of a gene encoding the extracellular domain of Flt3-ligand to an antigen gene may greatly enhance the potency of DNA vaccines via CD8-dependent pathways.

INTRODUCTION

Antigen-specific cancer immunotherapy has recently emerged as a promising approach for controlling cancer because it is capable of developing specific immunity against neoplastic cells while not attacking normal cells. Increasing evidence suggests that professional antigen presenting cells (APCs), particularly dendritic cells (DCs), are the central players for mediating cancer immunotherapy. An effective vaccine most likely requires a strategy that targets antigen to professional APCs to activate antigen-specific T cells (for review, see (1)).

Recently, DNA vaccines have become an attractive approach for generating antigen-specific immunotherapy. Naked DNA is safe, has low immunogenicity, and can be repeatedly administered. Furthermore, DNA vaccines can be easily prepared at a large scale with high purity and stability at lower cost compared to proteins (for review, see (2-4)). One of the concerns about DNA vaccines is their potency, since they do not have the intrinsic ability to amplify *in vivo* as viral vaccines do. We reasoned that a DNA vaccine encoding a fusion antigen that is directed to sites of immune induction, such as dendritic cells, may enhance vaccine potency. Previously, it has been demonstrated that the linkage of a GM-CSF gene to an antigen gene has enhanced the potency of DNA vaccines against HIV (5) and hepatitis C (6). It is believed that chimeric GM-CSF/antigen may act as an immunostimulatory signal to DCs, inducing differentiation from an immature DC form to a mature form and mobilizing active dendritic cells at the onset of a cell-mediated immune response (7). Since DCs and their precursor cells express high levels of GM-CSF receptors (GM-CSFR), chimeric GM-CSF/antigen may

target and concentrate the linked antigen to DCs and further improve DNA vaccine potency.

Another important molecule that also possesses a growth-stimulatory effect on DC precursors and has been shown to be capable of generating large numbers of DCs *in vivo* is Flt3-ligand (8, 9). Flt3-ligand has emerged as an important molecule for the development of tumor vaccines that augment the function and quantity of DCs *in vivo*. Flt3, a murine tyrosine kinase receptor, was first described in 1991 (10) and found to be a member of the same family of receptors as c-kit and c-fms receptors -- the type III receptor kinase family (for review see (11)). In hematopoietic tissues, the expression of Flt3 is restricted to the CD34-positive progenitors. Flt3 has been used to identify and subsequently clone the corresponding ligand, Flt3-ligand (12, 13).

The predominant form of Flt3-ligand is synthesized as a transmembrane protein from which the soluble form is generated, presumably by proteolytic cleavage. The soluble form of Flt3-ligand (extracellular domain) has been shown to be functionally similar to Flt3-ligand (12). These proteins function by binding to and activating unique tyrosine kinase receptors. Expression of the Flt3 receptor is primarily restricted, among hematopoietic cells, to the most primitive progenitor cells, including dendritic cell precursors. Several studies have shown that the soluble extracellular domain of Flt3-ligand generated strong antitumor effects against several murine model tumors including fibrosarcoma (14), breast cancer (15, 16), liver cancer (17), lung cancer (18), melanoma and lymphoma (19). Presently, Flt3-ligand has not been used in the form of chimeric DNA vaccines.

In our current study, we investigated whether linking a full-length E7 gene to a gene encoding the extracellular domain of Flt3-ligand would enhance the potency of DNA vaccines. We chose human HPV-16 E7 as a model antigen for vaccine development because HPVs, particularly HPV-16, are associated with most cervical cancers. The HPV oncogenic proteins, E6 and E7, are important in the induction and maintenance of cellular transformation and co-expressed in most HPV-containing cervical cancers. Vaccines or immunotherapies targeting E7 and/or E6 proteins may provide an opportunity to prevent and treat HPV-associated cervical malignancies. We compared DNA vaccines containing wild type HPV-16 E7 with DNA vaccines containing full-length E7 fused to FL for their generation of immune responses and their ability to protect animals against HPV-16 E7-expressing murine tumors (20). Our data indicated that linkage of gene encoding the extracellular domain of Flt3-ligand to E7 dramatically increases expansion and activation of E7-specific $CD8^+$ T cells, completely bypassing the CD4 arm. This strategy enhanced E7-specific $CD8^+$ T cell responses, resulting in potent antitumor immunity against established E7-expressing metastatic tumors.

MATERIALS AND METHODS

Plasmid DNA Constructs and Preparation

The generation of HPV-16 E7-expressing plasmid, pcDNA3-E7 has been described previously (21). For the generation of plasmid encoding the extracellular domain of mouse Flt3-ligand (FL), pcDNA3-FL, the DNA fragment encoding the signal peptide and extracellular domain of mouse Flt3-ligand (FL) was first amplified with PCR

using conditions as described previously (21) with a mouse Flt3-ligand DNA template, sfHAV-EO410 (ATCC, Manassas, VA) and a set of primers: 5'-gggtctagaatgacagtgctggcgccagc-3' and 5'-gggggatccctgcctgggccgaggctctgg-3'. The amplified product was then digested with XbaI and BamHI and further cloned into the XbaI and BamHI cloning sites of pcDNA3 vector (Invitrogen, Carlsbad, CA). For the generation of pcDNA3-FL-E7, the E7 DNA fragment was isolated from pcDNA3-E7 by digestion with BamHI and HindIII and gel-recovered. The isolated E7 DNA fragment was further cloned into the BamHI and HindIII cloning sites of pcDNA3-FL. For the generation of pcDNA3-GFP, DNA fragment encoding the green fluoresecnt protein (GFP) was first amplified with PCR using pEGFPN1 DNA (Clontech, Palo Alto, CA) and a set of primers: 5'-atcggatccatggtgagcaagggcgaggag-3'and 5'-gggaagctttacttgtacagctcgtccatg-3'. The amplified product was then digested with BamHI and HindIII and further cloned into the BamHI and HindIII cloning sites of pcDNA3 vector (Invitrogen). For the generation of pDNA3-E7-GFP, DNA fragment encoding HPV-16 E7 first amplified with PCR using pcDNA3-E7 as template and a set of primers: 5'-ggggaattcatgcatggagatacaccta-3' and 5'-ggtggatccttgagaacagatgg-3'. The amplified product was then digested with EcoRI and BamHI and further cloned into the EcoRI and BamHI cloning sites of pcDNA3-GFP vector. For the generation of pcDNA3-FL-E7-GFP, the DNA encoding the signal peptide and extracellular domain of Flt3-ligand was amplified with PCR using pcDNA3-FL as a DNA template and a set of primers: 5'-gggtctagaatgacagtgctggcgccagc-3' and 5'-cgagaattcctgcctgggccgaggctctg-3'. The amplified product was then digested with XbaI and EcoRI and further cloned into the XbaI and EcoRI cloning sites of pcDNA3-E7-GFP vector. The accuracy of these

constructs was confirmed by DNA sequencing. pcDNA3 DNA with FL, E7, FL-E7, E7-GFP or FL-E7-GFP gene insert and the "empty" plasmid, pcDNA3 vector were transfected into subcloning efficient DH5™ cells (Life Technologies, USA). The DNA was then amplified and purified as described previously (21). The integrity of plasmid DNA and the absence of Escherichia coli DNA or RNA was checked in each preparation using 1% agarose gel electrophoresis. DNA concentration was determined by the optical density measured at 260 nm. The presence of inserted E7 fragment was confirmed by restriction enzyme digestion and gel electrophoresis.

Cell Lines

The production and maintenance of TC-1 cells has been described previously (20). On the day of tumor challenge, TC-1 cells were harvested by trypsinization, washed twice with 1X Hanks buffered salt solution (HBSS) and finally resuspended in 1X HBSS to the designated concentration for injection. A human embryonic kidney 293 cell line expressing the D^b and K^b (293 D^b,K^b) (22), two of C57BL/6 mouse MHC class I molecules , was a gift from Dr. JC Yang (National Cancer Institute, National Institutes of Health, Bethesda). It was grown in DMEM medium containing 10% heat-inactivated fetal calf serum, 0.3% glutamine, 0.01 M HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin and 400 µg/ml G418.

Confocal Fluoresence Microscopy

293 D^b,K^b Cells transfected with pcDNA E7-GFP and pcDNA FL-E7-GFP DNA were cultured for 24-36 hr, then cytospined to glass slides. Cells were fixed for 30 min at

room temperature with 4 % paraformaldehyde in PBS, permeablized with PBS containing 0.05% saponin and 1% BSA, then incubated for 30 min at room temperature, with primary antibody. (Anti-calnexin antibody was from Streegen Biotecnologies.) Unbound antibodies were remove by washing in the same medium. Cells then incubated for 30 min with Cy3-conjugated F(ab')2 fragment goat anti-mouse IgG(Jackson ImmunoReseach labratories) in the concentration of 10 ug/ml, and washed with PBS containing and 1% BSA. Coverslips were mounted onto glass slides in anti-fading medium (Mowiol 4-88, Calbichem Inc. La Jolla, CA) . Samples were immaged on a confocal laser scanning microscopy.

Mice

6- to 8-week-old female C57BL/6 mice from the National Cancer Institute (Frederick, Maryland) were purchased and kept in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, Maryland). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

DNA Vaccination

Preparation of DNA-coated gold particles and gene gun particle-mediated DNA vaccination was performed using a helium-driven gene gun (Bio-rad, Hercules, CA) according to a previously described protocol (21). DNA coated gold particles (1 μg DNA/bullet) were delivered to the shaved abdominal region of the mice using a helium-driven gene gun (Bio-rad, Hercules, CA) with a discharge pressure of 400 p.s.i..

Intracytoplasmic Cytokine Staining and Flow Cytometry Analysis

Splenocytes from naïve or vaccinated groups of mice were incubated either with the E7 peptide (aa 49-57) that contains MHC class I epitope (23) for detecting E7-specific $CD8^+$ T cell precursors or the E7 peptide (aa 30-67) that contains MHC class II peptide (24) for detecting E7-specific $CD4^+$ T helper cell precursors. The E7 peptide was added at a concentration of 2 μg/ml for 20 hours. Golgistop (Pharmigen, San Diego, CA) was added 6 hours before harvesting the cells from the culture. Cells were then washed once in FACScan buffer and stained with phycoerythrin (PE)-conjugated monoclonal rat anti-mouse CD8 or CD4 antibody (PharMingen, San Diego, CA). Cells were subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit according to the manufacturer's instructions (PharMingen). FITC-conjugated anti-IFN-γ antibody and the immunoglobulin isotype control antibody (rat IgG1) were all purchased from PharMingen. Analysis was done on a Becton Dickinson FACScan with CELLQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA).

ELISA

The anti-HPV 16 E7 antibodies in the sera were determined by a direct ELISA as described previously (25). Briefly, a 96 microwell plate was coated with 100 μl 5 μg/ml bacteria-derived HPV-16 E7 proteins and incubated at 4^{0}C overnight. The wells were then blocked with PBS containing 20% fetal bovine serum. Sera were prepared from the mice on day 14 post-immunization, serially diluted in PBS, added to the ELISA wells,

and incubated on 37 ^{0}C for 2 hr. After washing with PBS containing 0.05% Tween-20, the plate was incubated with 1/2000 dilution of a peroxidase-conjugated rabbit anti-mouse IgG antibody (Zymed, San Francisco, CA) at room temperature for one hour. The plate was washed 6 times, developed with TMB (Pierce, Rockford, IL), and stopped with 1M H_2SO_4. The ELISA plate was read with a standard ELISA reader at 450 nm.

In Vivo Tumor Protection Experiments

For the tumor protection experiment, mice (5 per group) were vaccinated via gene gun with 2 µg of FL DNA, E7 DNA, FL-E7 DNA or unvaccinated. One week later, the mice were boosted with the same regimen as the first vaccination. One week after the last vaccination, mice were subcutaneously challenged with 1 x 10^4 cells/mouse TC-1 tumor cells in the right leg and then monitored twice a week.

In Vivo Tumor Treatment Experiments

The tumor cells and DNA vaccines were prepared as described earlier. Mice were intravenously challenged with 1 x 10^4 cells/mouse TC-1 tumor cells via tail vein on D0. Three days (D3) after challenge with TC-1 tumor cells, mice were given 2 µg of FL DNA, E7 DNA, FL-E7 DNA via gene gun or unvaccinated. One week later, these mice were boosted with the same regimen as the first vaccination. The mice were sacrificed on D21. The number of pulmonary metastatic nodules of each mouse were evaluated and counted by experimenters blinded to sample identity.

In Vivo Antibody Depletion Experiments

In vivo antibody depletions have been described previously (20). Briefly, mice were vaccinated with 2 μg FL-E7 DNA via gene gun, boosted one week later, and challenged with 5 x 10^4 cells/mouse TC-1 tumor cells. Depletions were started one week prior to tumor challenge. MAb GK1.5 (26) was used for CD4 depletion, MAb 2.43 (27) was used for CD8 depletion, and MAb PK136 (28) was used for NK1.1 depletion. Flow cytometry analysis revealed that the >95% of the appropriate lymphocytes subset were depleted with normal levels of other subsets. Depletion was terminated on day 40 after tumor challenge.

Generation of Dentritic Cells (DCs)

DCs were generated by culture of bone marrow cells in the presence of GM-CSF as described previously (29). Briefly, bone marrow was collected from the tibias of mice. Erythrocytes were lysed, and the remaining cells were passed through a nylon mesh to remove small pieces of bone and debris. The cells were collected and 1x10^6 cells/ml were placed in 24-well plates in RMPI 1640, supplemented with 5% FCS, 2mM β-mercaptoethanol, 1% nonessential amino acids, 100 U/ml penicilin and 100 μg/ml streptomicin (Life Technologies, Rockville, MD), and 100 U/ml GM-CSF (PharMingen, San Diego, CA). Two-thirds of the medium was replaced every 2 days, and nonadherent cells were harvested on day 7. The collected cells were characterized by flow cytometry analysis for DC markers as previously described (30).

Generation of E7-Specific CD8⁺ T Cell Lines

Generation of E7-specfic CD8+ cell lines has been described previously (30). Briefly, female C57BL/6 (H-2b) mice were immunized by intraperitoneal injection of vaccina-Sig/E7/LAMP-1. Splenocytes were harvested on day 8. For initial in vitro stimulation, splenocytes were pulsed with IL-2 at a concentration of 20 U/ml and 1 μM E7 peptide (amino acids 49-57) for 6 days. Propagation of the E7-specific CTL cell line was performed in 24-well plates by mixing (2 ml/well) $1x10^6$ splenocytes containing E7-specific CTLs with $3x10^6$ irradiated splenocytes and pulsing them with IL-2 at a concentration of 20 U/ml and 1 μM E7 peptide (amino acids 49-57). This procedure was repeated every 6 days. The specificity of the E7 CTL line was characterized by the CTL assay. Flow cytometry was performed to demonstrate the expression of the CD8 marker.

Cytotoxic T Lymphocyte (CTL) Assays

CTL assays were performed in 96-well round-bottom platesas described by Corr et al. (31). Cytolysis was determined by quantitative measurements of lactate dehydrogenase (LDH) as previous report (31). Effector cells and targets cells were mixed together with various ratios in a final volume of 200 μl. After 5 h incubation at 37^0C, 50 μl of the cultured media were collected to assess the amount of LDH in the cultured media using CytoTox assay kits (Promega, Madison, WI) according to the manufacturer's protocol. The percentage of lysis was calculated from the following equation: 100 X (A-B)/(C-D) [where A is the reading of experimental-effector signal

value, B is the effector spontaneous background signal value, C is maximum signal value from target cells, D is the target spontaneous background signal value].

CTL Assay using Transfected 293 D^b,K^b Cells as Target Cells

$5x10^6$ 293 D^b,K^b cells were transfected with 20 μg of pcDNA3 (empty plasmid), E7, FL, or FL-E7 DNA vaccines via lipofactamine 2000 (Life Technologies, Rockville, MD) according to vendor's manual. The 293 D^b,K^b cells were trypsinized and centrifugated after 40-44 hr of transfection. The levels of E7 protein expression determined by ELISA are approximately similar in E7 and FL-E7 transfected 293 D^b,K^b. Transfected 293 D^b,K^b cells were used as target cells while E7-specific $CD8^+$ T cells served as effector cells. CTL assays with various E/T ratios (1:1, 3:1, 9:1, and 27:1) were performed and cytolysis was determined by quantitative measurements of LDH as described above.

CTL Assay Using DCs Pulsed with Lysates of Transfected 293 D^b,K^b Cells as Target Cells

CTL assays using DCs pulsed with cell lysates as target cells were performed as described by Uger and Barber (32) with modification. Briefly, $5x10^6$ 293 D^b,K^b cells were first transfected with 20 μg of pcDNA3 (empty plasmid), E7, FL, or FL-E7 DNA vaccines via lipofactamine 2000 (Life technologies, Rockville, MD) according to vendor's manual. 293 D^b,K^b cells were trypsinized and centrifugated after 40-44 hr of transfection. The transfected 293 D^b,K^b cells then received three cycles of freeze-thaw.

The protein concentration was determined using the BioRad protein assay (Bio-rad, Hercules, CA) using vendor's protocol. The quantity of E7 protein was determined using ELISA and the cell lysates from E7 or FL-E7 DNA transfected 293 D^b,K^b cells were standardized for E7 protein concentration. The DCs were used as target cells and prepared by pulsing 1 million of DCs with different concentration of cell lysates (50 μg/ml, 10 μg/ml, 2 μg/ml and 0.4 μg/ml) in a final volume of 2 ml for 16-20 hrs. E7-specific $CD8^+$ T cells were used as effector cells. CTL assays was performed at fixed E/T (9/1) ratio with $9x10^4$ of E7-specific T cells mixed with $1x10^4$ of prepared DCs in a final volume of 200 μl. CTL assays was determined by quantitative measurements of LDH as described above.

RESULTS

Generation and Characterization of FL-E7 Fusion DNA Vaccine

A schematic diagram showing the domains of Flt3-ligand and the construct of chimeric FL-E7 is presented in **Figure 1A**. The DNA fragments encoding both the signal peptide with the extracelluar domain of Flt3-ligand (aa 1-189) is depicted in black and HPV-16 E7 (aa 1-96) is depicted in white. **Figure 1B** shows the sequence of the chimeric FL-E7 construct. The underlined area represents the sequence of HPV-16 E7. The linker between the extracellular domain of Flt3-ligand and E7 is indicated in italics. The additional sequence encoded by the pcDNA vector before the stop codon is indicated in bold.

Linkage of the Extracellular Domain of Flt3-ligand to HPV-16 E7 Protein Partially Re-routes E7 into the Endoplasmic Reticulum

In order to determine the expression and localization of wild type and modified HPV-16 E7 protein, we have added gene encoding green fluorescent protein (GFP) to 3' end of E7 gene and chimeric FL-E7 genes as a tag. Transfection and subsequent examination with fluorescent microscope were used to determine expression and localization of wild type and modified HPV-16 E7 protein. As shown in Figure 2, the level of protein expression was quite similar between cells transfected with E7-GFP or FL-E7-GFP. As expected, cells transfected with the wild type E7 showed homogeneous cytoplasmic/nuclear distribution (**Figure 2A**). In comparison, cells transfected with the chimeric FL-E7 construct displayed a netlike pattern, different from the pattern observed in cells transfected with wild type E7 (**Figure 2B**). To explore if E7 and FL-E7 chimera have been distributed to the endoplamic reticulum (ER) compartments, we performed double labelling experiments of cells stably transfected with either wild type E7-GFP or FL-E7-GFP using antibodies against calnexin, a well characterized marker for the ER compartments. As shown in **Figure 2C** and **2D**, co-localization of E7 and the calnexin protein was only observed in cells transfected with FL-E7 but not wild type E7, indicating that at least some of the FL-E7 chimera was targeted to the ER compartments. Controls omitting primary antibodies did not show specific staining (data not shown). These data indicated that the addition of the extracellular domain of Flt3-ligand to E7 facilitate the entry of E7 into ER compartments.

Vaccination with FL-E7 Fusion DNA Significantly Enhances E7-Specific $CD8^+$ T Cell-Mediated Immune Responses

$CD8^+$ T lymphocytes are one of the most crucial effectors for inducing anti-tumor immunity. To determine the quantity of E7-specific $CD8^+$ T cell precursors generated by the FL-E7 DNA vaccine, intracellular cytokine staining was used. This is a sensitive functional assay for measuring IFN-γ production at the single-cell level that can be applied to quantify antigen-specific $CD8^+$ T cells (33). As shown in **Figure 3**, mice vaccinated with FL-E7 DNA generated the highest number of E7-specific IFN-γ^+ $CD8^+$ T cell precursors ($94/3x10^5$ splenocytes) using flow cytometry analysis, whereas mice vaccinated with E7 DNA generated fewer precursors ($12/3x10^5$ splenocytes) ($p<0.01$). FL-E7 DNA immunization led to a nearly 8-fold increase in the number of E7-specific $CD8^+$ T cell precursors. These results also indicated that fusion of E7 to FL was required for enhancement of CD8+ T cell activity, since FL mixed to E7 (FL + E7 DNA) did not generate enhancement of CD8+ T cell activity.

Vaccination with FL-E7 Fusion DNA Does Not Enhance E7-Specific $CD4^+$ T Cell-Mediated Immune Responses

To examine the generation of E7-specific $CD4^+$T precursor cells and cytokine profiles by each of these vaccines, we performed double staining for CD4 surface marker and intracellular IFN-γ or IL-4 on splenocytes obtained from immunized mice, followed by flow cytometry analysis. The splenocytes were cultured *in vitro* with E7 peptide (aa 30-67) overnight and stained for both CD4 and intracellular IFN-γ or IL–4. The E7

peptide (aa 30-67) contains a major T helper epitope in the E7 open reading frame protein of HPV-16 (24). The percentage of IFN-γ or IL-4 secreting $CD4^+$ T cells was analyzed using flow cytometry. As shown in **Figure 4A**, mice vaccinated with FL-E7 DNA generated a similar number of $CD4^+$ IFN-γ^+ double positive cells compared to mice vaccinated with wild-type E7 DNA ($6/3x10^5$ splenocytes versus $4/3x10^5$ splenocytes, $p>0.05$) or other DNA groups. There was no significant difference in the number of E7-specific $CD4^+$ IFN-γ^+ cells observed using flow cytometry staining among naïve mice or mice vaccinated with empty plasmid, FL, E7, FL + E7, or FL-E7 DNA.

We then analyzed IL-4-secreting E7-specific $CD4^+$ T cells in mice vaccinated with various DNA vaccines. IL-4-secreting activated mouse splenocytes (MiCK-2, PharMingen) were used as positive controls to assure the success of intracellular IL-4 staining for this study. The specificity of the IL-4 staining was demonstrated by the absence of $CD4^+$ IL-4^+ T cells when the IL-4 antibody was omitted. As shown in **Figure 4B**, no significant $CD4^+$ IL-4^+ double-positive cells were identified in the mice vaccinated with FL-E7, FL DNA, wild type E7 DNA, plasmid DNA vaccination or in the naïve mice without vaccination. In addition, no significant variation was observed in the frequency of IL-4-secreting $CD4^+$ IL-4^+ T cells in the different vaccination groups.

Also, the quantity of anti-HPV 16 E7 antibodies in the sera of the vaccinated mice was determined using a direct ELISA 2 weeks after the last vaccination and read at 450 nm. No significant E7-specific antibody responses were detected in mice vaccinated with chimeric FL-E7 DNA, FL, or empty plasmid, or in naïve mice. (data not shown)

Vaccination with Chimeric FL-E7 DNA Vaccine Enhances Protection of Mice Against the Growth of TC-1 Tumors

To determine whether vaccination with various DNA vaccine constructs protects mice against E7-expressing tumors, *in vivo* tumor protection experiments were performed. Mice were vaccinated with 2 μg naked DNA/mouse via gene gun and boosted with the same dose one week later. Mice were then challenged with 1×10^4 TC-1/mouse subcutaneously in the right leg 7 days after the last vaccination. As shown in **Figure 5**, 100% of those receiving FL-E7 DNA vaccination remained tumor-free 70 days after TC-1 challenge. In contrast, only 20% of mice receiving wild-type E7 remained tumor free after day 32 and all unvaccinated mice, or FL DNA developed tumor growth within 20 days after tumor challenge. These results also indicated that fusion of E7 to FL was required for antitumor immunity, since only 20% of mice receiving FL mixed with E7 (FL + E7 DNA) remained tumor free after 32 days. Therefore, FL-E7 fusion DNA significantly enhanced the anti-tumor immunity against the growth of TC-1 tumors.

Treatment with FL-E7 Fusion DNA Eradicates Established E7-expressing Tumors in the Lungs

To determine the therapeutic potential of a chimeric FL-E7 DNA construct in treating TC-1 tumor metastases in the lungs, mice were first challenged with 1×10^4 TC-1 tumor cells per mouse via intravenous injection in the tail vein (a previously described lung metastasis model) (34, 35). Mice were then treated with 2 μg naked DNA via gene gun 3 days later and boosted with the same dose 1 week later. Mice were sacrificed 25

days after tumor challenge. As shown in **Figure 6A**, mice vaccinated with FL-E7 DNA demonstrated the lowest mean pulmonary nodule numbers (5.8±3.6) compared to mice vaccinated with wild-type E7 DNA (67.5±3.5), FL mixed with E7 DNA (68±15),or FL DNA (65±5.0) or unvaccinated mice (control) (50.66±7.3) (one-way ANOVA, P<0.001). Data are expressed as mean number of pulmonary metastatic tumor nodules ±SEM. Furthermore, mice vaccinated with FL-E7 DNA also had the lowest mean lung weight (g) (0.158±0.025) compared to mice vaccinated with wild-type E7 DNA (0.462±0.02), FL plus E7 DNA (0.469±0.08),or FL DNA (0.6±0.03), or unvaccinated mice (control) (0.645±0.08) (one-way ANOVA, P<0.001) (**Figure 6B**). Data are expressed as mean lung weight ±SEM. Representative gross photographs of the lung tumors are shown in **Figure 7**.

$CD8^+$ T Cells But Not $CD4^+$ T cells are Essential for the Anti-tumor Effect Generated by DNA Vaccine with E7 Fused to FL

To determine the subset of lymphocytes that are important for the rejection of E7-positive tumor cells, we performed in vivo antibody depletion experiments (20, 36). Depletion of lymphocyte subsets was assessed on the day of tumor injection, and weekly thereafter by flow cytometry analysis of spleen cells. More than 99% depletion of the appropriate subset was achieved with normal levels of the other subsets (data not shown). As shown in **Figure 8**, all naïve mice and all of the mice depleted of $CD8^+$ T cells grew tumors within 14 days after tumor challenge. In contrast, all of the non-depleted mice and all of the mice depleted of $CD4^+$ T cells remained tumor-free 60 days after tumor challenge. 40% of NK1.1-depleted mice grew tumors 6 weeks after tumor injections.

These results suggest that $CD8^{+}$ T cells are essential for E7-specific anti-tumor immunity generated by the FL-E7 DNA vaccine. NK1.1 cells may also contribute to the antitumor effect generated by the FL-E7 DNA vaccine.

Enhanced Presentation of E7 through the MHC Class I Pathway in Cells Transfected with FL-E7 DNA

From the immunological assays of vaccinated mice, we observed that mice vaccinated with FL-E7 generated the highest number of E7-specific CD8+ T cell precursors (**Figure 3**). In order to determine the mechanism that accounts for such a phenomenon, we first tested if there is enhanced MHC class I presentation ability of E7 in target cells (in this case, human embryonic kidney 293 cells expressing D^{b} and K^{b} transfected with FL-E7. E7-specific $CD8^{+}$ T cells served as effector cells. Human embryonic kidney 293 cells expressing K^{b} and D^{b}, two mouse MHC molecules, were kindly provided by Dr. JC Yang (National Cancer Institute, National Institutes of Health, Bethesda) and used as target cells. 293 D^{b},K^{b} cells have been shown to have a stable transfection efficiency. In addition, the level of E7 expression in 293 D^{b},K^{b} cells is similar in cells transfected with different E7-containing DNA constructs (data not shown). A CTL assay was performed using naïve 293 D^{b},K^{b} cells and 293 D^{b},K^{b} cells transfected with empty plasmid, FL, E7, or FL-E7 DNA with various E/T ratios (1:1, 3:1, 9:1, 27:1) using an E7-specific T cell line. As shown in **Figure 9**, 293 D^{b},K^{b} cells transfected with FL-E7 DNA generated significantly higher percentages of specific lysis on the 9:1 (21.03±3.62% versus 12.5±1.12%, $P<0.001$) and 27:1 (44.52±2.75% versus 22.66±1.71%, $P<0.001$) E/T ratios compared to mice vaccinated with wild-type E7 DNA

vaccine. These results indicate that cells transfected with FL-E7 DNA are capable of presenting E7 antigen through the MHC class I pathway in a more efficient manner than cells transfected with wild-type E7 DNA.

Enhanced Presentation of E7 Through the MHC Class I Pathway in Dendritic Cells Pulsed With Chimeric FL-E7 Protein

Another potential mechanism for the enhanced E7-specific CD8+ T cell immune responses *in vivo*, is the presentation of E7 through the MHC class I pathway by uptake of lysed cells expressing various DNA constructs, also called "cross-priming". A cross priming experiment was performed to characterize the MHC class I presentation of E7 dendritic cells pulsed with cell lysates of 293 D^b,K^b cells transfected with empty plasmid, FL, E7, or FL-E7 DNA. As mentioned previously, 293 cells have been shown to have stable transfection efficiency and similar E7 expression among cells transfected with different E7-containing DNA constructs. Lysates of transfected 293 D^b,K^b cells were obtained from cycles of freeze-thaw. One million of Bone marrow-derived DCs were pulsed with a serial dilution of lysates derived from various transfected 293 D^b,K^b cells (50 μg, 10 μg, 2μg, 0.4 μg). DCs were used as target cells while E7-specific $CD8^+$ T cells served as effector cells. CTL assays were performed with a fixed E/T ratio (9/1). As shown in **Figure 10**, DCs pulsed with lysates from 293 D^b,K^b cells transfected with FL-E7 DNA generated significantly higher percentages of specific lysis as compared to DCs pulsed with lysates from 293 D^b,K^b cells transfected with the other DNA constructs and naïve DCs. The FL-E7 DNA generated significantly higher percentages of specific lysis when target cells, DCs, were the 1/10 dilution ($76.67 \pm 5.81\%$ versus $25.44 \pm 4.2\%$,

P<0.001) and 1/50 dilution (20.0±2.11% versus 5.25±2.4%, P<0.001) as compared with cell transfected with wild-type E7 DNA vaccine. These results revealed that dendritic cells pulsed with FL-E7 fusion protein are capable of presenting E7 antigen through the MHC class I pathway in a more efficient manner than dendritic cells pulsed with wild-type E7 protein. Our data suggest that the fusion of FL to E7 may enhance E7-specific CD8+ T cell immune responses via both direct and cross priming effects.

Vaccination with FL, FL-E7 Fusion DNA Does Not Increase the Number of DCs or NK cells in the Spleen

Since Flt3-ligand has been shown to significantly expand DCs in animals (37-39), we investigated if mice vaccinated with FL-E7 or FL DNA vaccines would exhibit expansion of DCs in vivo. C57BL/7 mice immunized intradermerally with gene gun with FL-E7, E7, FL. Splenocytes were analyzed 7 days from the last vaccination. The percentages of CD11c$^+$ DCs in splenocytes were measured using flow cytometry analysis. Gates for the population were based on the control bone marrow dendritric cells. As shown in **Figure 11A**, no significant increase in percentages of DC cells was observed in mice vaccinated with FL-E7 or FL compared to mice vaccinated with E7 or unvaccinated mice. Furthermore, DCs derived from the draining lymph nodes of vaccinated mice did not show significant difference in mice vaccinated with different DNA vaccines (data not shown). These data indicated that mice vaccinated with FL or FL-E7 DNA did not increase the number of DCs *in vivo*.

Flt3-ligand has also been shown to significantly expand NK cells in animals (17, 40). Therefore, we investigated if mice vaccinated with FL-E7 or FL DNA vaccines

would increase the number of NK cells *in vivo*. For the determination of $CD3^-$,$NK1.1^+$ cells in the spleen of vaccinated mice, cells were stained simultaneously with FITC-conjugated anti-CD3 and PE-conjugated anti-NK1.1 mAbs and analyzed by flow cytometry. As shown in **Figure 11B**, no significant increase of the number of $CD3^-$,$NK1.1^+$ cells in the splenocytes of mice vaccinated with different DNA vaccines was observed. These data indicated that mice vaccinated with FL or FL-E7 DNA did not increase the number of NK cells *in vivo*.

DISCUSSION

In this study we demonstrated that linkage of the extracellular domain of Flt3-ligand to E7 can significantly enhance the potency of HPV-16 E7-expressing DNA vaccines. DNA vaccines with FL fused to HPV-16 E7 generated significant $CD8^+$ T cell-dependent preventive effects against HPV-16 E7-expressing murine tumors. Furthermore, the chimeric FL-E7 DNA vaccine was capable of controlling lethal pulmonary metastatic tumors.

Our data demonstrated that the incorporation of FL can preferentially enhance $CD8^+$ T cell responses of E7 DNA vaccines in vaccinated mice. In contrast, E7-specific $CD4^+$ T cell responses were not significantly enhanced by FL-E7 DNA vaccine. We found that the linkage of FL to E7 directly enhanced MHC class I presentation of E7 compared to wild type E7 in transfected cells *in vitro* (**Figure 9**). Since ballistic DNA delivery can introduce DNA directly into dermal professional antigen presenting cells (APCs), the FL-E7 DNA-transfected APCs may directly enhance the presentation of E7

through MHC class I pathway to $CD8^+$ T cells and contribute to the generation of E7-specific $CD8^+$ T cell precursors *in vivo*.

Although it is not clear how the linkage of FL to E7 can directly enhance MHC class I presentation of E7, one of the possible mechanisms for the enhancement of MHC class I presentation of E7 may be related the chaperone effect of FL. Flt3-ligand expressed in cells has been distributed to the ER and Golgi apparatus (41). In our study, we have used fluorescent microscopic examination to investigate the distribution of E7 and FL-E7 proteins linked to green fluorescent protein (GFP) within transfected 293 D^b,K^b cells. In cells transfected with FL-E7-GFP, some of the FL-E7-GFP protein showed co-localization with calnexin in the ER (**Figure 2**). Thus, the addition of the extracellular domain of Flt3-ligand to E7 may facilitate the entry of E7 into the ER. Several studies have demonstrated that ER targeting may lead to enhanced antigen-specific MHC class I-restricted CTL activity (42-44).

Another important mechanism that may contribute to enhanced E7-specific $CD8^+$ T cell immune responses *in vivo* is the so-called "cross-priming" effect, whereby lysis of cells expressing FL-E7 antigen can release exogenous protein to be taken up and processed by other antigen-presenting cells via the MHC-I-restricted pathway. Our data suggested that dendritic cells pulsed with FL-E7 fusion protein are capable of presenting E7 antigen through the MHC class I pathway in a more efficient manner than dendritic cells pulsed with wild-type E7 protein. (**Figure 10**). However, the "cross-priming" of chimeric FL-E7 probably does not play a major role in the gene gun-mediated FL-E7 DNA vaccines. It has been shown that direct priming of $CD8^+$ T cells by gene-transfected DCs is the key event in gene gun-mediated DNA immunization (45, 46), while cross-

priming of DCs is not an major mechanism for gene gun-mediated DNA vaccination (45, 46). However, we can not completely rule out the possibility of cross-priming, because FL-E7 might be released from other cell types, such as keratinocytes (which were also transfected by gene gun vaccination), and then enter the DCs via the cross-priming mechanism.

In this study, we failed to detect significant E7-specific IFN-γ or IL-4-secreting $CD4^+$ T cells in FL-E7 vaccinated mice (**Figure 4**). The observed enhancement of E7-specific CD8+ T cell immune responses was most likely not facilitated by E7-specific $CD4^+$ T helper cells. However, the generation of FL-specific $CD4^+$ T cells by FL-E7 DNA is a possibility, which may contribute to the generation and expansion of E7-specific $CD8^+$T cells. A previous study has shown that cross-reactive T helper cells (in this case FL-specific $CD4^+$ T cells) can exert a strong helper effect by reacting to conjugated peptides.

We have observed that NK cells contribute to the anti-tumor effect generated by the FL-E7 vaccine since depletion of NK cells in vivo compromised the antitumor effects induced by FL-E7 vaccine. Flt3 ligand-expanded DCs have been shown to promote NK cell-dependent anti-tumor effects. For example, Fernandez et al. have demonstrated that cell-to-cell contact between DC and resting NK cells may result in a substantial increase in both NK cell cytolytic activity and IFN-γ production in an *in vitro* studies (29). Since NK cells do not have specificity for tumor antigens, NK cells most likely aid in mediating anti-tumor immunity through interaction with other effector cells such as E7-specific $CD8^+$ T cells. It is quite possible that the IFN-γ released from activated E7-specific $CD8^+$

T cells around E7-expressing tumor cells may further enhance NK cell activities around the tumor cells and consequently facilitate the elimination of E7-expressing tumor cells *in vivo*.

Although Flt3-ligand has been shown to significantly expand DCs (17) and NK cells (40, 47), we did not detect a significant increase in the number of DCs or NK cells in the spleens of mice vaccinated with FL-E7 DNA vaccines. This may be related to a low quantity of FL-E7 released in blood circulation after DNA vaccination. We were not able to detect any FL-E7 protein in the sera derived from mice vaccinated with FL-E7 DNA (data not shown). This finding raises the issue of the source of FL-E7 protein for cross-priming. One possibility is that FL-E7 protein from the lysis of transfected keratinocytes may be taken up by Langerhans' cells and further processed in the draining lymph nodes without involving blood circulation.

Since antigen-specific CD4+ and CD8+ T cells have been shown to be important in the generation of antitumor immunity, co-administration of vaccines that directly enhance MHC class I and class II presentation may be a strategy to further enhance vaccine potency. We demonstrated that the FL-E7 vaccine in this paper generated potent CD8+ T cell-mediated immune responses. Additionally, we have previously developed a chimeric Sig/E7/LAMP-1 DNA vaccine that uses the LAMP-1 endosomal/lysosomal targeting signal for enhancing the MHC class II presentation pathway of E7 (25). The FL-E7 vaccine described here may be used in conjunction with a MHC class II-targeting vaccine such as Sig/E7/LAMP-1 to activate multiple arms of the immune system in a synergistic fashion, leading to significantly enhanced CD4+ and CD8+ T cell responses and potent antitumor effects.

The FL-E7 DNA vaccine may raise certain safety issues that need to be addressed before it is used for widespread vaccination. First, there is the concern that DNA may integrate into the host genome, resulting in the inactivation of tumor suppresser genes or the activation of oncogenes and leading to malignant transformation of the host cell. Fortunately, it is estimated that the frequency of integration is much lower than that of spontaneous mutation and integration should not pose any real risk (48). The second issue concerns potential risks associated with the presence of HPV-16 E7 protein in host cells. E7 is an oncoprotein that disrupts cell cycle regulation by binding to tumor suppressor pRB protein in nuclei (49). Thus, the presence of E7 in host cells may lead to accumulation of genetic aberrations and eventual malignant transformation in the host cells. The oncogenicity of E7 can be eliminated by introducing mutations into E7 DNA so that the resulting E7 protein cannot bind with pRB (50) but still maintains most of its antigenicity. The third issue is the concern over the generation of autoimmunity that may be caused when FL leads to excessive expansion of dendritic cells *in vivo*. However, we did not observe any significant increase in the number of DCs in the spleen or lymph nodes of mice vaccinated with FL or FL-E7 DNA vaccines. Furthermore, we performed pathological examination of the vital organs in the FL-E7-vaccinated mice. We did not observe any significant pathology, indicating that FL-E7 is a potent vaccine with no detectable side effects.

In summary, our results indicate that fusion of FL to HPV-16 E7 gene can generate stronger E7-specific CD8+ T cell-mediated immune responses and antitumor effects against HPV-16 E7-expressing murine tumors generated by E7 DNA vaccines. Our results indicate that fusion of FL to an antigen gene may greatly enhance the potency

of DNA vaccines and can potentially be applied to other cancer systems with known tumor-specific antigens.

ACKNOWLEDGEMENTS

We would like to thank Drs, Keerti V. Shah, Robert J. Kurman, Elizabeth Jaffee, Drew M. Pardoll for insightful discussions. We also would like to thank Dr. Richard Roden, for critical review of the manuscript. We greatly appreciate Yanqin Yang for her excellent technical assistance. This work was supported by NIH 5 POI 34582-01, U19 CA 72108-02, RO1 CA 72631-01, the Richard W. TeLinde fund and the Alexander and Margaret Stewart Trust grant.

REFERENCES

1. Chen, C. H. and Wu, T. C. Experimental vaccine strategies for cancer immunotherapy, J Biomed Sci. *5:* 231-52, 1998.

2. Robinson, H. L. and Torres, C. A. DNA vaccines, Semin Immunol. *9:* 271-83, 1997.

3. Pardoll, D. M. and Beckerleg, A. M. Exposing the immunology of naked DNA vaccines, Immunity. *3:* 165-9, 1995.

4. Donnelly, J. J., Ulmer, J. B., Shiver, J. W., and Liu, M. A. DNA vaccines, Annu Rev Immunol. *15:* 617-48, 1997.

5. Lee, A. H., Suh, Y. S., and Sung, Y. C. DNA inoculations with HIV-1 recombinant genomes that express cytokine genes enhance HIV-1 specific immune responses, Vaccine. *17:* 473-9, 1999.

6. Lee, S. W., Cho, J. H., and Sung, Y. C. Optimal induction of hepatitis C virus envelope-specific immunity by bicistronic plasmid DNA inoculation with the granulocyte-macrophage colony-stimulating factor gene, J Virol. *72:* 8430-6, 1998.

7. Banchereau, J. and Steinman, R. M. Dendritic cells and the control of immunity, Nature. *392:* 245-52, 1998.

8. Maraskovsky, E., Brasel, K., Teepe, M., Roux, E. R., Lyman, S. D., Shortman, K., and McKenna, H. J. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified, J Exp Med. *184.* 1953-62, 1996.

9. Shurin, M. R., Pandharipande, P. P., Zorina, T. D., Haluszczak, C., Subbotin, V. M., Hunter, O., Brumfield, A., Storkus, W. J., Maraskovsky, E., and Lotze, M. T. FLT3 ligand induces the generation of functionally active dendritic cells in mice, Cell Immunol. *179:* 174-84, 1997.

10. Rosnet, O., Marchetto, S., deLapeyriere, O., and Birnbaum, D. Murine Flt3, a gene encoding a novel tyrosine kinase receptor of the PDGFR/CSF1R family, Oncogene. *6:* 1641-50, 1991.

11. Lyman, S. D. Biologic effects and potential clinical applications of Flt3 ligand, Curr Opin Hematol. *5:* 192-6, 1998.

12. Lyman, S. D., James, L., Vanden Bos, T., de Vries, P., Brasel, K., Gliniak, B., Hollingsworth, L. T., Picha, K. S., McKenna, H. J., Splett, R. R., and et al. Molecular cloning of a ligand for the flt3/flk-2 tyrosine kinase receptor: a proliferative factor for primitive hematopoietic cells, Cell. *75:* 1157-67, 1993.

13. Hannum, C., Culpepper, J., Campbell, D., McClanahan, T., Zurawski, S., Bazan, J. F., Kastelein, R., Hudak, S., Wagner, J., Mattson, J., and et al. Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of haematopoietic stem cells and is encoded by variant RNAs, Nature. *368:* 643-8, 1994.

14. Lynch, D. H., Andreasen, A., Maraskovsky, E., Whitmore, J., Miller, R. E., and Schuh, J. C. Flt3 ligand induces tumor regression and antitumor immune responses in vivo, Nat Med. *3:* 625-31, 1997.

15. Chen, K., Braun, S., Lyman, S., Fan, Y., Traycoff, C. M., Wiebke, E. A., Gaddy, J., Sledge, G., Broxmeyer, H. E., and Cornetta, K. Antitumor activity and immunotherapeutic properties of Flt3-ligand in a murine breast cancer model, Cancer Res. *57:* 3511-6, 1997.

16. Braun, S. E., Chen, K., Blazar, B. R., Orchard, P. J., Sledge, G., Robertson, M. J., Broxmeyer, H. E., and Cornetta, K. Flt3 ligand antitumor activity in a murine breast cancer model: a comparison with granulocyte-macrophage colony-stimulating factor and a potential mechanism of action, Hum Gene Ther. *10:* 2141-51, 1999.

17. Peron, J. M., Esche, C., Subbotin, V. M., Maliszewski, C., Lotze, M. T., and Shurin, M. R. FLT3-ligand administration inhibits liver metastases: role of NK cells, J Immunol. *161:* 6164-70, 1998.

18. Chakravarty, P. K., Alfieri, A., Thomas, E. K., Beri, V., Tanaka, K. E., Vikram, B., and Guha, C. Flt3-ligand administration after radiation therapy prolongs survival in a murine model of metastatic lung cancer, Cancer Res. *59:* 6028-32, 1999.

19. Esche, C., Subbotin, V. M., Maliszewski, C., Lotze, M. T., and Shurin, M. R. FLT3 ligand administration inhibits tumor growth in murine melanoma and lymphoma, Cancer Res. *58:* 380-3, 1998.

20. Lin, K.-Y., Guarnieri, F. G., Staveley-O'Carroll, K. F., Levitsky, H. I., August, T., Pardoll, D. M., and Wu, T.-C. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen., Cancer Research. *56:* 21-26., 1996.

21. Chen, C.-H., Wang, T.-L., Hung, C.-F., Yang, Y., Young, R. A., Pardoll, D. M., and Wu, T.-C. Enhancement of DNA vaccine potency by linkage of antigen gene to an HSP70 gene, Cancer Research. *60:* 1035-1042., 2000.

22. Bloom, M. B., Perry-Lalley, D., Robbins, P. F., Li, Y., el-Gamil, M., Rosenberg, S. A., and Yang, J. C. Identification of tyrosinase-related protein 2 as a tumor rejection antigen for the B16 melanoma, J Exp Med. *185:* 453-9, 1997.

23. Feltkamp, M. C., Smits, H. L., Vierboom, M. P., Minnaar, R. P., de, J. B., Drijfhout, J. W., ter, S. J., Melief, C. J., and Kast, W. M. Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells, Eur J Immunol. *23:* 2242-9, 1993.

24. Tindle, R. W., Fernando, G. J., Sterling, J. C., and Frazer, I. H. A "public" T-helper epitope of the E7 transforming protein of human papillomavirus 16 provides cognate help for several E7 B-cell epitopes from cervical cancer-associated human papillomavirus genotypes, Proc Natl Acad Sci U S A. *88:* 5887-91, 1991.

25. Wu, T.-C., Guarnieri, F. G., Staveley-O'Carroll, K. F., Viscidi, R. P., Levitsky, H. I., Hedrick, L., Cho, K. R., August, T., and Pardoll, D. M. Engineering an

intracellular pathway for MHC class II presentation of HPV-16 E7, Proc. Natl. Acad. Sci. *92:* 11671-11675., 1995.

26. Dialynas, D. P., Wilde, D. B., Marrack, P., Pierres, A., Wall, K. A., Havran, W., Otten, G., Loken, M. R., Pierres, M., Kappler, J., and et al. Characterization of the murine antigenic determinant, designated L3T4a, recognized by monoclonal antibody GK1.5: expression of L3T4a by functional T cell clones appears to correlate primarily with class II MHC antigen-reactivity, Immunol Rev. *74:* 29-56, 1983.

27. Sarmiento, M., Glasebrook, A. L., and Fitch, F. W. IgG or IgM monoclonal antibodies reactive with different determinants on the molecular complex bearing Lyt 2 antigen block T cell-mediated cytolysis in the absence of complement, J. Immunol. *125:* 2665, 1980.

28. Koo, G. C., Dumont, F. J., Tutt, M., Hackett, J., Jr., and Kumar, V. The NK-1.1(-) mouse: a model to study differentiation of murine NK cells, J Immunol. *137:* 3742-7, 1986.

29. Fernandez, N. C., Lozier, A., Flament, C., Ricciardi-Castagnoli, P., Bellet, D., Suter, M., Perricaudet, M., Tursz, T., Maraskovsky, E., and Zitvogel, L. Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo, Nat Med. *5:* 405-11, 1999.

30. Wang, T.-L., Ling, L., Shih, I.-M., Pham, T., Pai, S. I., Lu, L., Kurman, R. J., Pardoll, D. M., and Wu, T.-C. Intramuscular Administration of E7-Transfected Dendritic Cells Generates the Most Potent E7-Specific Anti-Tumor Immunity, Gene Therapy (in press), 2000.

31. Corr, M., von Damm, A., Lee, D. J., and Tighe, H. In vivo priming by DNA injection occurs predominantly by antigen transfer, J Immunol. *163:* 4721-7, 1999.

32. Uger, R. A. and Barber, B. H. Creating CTL targets with epitope-linked beta 2-microglobulin constructs, J Immunol. *160:* 1598-605, 1998.

33. Murali-Krishna, K., Altman, J. D., Suresh, M., Sourdive, D. J., Zajac, A. J., Miller, J. D., Slansky, J., and Ahmed, R. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection, Immunity. *8:* 177-87, 1998.

34. Ji, H., Chang, E. Y., Lin, K. Y., Kurman, R. J., Pardoll, D. M., and Wu, T. C. Antigen-specific immunotherapy for murine lung metastatic tumors expressing human papillomavirus type 16 E7 oncoprotein, Int J Cancer. *78:* 41-5, 1998.

35. Chen, C.-H., Ji, H., Suh, K. W., Choti, M. A., Pardoll, D. M., and Wu, T.-C. Gene gun-mediated DNA vaccination induces antitumor immunity against human papillomavirus type 16 E7-expressing murine tumor metastases in the liver and lungs, Gene Therapy (in press), 1999.

36. Wu, T.-C., Huang, A. Y. C., Jaffee, E., Levitsky, H., and Pardoll, D. M. A reassessment of the role of B7-1 in anti-tumor immunity., J. Exp. Med. (in press), 1995.

37. Drakes, M. L., Lu, L., Subbotin, V. M., and Thomson, A. W. In vivo administration of flt3 ligand markedly stimulates generation of dendritic cell progenitors from mouse liver, J Immunol. *159:* 4268-78, 1997.

38. Lynch, D. H. Induction of dendritic cells (DC) by Flt3 Ligand (FL) promotes the generation of tumor-specific immune responses in vivo, Crit Rev Immunol. *18:* 99-107, 1998.

39. Borges, L., Miller, R. E., Jones, J., Ariail, K., Whitmore, J., Fanslow, W., and Lynch, D. H. Synergistic action of fms-like tyrosine kinase 3 ligand and CD40 ligand in the induction of dendritic cells and generation of antitumor immunity in vivo, J Immunol. *163:* 1289-97, 1999.

40. Williams, N. S., Moore, T. A., Schatzle, J. D., Puzanov, I. J., Sivakumar, P. V., Zlotnik, A., Bennett, M., and Kumar, V. Generation of lytic natural killer 1.1+, Ly-49- cells from multipotential murine bone marrow progenitors in a stroma-free culture: definition of cytokine requirements and developmental intermediates, J Exp Med. *186:* 1609-14, 1997.

41. Chklovskaia, E., Jansen, W., Nissen, C., Lyman, S. D., Rahner, C., Landmann, L., and Wodnar-Filipowicz, A. Mechanism of flt3 ligand expression in bone marrow failure: translocation from intracellular stores to the surface of T lymphocytes after chemotherapy-induced suppression of hematopoiesis, Blood. *93:* 2595-604, 1999.

42. Shiver, J. W., Davies, M. E., Perry, H. C., Freed, D. C., and Liu, M. A. Humoral and cellular immunities elicited by HIV-1 vaccination, J Pharm Sci. *85:* 1317-24, 1996.

43. Boyle, J. S., Koniaras, C., and Lew, A. M. Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: cytotoxic T lymphocyte and antibody responses are suboptimal when antigen is cytoplasmic after intramuscular DNA immunization, Int Immunol. *9:* 1897-906, 1997.

44. Hsu, S. C., Obeid, O. E., Collins, M., Iqbal, M., Chargelegue, D., and Steward, M. W. Protective cytotoxic T lymphocyte responses against paramyxoviruses

induced by epitope-based DNA vaccines: involvement of IFN-gamma, Int Immunol. *10:* 1441-7, 1998.

45. Porgador, A., Irvine, K. R., Iwasaki, A., Barber, B. H., Restifo, N. P., and Germain, R. N. Predominant role for directly transfected dendritic cells in antigen presentation to CD8+ T cells after gene gun immunization, J Exp Med. *188:* 1075-82, 1998.

46. Akbari, O., Panjwani, N., Garcia, S., Tascon, R., Lowrie, D., and Stockinger, B. DNA vaccination: transfection and activation of dendritic cells as key events for immunity, J Exp Med. *189:* 169-78, 1999.

47. Shaw, S. G., Maung, A. A., Steptoe, R. J., Thomson, A. W., and Vujanovic, N. L. Expansion of functional NK cells in multiple tissue compartments of mice treated with Flt3-ligand: implications for anti-cancer and anti- viral therapy, J Immunol. *161:* 2817-24, 1998.

48. Nichols, W. W., Ledwith, B. J., Manam, S. V., and Troilo, P. J. Potential DNA vaccine integration into host cell genome., Annals of NY Academy of Science. *772:* 30-39., 1995.

49. Lukas, J., Muller, H., Bartkova, J., Spitkovsky, D., Kjerulff, A. A., Jansen, D. P., Strauss, M., and Bartek, J. DNA tumor virus oncoproteins and retinoblastoma gene mutations share the ability to relieve the cell's requirement for cyclin D1 function in G1, J Cell Biol. *125:* 625-38, 1994.

50. Heck, D. V., Yee, C. L., Howley, P. M., and Munger, K. Efficiency of binding the retinoblastoma protein correlates with the transforming capacity of the E7

oncoproteins of the human papillomaviruses, Proc Natl Acad Sci U S A. *89:* 4442-6, 1992.

Figure Legends

Figure 1. Schematic diagram depicting the Flt3-ligand and chimeric FL-E7 constructs and nucleotide sequences.

(A) Diagram of Flt3-ligand. The signal peptide and extracellular domain (EX, aa 1-189) is black, followed by the transmembrane domain (TM) and cytoplasmic domain (CY). Diagram of chimeric FL-E7. The extracellular domain of Flt3-ligand (FL) is chimerically linked to HPV-16 E7 (aa 1-96). (B) Nucleotide sequence of chimeric FL-E7. The sequence of HPV-16 E7 is underlined. The linker between the extracellular domain of Flt3-ligand and E7 is italicized. The additional sequence encoded by the pcDNA vector before the stop codon is bolded.

Figure 2. Confocal fluorescent microscopic examination to demonstrate the expression and distribution of E7 and chimeric FL-E7 proteins in cells transfected with either pcDNA3-E7-GFP or pcDNA3-FL-E7-GFP DNA

293 D^b,K^b cells were transfected with pcDNA3 E7-GFP (A and C) or pcDNA3 FL-E7-GFP DNA (B and D) with lipofectamine. Immunofluorescent stainings were performed by incubating transfected 293 D^b,K^b cells with mouse anti-calnexin monoclonal antibody followed by Cy3-conjugated goat anti-mouse IgG secondary antibodies. For the detection of GFP protein, green fluorescent was noted (A and B). For the detection of endogenous calnexin protein, we observed the areas fluorescently stained by Cy3- (C and D). Controls which omitted primary antibodies did not show specific staining (data not shown).

Figure 3. Intracellular cytokine staining with flow cytometry analysis to determine E7-specific CD8⁺ T cell precursors in C57BL/6 mice

Mice were immunized with FL DNA, E7 DNA (E7), FL-E7 DNA or FL mixed with E7 DNA (FL + E7) via gene gun, or received no vaccination. For vaccinated mice, 2 μg DNA /mouse was administered twice. Splenocytes were harvested 7 days after the last DNA vaccination. E7-specific $CD8^+$ T cells. (A) Splenocytes from vaccinated mice were cultured *in vitro* with E7 peptide (aa 49-57) overnight and were stained for both CD8 and intracellular IFN-γ. The number of IFN-γ secreting $CD8^+$ T cell precursors in mice immunized with various recombinant DNA vaccines was analyzed by flow cytometry. Mice vaccinated with FL-E7 DNA generated the highest IFN-γ^+ $CD8^+$ double positive T cells compared to other groups. (B) The number of IFN-γ-producing E7-specific $CD8^+$ T cells was determined using flow cytometry in the presence (solid columns) and absence (open columns) of E7 peptide (aa 49-57). Data are expressed as mean number of $CD8^+$ IFN-γ^+ cells/$3x10^5$ splenocytes ± SEM. The data from intracellular cytokine staining shown here are from one representative experiment of two performed.

Figure 4. Flow cytometry analysis of IFN-γ–secreting and IL-4-secreting E7-specific $CD4^+$ cells in mice vaccinated with various recombinant DNA vaccines.

Mice were immunized as described in **Figure Legend** 3. (A) Splenocytes from vaccinated mice were cultured *in vitro* with E7 peptide (aa 30-67) overnight and were stained for both CD4

and intracellular IFN-γ. The number of IFN-γ secreting $CD4^+$ T cells was analyzed using flow cytometry. No significant difference in the frequency of E7-specific IL-4-secreting $CD4^+$ cells was observed in mice immunized with various recombinant DNA vaccines. (B) Splenocytes from vaccinated mice were cultured *in vitro* with E7 peptide (aa 30-67) overnight and stained for both CD4 and intracellular IL-4. The percentage of IL-4 secreting $CD4^+$ T cells was analyzed by flow cytometry. The IL-4 secreting activated mouse splenocytes (MiCK-2) from PharMingen were used as positive controls to assure the success of intracytoplasmic IL-4 staining for this study. The specificity of the IL-4 staining was demonstrated by the absence of $CD4^+$ $IL\text{-}4^+$ T cells when the IL-4 antibody was omitted. No significant difference in the frequency of IL-4 secreting E7-specific $CD4^+$ cells was observed in mice immunized with various recombinant DNA vaccines. The intracellular cytokine staining shown here are from one representative experiment of two performed.

Figure 5. *In vivo* tumor protection experiments against the growth of TC-1 tumors.

Mice were immunized with FL DNA , E7 DNA , FL-E7 DNA or FL mixed with E7 DNA (FL + E7) via gene gun and boosted with the same regimen one week later. One week after the last vaccination, mice were challenged with $1x10^4$ TC-1 cells/ mouse subcutaneously. Mice were monitored for evidence of tumor growth by palpation and inspection twice a week. 100% of mice receiving FL-E7 DNA vaccination remained tumor-free 60 days after TC-1 challenge. The data collected from the in vivo tumor protection experiments shown here are from one representative experiment of two performed.

Figure 6. *In vivo* tumor treatment experiments against pre-existing metastatic TC-1 tumor cells.

The mice were intravenously challenged with $1x10^4$ cells/mouse TC-1 tumor cells in the tail vein on D0. Three days (D3) after challenge with TC-1 tumor cells, mice received 2 µg of FL DNA, E7 DNA, FL-E7 DNA, FL mixed with E7 (FL+E7), via gene gunor unvaccinated. One week later, these mice were boosted with the same regimen as the first vaccination. The mice were sacrificed on D25. The FL-E7 group has (A) the least number of pulmonary metastatic nodules and (B) the lowest lung weight as compared with the other vaccinated groups (one-way ANOVA, P<0.001). The data obtained from these in vivo treatment experiments are from one representative experiment of two performed.

Figure 7. Representative gross pictures of the lung tumors in each vaccinated group.

C57BL/6 mice were challenged with TC-1 tumor intravenously and E7 DNA, FL or FL-E7 DNA via gene gun as described in **Figure Legend 7**. Mice were sacrificed 25 days after tumor challenge. There are multiple grossly visible lung tumors in unvaccinated control mice and mice vaccinated with FL, wild type E7 DNA or FL mixed with E7 DNA. The lung tumors in FL-E7 vaccinated group cannot be seen at the magnification provided in this figure.

Figure 8. *In vivo* antibody depletion experiments to determine the effect of lymphocyte subset on the potency of FL-E7 DNA vaccine.

Mice were immunized with 2 μg FL-E7 DNA via gene gun and boosted with 2 μg FL-E7 DNA one week later. One week after the last vaccination, mice were challenged with 1×10^4 TC-1 cells/ mouse subcutaneously. CD4, CD8 and NK1.1 depletions were initiated one week prior to tumor challenge and lasted 40 days after tumor challenge. All naïve mice and all of the mice depleted of $CD8^+$ T cells grew tumors within 14 days after tumor challenge. The data of antibody depletion experiments shown here are from one representative experiment of two performed.

Figure 9. CTL assays to demonstrate enhanced presentation of E7 through the MHC class I pathway in cells transfected with FL-E7 DNA

293 D^b,K^b cells were transfected with various DNA vaccines with lipofectamine and then trypsinized and centrifugated after 40-44 hr of transfection. Transfected 293 D^b,K^b cells were used as target cells while E7-specific $CD8^+$ T cells served as effector cells. CTL assays with various E/T ratios were performed. Note: The FL-E7 DNA generated significantly higher percentages of specific lysis on the 9:1 and 27:1 (both Ps<0.001) E/T ratios as compared with mice vaccinated with other DNA vaccines. CTL assays shown here are from one representative experiment of two performed.

Figure 10. CTL assays to demonstrate enhanced MHC class I presentation of E7 in bone marrow derived dendritic cells pulsed with cell lysats containing chimeric FL-E7 protein.

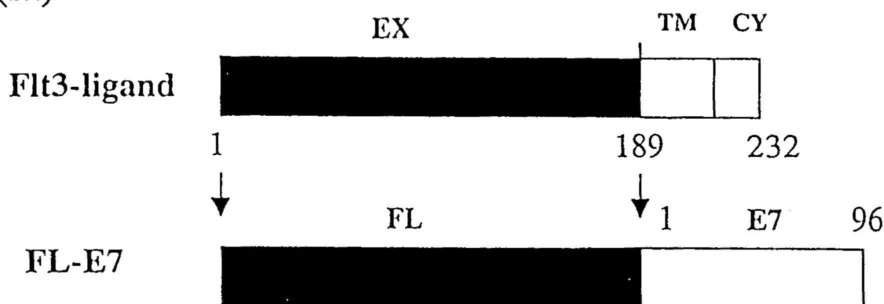
Bone marrow-derived DCs were pulsed with cell lysates from 293 D^b,K^b cells transfected with various DNA vaccines in different concentration (50 μg/ml, 10 μg/ml, 2 μg/ml, and 0.4 μg/ml) for 16-24 hrs. E7-specific $CD8^+$ T cells were used as effector cells. CTL assays was performed at fixed E/T (9/1) ratio with $9x10^4$ of E7-specific T cells mixed with $1x10^4$ of prepared DCs in a final volume of 200 μl. CTL assays was determined by quantitative measurements of LDH as described in Materials and Methods. Note: CTL using DC target cells pulsed with cells transfected with FL-E7 DNA generated significantly higher percentages of specific lysis compared to DC target cells pulsed with cells transfected with other DNA vaccines. CTL assays shown here are from one representative experiment of two performed.

Figure 11. Flow cytometry analysis to determine percentages of dendritic cells and NK cells in the spleens of mice immunized with various DNA vaccines.

C57BL/6 mice were immunized with wild-type E7, FL, or FL-E7 DNA via gene gun. Splenocytes were obtained from immunized mice 7 days after vaccination. (A) The portion of CD11c positive DC cells in splenocytes are indicated in percentages. Gates for the population were based on the control bone marrow dendritric cells. (B) The splenocytes were stained simultaneously with FITC-conjugated anti-CD-3 and PE-conjugated anti-NK1.1 mAbs and analyzed by flow cytometry. Two-color contour plot and percentage cells are presented in the upper left corner with $CD3^-$,$NK1.1^+$ natural killer cells. The data shown here are one representative experiment of two performed.

Figure 1

(A)



(B)

```
1/1                                        31/11
atg aca gtg ctg gcg cca gcc tgg agc cca aat tcc tcc ctg ttg ctg ctg ttg ctg ctg
Met thr val leu ala pro ala trp ser pro asn ser ser leu leu leu leu leu leu leu
61/21                                      91/31
ctg agt cct tgc ctg cgg ggg aca cct gac tgt tac ttc agc cac agt ccc atc tcc tcc
leu ser pro cys leu arg gly thr pro asp cys tyr phe ser his ser pro ile ser ser
121/41                                     151/51
aac ttc aaa gtg aag ttt aga gag ttg act gac cac ctg ctt aaa gat tac cca gtc act
asn phe lys val lys phe arg glu leu thr asp his leu leu lys asp tyr pro val thr
181/61                                     211/71
gtg gcc gtc aat ctt cag gac gag aag cac tgc aag gcc ttg tgg agc ctc ttc cta gcc
val ala val asn leu gln asp glu lys his cys lys ala leu trp ser leu phe leu ala
241/81                                     271/91
cag cgc tgg ata gag caa ctg aag act gtg gca ggg tct aag atg caa acg ctt ctg gag
gln arg trp ile glu gln leu lys thr val ala gly ser lys met gln thr leu leu glu
301/101                                    331/111
gac gtc aac acc gag ata cat ttt gtc acc tca tgt acc ttc cag ccc cta cca gaa tgt
asp val asn thr glu ile his phe val thr ser cys thr phe gln pro leu pro glu cys
361/121                                    391/131
ctg cga ttc gtc cag acc aac atc tcc cac ctc ctg aag gac acc tgc aca cag ctg ctt
leu arg phe val gln thr asn ile ser his leu leu lys asp thr cys thr gln leu leu
421/141                                    451/151
gct ctg aag ccc tgt atc ggg aag gcc tgc cag aat ttc tct cgg tgc ctg gag gtg cag
ala leu lys pro cys ile gly lys ala cys gln asn phe ser arg cys leu glu val gln
481/161                                    511/171
tgc cag ccg gac tcc tcc acc ctg ctg ccc cca agg agt ccc ata gcc cta gaa gcc acg
cys gln pro asp ser ser thr leu leu pro pro arg ser pro ile ala leu glu ala thr
541/181                                    571/191
gag ctc cca gag cct cgg ccc agg cag gga tcc atg cat gga gat aca cct aca ttg cat
glu leu pro glu pro arg pro arg gln gly ser met his gly asp thr pro thr leu his
601/201                                    631/211
gaa tat atg tta gat ttg caa cca gag aca act gat ctc tac tgt tat gag caa tta aat
glu tyr met leu asp leu gln pro glu thr thr asp leu tyr cys tyr glu gln leu asn
661/221                                    691/231
gac agc tca gag gag gag gat gaa ata gat ggt cca gct gga caa gca gaa ccg gac aga
asp ser ser glu glu glu asp glu ile asp gly pro ala gly gln ala glu pro asp arg
721/241                                    751/251
gcc cat tac aat att gta acc ttt tgt tgc aag tgt gac tct acg ctt cgg ttg tgc gta
ala his tyr asn ile val thr phe cys cys lys cys asp ser thr leu arg leu cys val
781/261                                    811/271
caa agc aca cac gta gac att cgt act ttg gaa gac ctg tta atg ggc aca cta gga att
gln ser thr his val asp ile arg thr leu glu asp leu leu met gly thr leu gly ile
841/281                                    871/291
gtg tgc ccc atc tgt tct cag gat aag ctt aag ttt aaa ccg ctg atc agc ctc gac tgt
val cys pro ile cys ser gln asp lys leu lys phe lys pro leu ile ser leu asp cys
901/301
gcc ttc tag
ala phe AMB
```

Figure 2

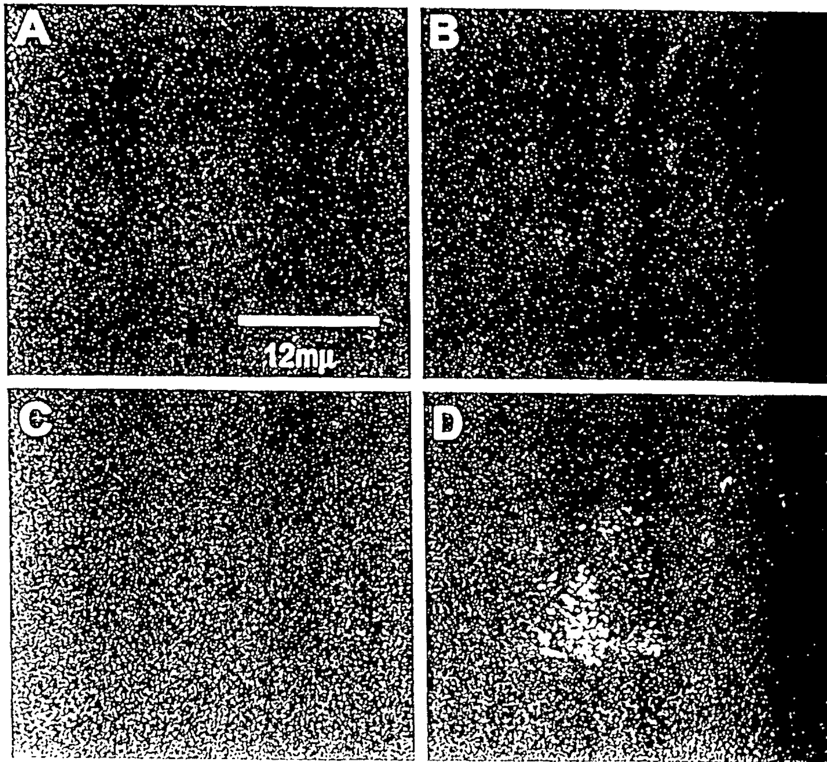


Figure 3A

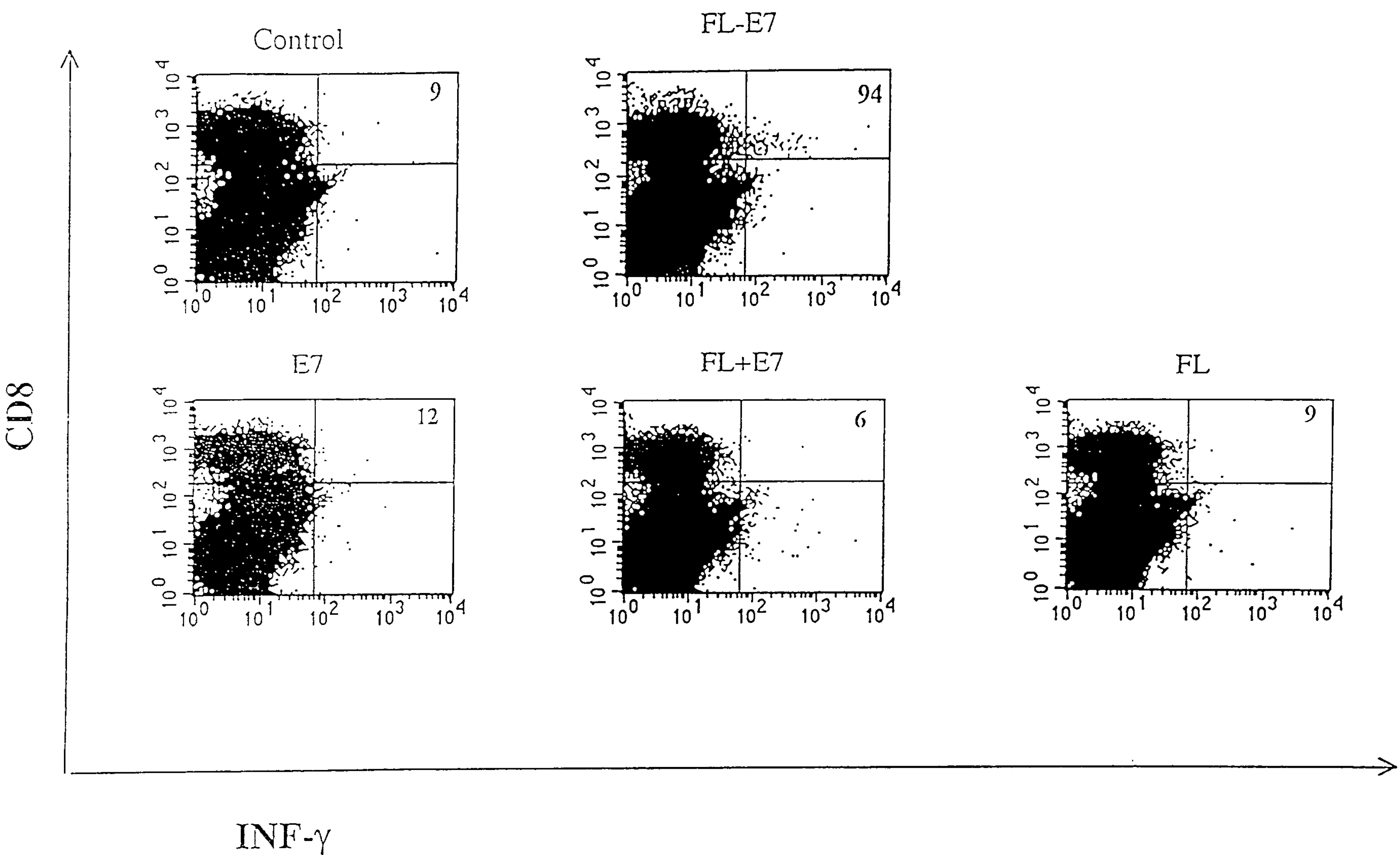


Figure 3B

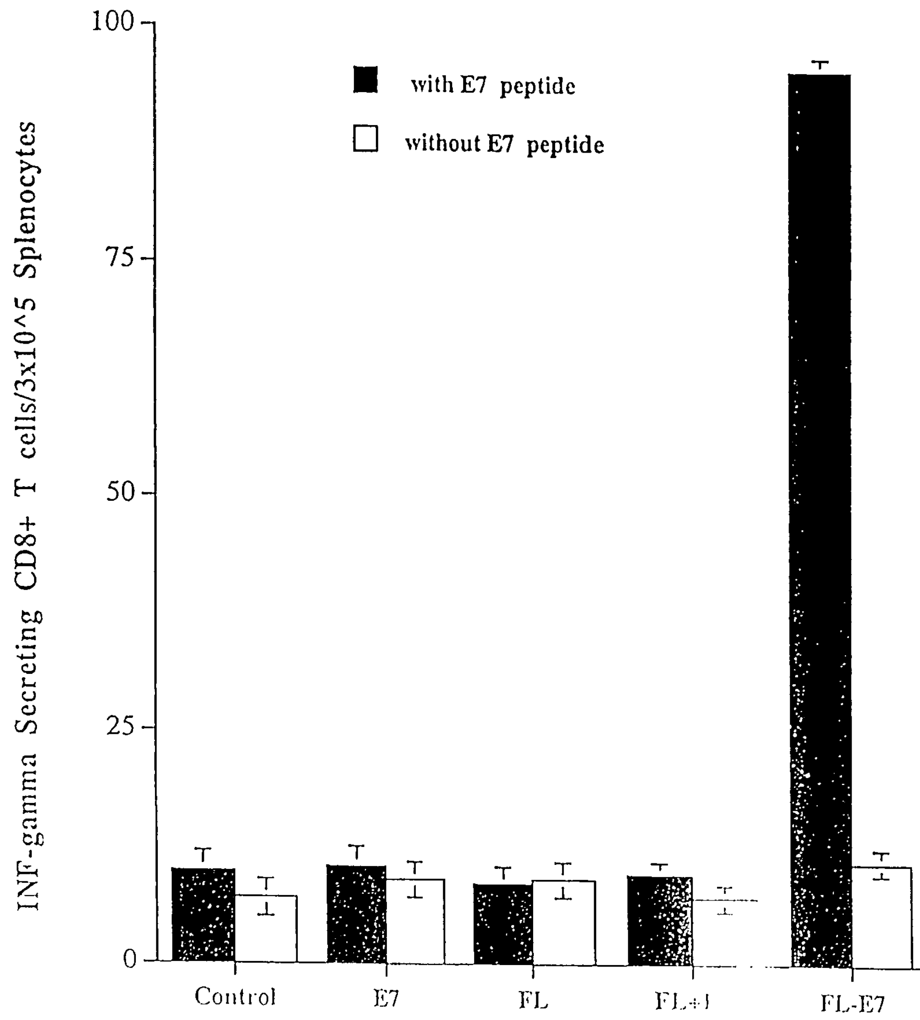


Figure 4

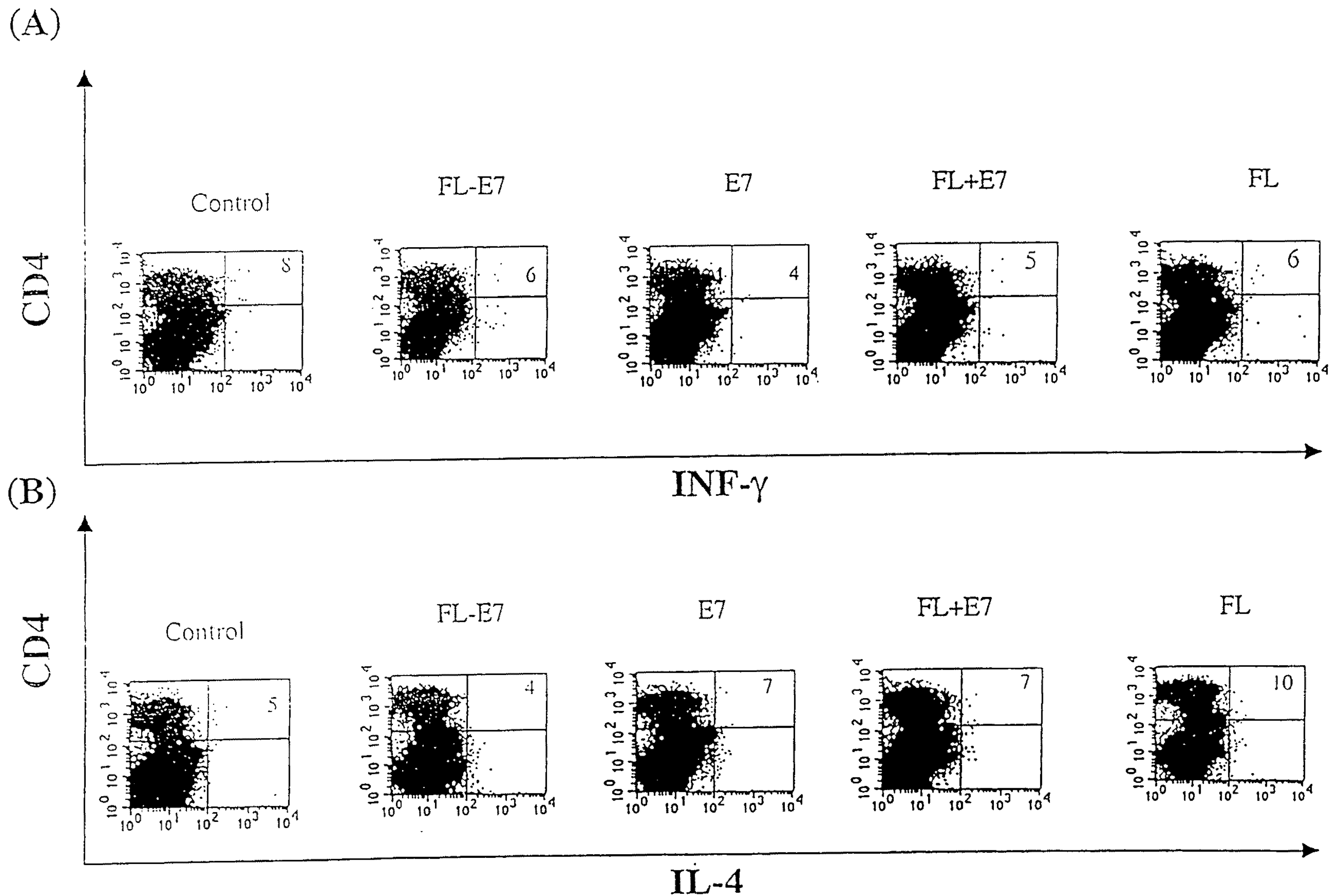


Figure 5

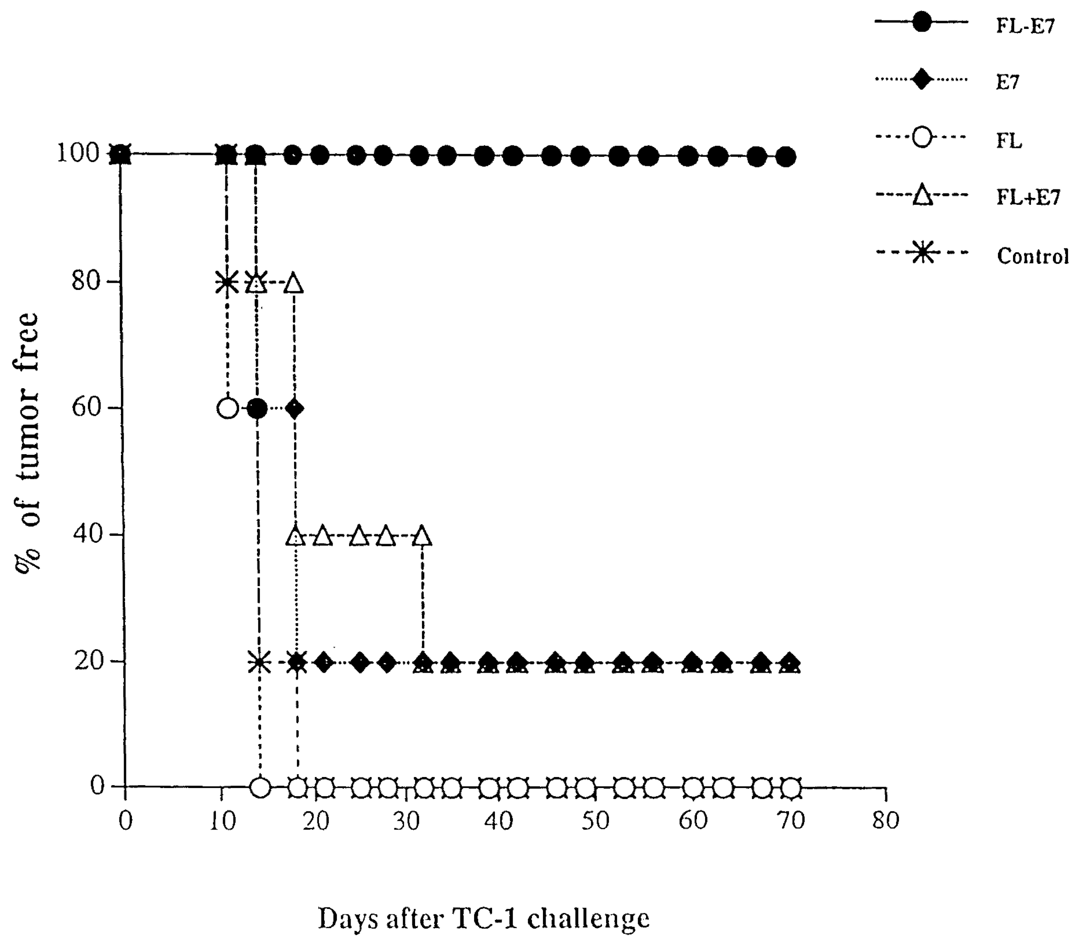
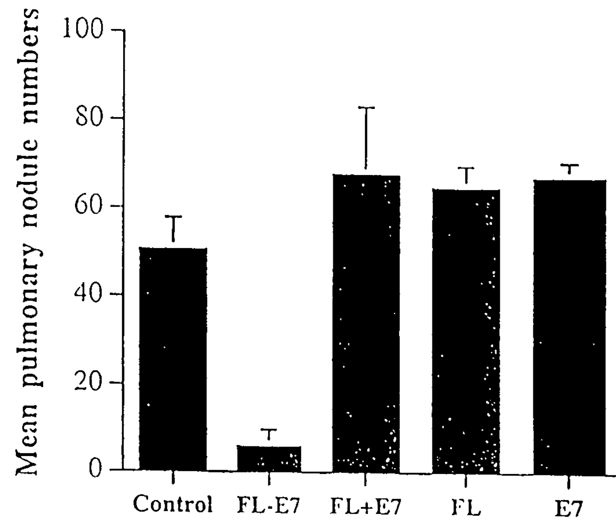


Figure 6

(A)



(B)

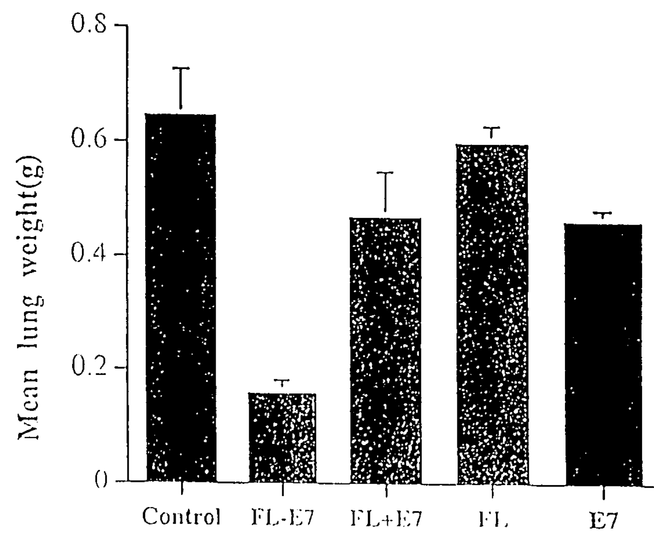


Figure 7

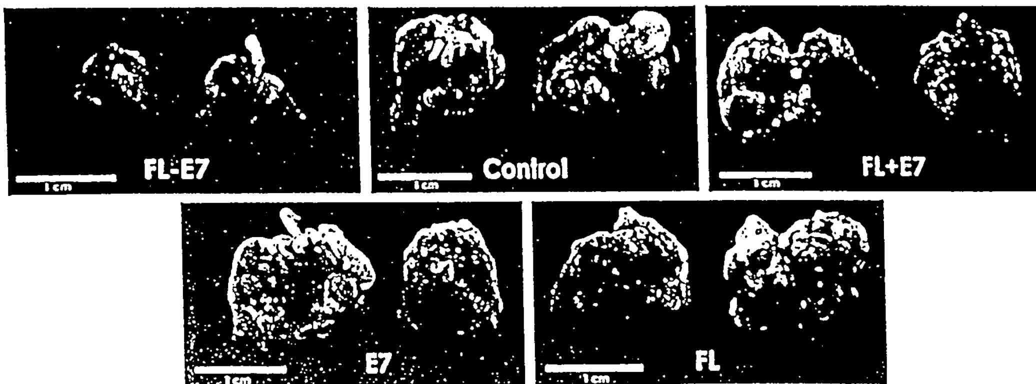


Figure 8

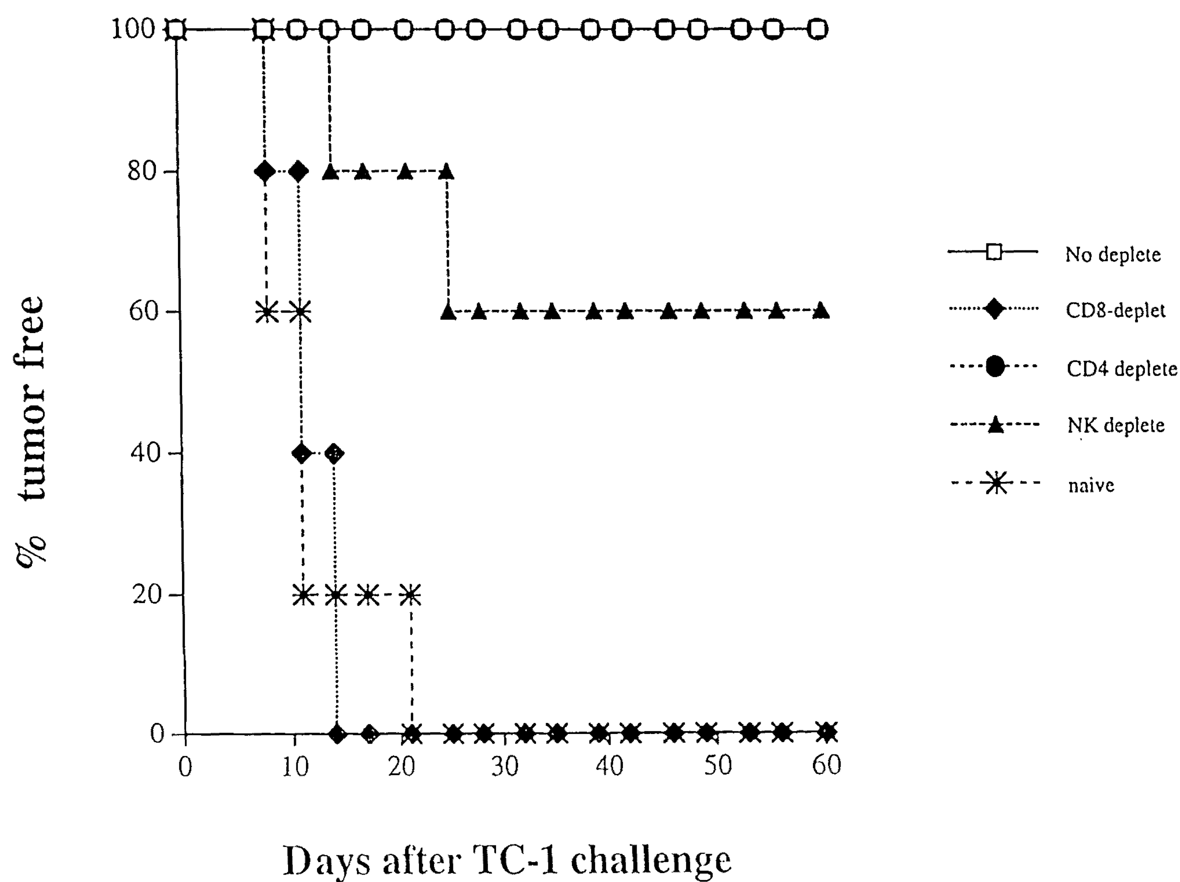


Figure 9

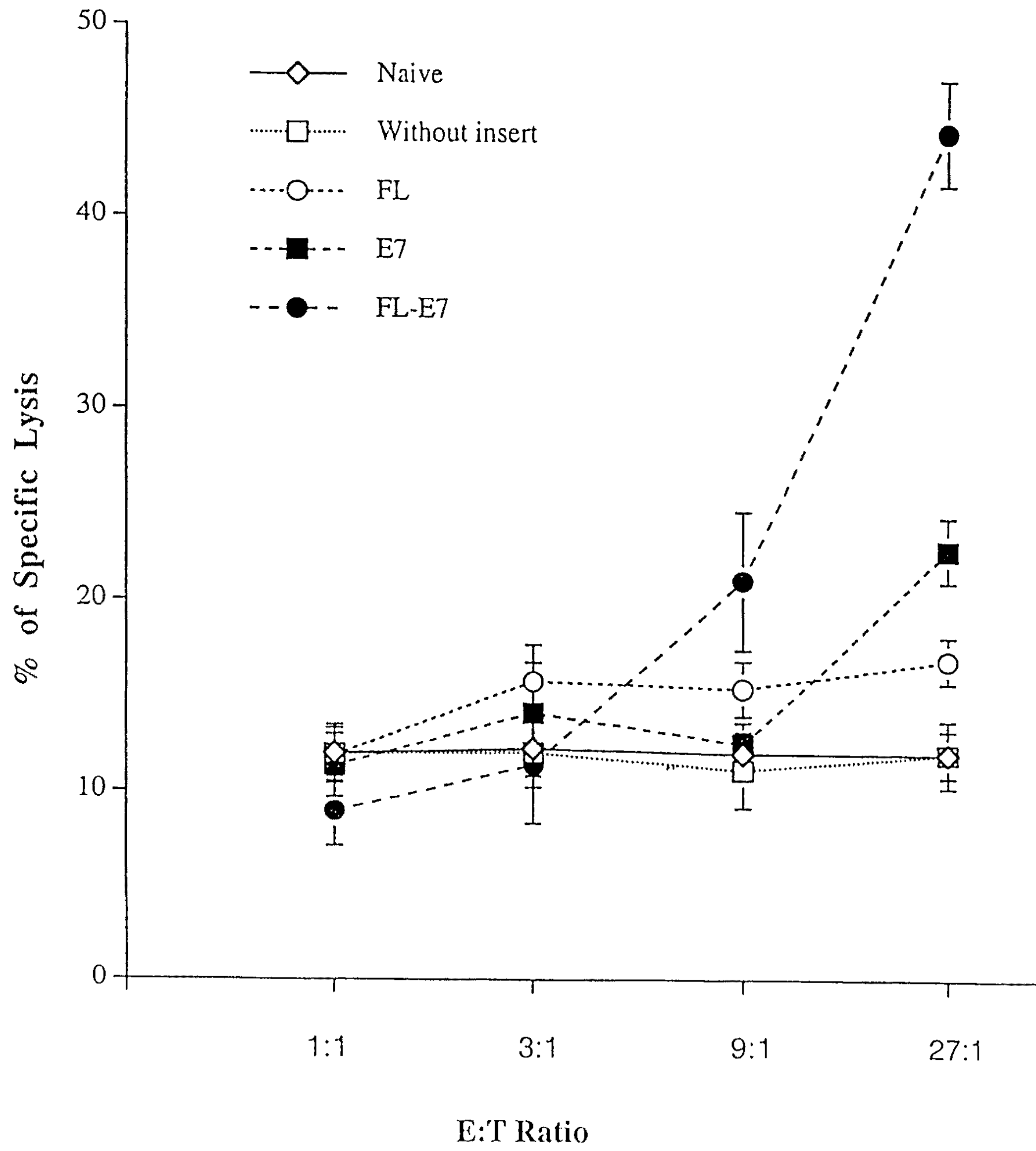


Figure 10

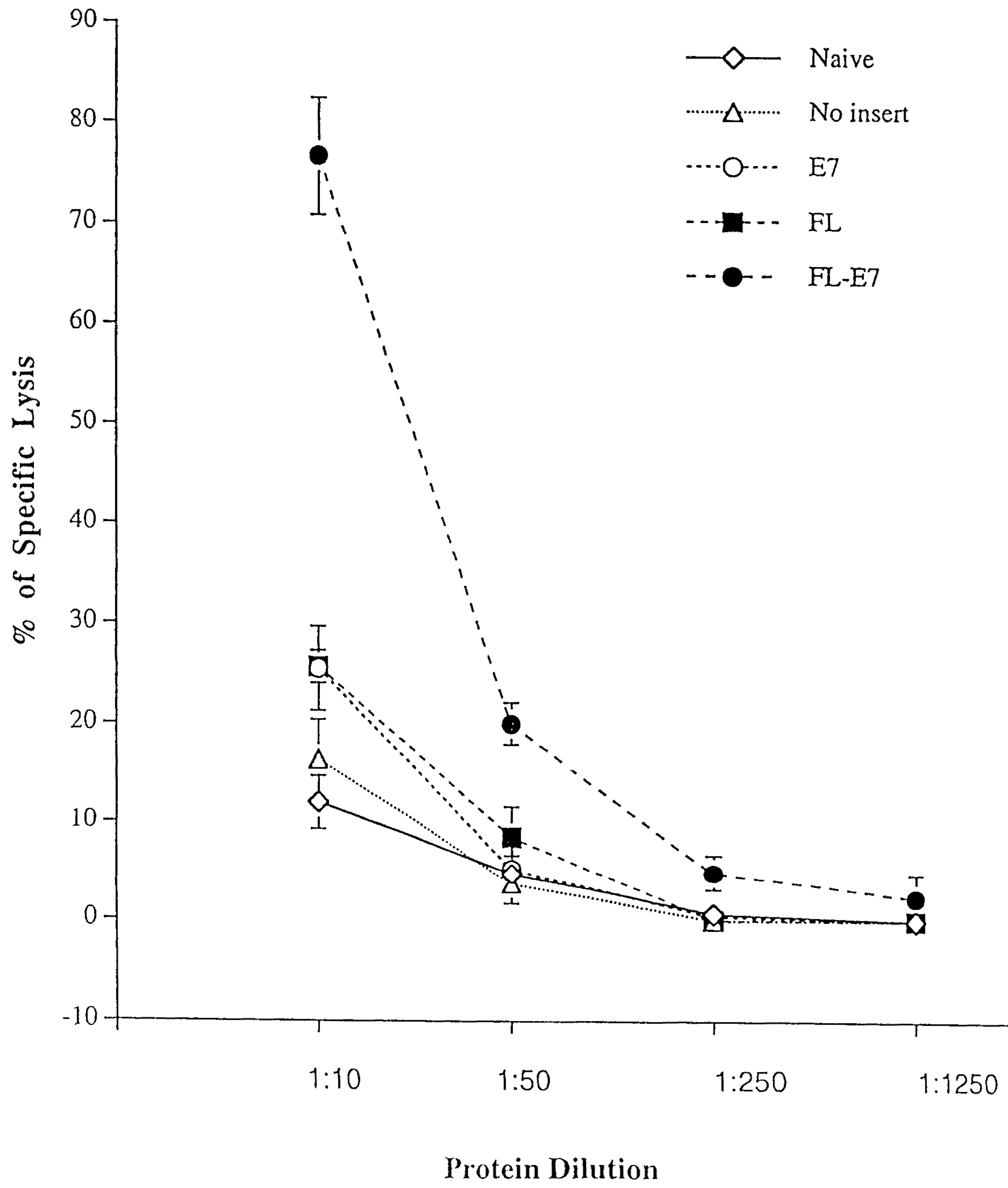
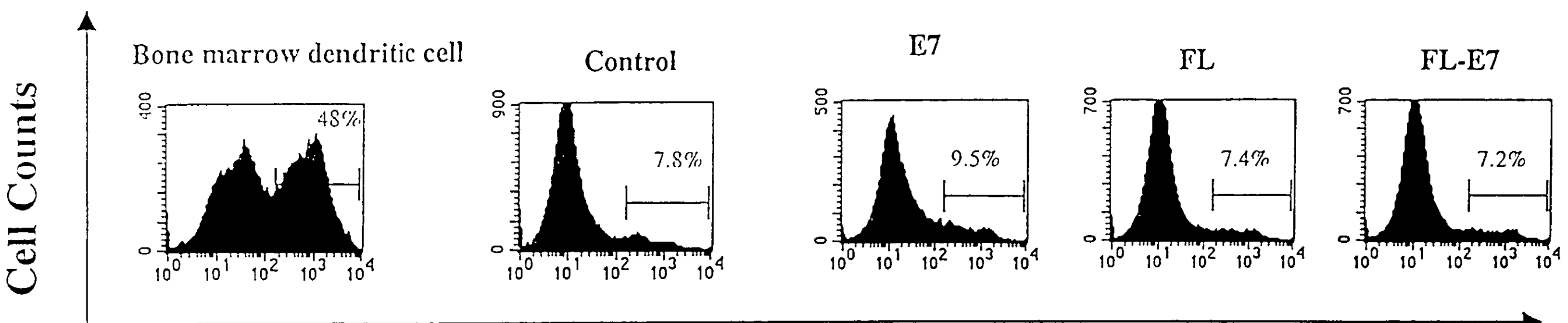


Figure 11

(A)



(B)