

PD-1-expressing tumor-infiltrating T cells are a favorable prognostic biomarker in HPV-associated head and neck cancer.

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ABSTRACT

Head and neck cancers positive for human papilloma virus (HPV) have a more favorable clinical outcome than HPV-negative cancers, but it is unknown why this is the case. We hypothesized that prognosis was affected by intrinsic features of HPV-infected tumor cells or differences in host immune response. In this study, we focused on a comparison of regulatory Foxp3⁺T cells and PD-1⁺T cells in the microenvironment of tumors that were positive or negative for HPV, in two groups that were matched for various clinical and biological parameters. HPV-positive head and neck cancers were more heavily infiltrated by regulatory T cells and PD-1⁺ T cells and the latter were positively correlated with a favorable clinical outcome. In explaining this paradoxical result, we showed that these PD-1⁺T cells expressed activation markers and were functional after blockade of the PD-1-PDL-1 axis *in vitro*. Approximately 50% of PD-1⁺ tumor-infiltrating T cells lacked Tim-3 expression and may indeed represent activated T cells. In mice, administration of a cancer vaccine increased PD-1 on T cells with concomitant tumor regression. In this setting, PD-1 blockade synergized with vaccine in eliciting antitumor efficacy. Our findings prompt a need to revisit the significance of PD-1-infiltrating T cells in cancer, where we suggest that PD-1 detection may reflect a previous immune responses against tumors that might be reactivated by PD-1/PD-L1 blockade.

INTRODUCTION

Recent studies have detected oncogenic HPV in 25.9% of head and neck cancers (mostly HPV16) and this prevalence increases to more than 50% for cancers of the oropharynx (tonsil, base of tongue, etc) with a growing world wide incidence (1).

Although these tumors frequently have aggressive histopathological features (2), the presence of HPV DNA is a favorable prognostic factor with regard to recurrence and survival (3-5).

The mechanisms underlying the more favorable outcome of HPV-associated head and neck cancer have not been clearly elucidated.

These tumors are more responsive to chemotherapy and radiotherapy, but even patients with HPV-positive oropharyngeal cancers treated by surgery alone, have a better prognosis than those with HPV-negative tumors after adjustment for tumor stage (6, 7).

HPV E6-E7 expressing tumors grew slower in immunocompetent mice but not in nude mice, than HPV negative tumors suggesting a role of adaptive immunity and not simply an intrinsic biological property of HPV positive tumor cells to explain this difference (8).

In human, T cells directed against HPV derived proteins (E2, E6, E7) have been described in the blood and in the tumor of patients with head and neck cancers (9-12). However, the capacity of tumor-infiltrating lymphocytes (TILs) to act as effector cells may be affected by the tumor microenvironment. Immunosuppressive cells or molecules (13, 14) or chronic inflammation in head and neck cancer predispose to a tumor promoting microenvironment (15, 16). Analysis of differences in the type of immunity within the tumor microenvironment of HPV-associated head and neck cancer compared to HPV-negative tumors may therefore provide new clues to explain the more favorable clinical behavior of this type of cancer. In the present study, we addressed the clinical significance of regulatory Foxp3⁺T cells and PD-1-positive T cell infiltration in the tumor microenvironment of HPV-positive and HPV-negative head and neck cancers. These immunosuppressive cells were selected because regulatory

CD4⁺CD25⁺Foxp3⁺T cells have emerged as the dominant T cell population governing peripheral tolerance by inhibiting effector T cells. We and other authors have shown that the frequency of intratumoral Treg is increased in head and neck cancer patients and in HPV-associated cervical carcinoma (17, 18). Programmed death-1 (PD-1), a member of the CD28 receptor family, is expressed by activated lymphocytes and inhibits their proliferation and effector functions after binding to PD-1 ligands such as B7-H1 (PD-L1)(19). PD-1 expression is indicative of chronic antigen stimulation and contributes to T cell dysfunction (exhaustion)(19). Interference with PD-1-PD-L1 signaling, via either antibody blockade or PD-1 deficiency has been shown to improve clinical outcome and restore functional T cell responses in chronic viral diseases and in several types of cancer (20). Preliminary results documented that more than 60% of freshly isolated human head and neck tumors express PD-L1 (21) and in early stage tongue cancer, intraepithelial T cells frequently express PD-1 (22). All these data therefore support a comprehensive analysis of PD-1-PD-L1 expression in head and neck cancers associated with chronic viral infection.

As expected, HPV-associated head and neck cancers were found to be associated with a good prognosis compared to HPV negative tumors. HPV-positive tumors were more heavily infiltrated by regulatory T cells and PD-1 expressing T cells than HPV negative tumors. Surprisingly, levels of PD-1⁺T cell infiltration positively correlated with a favorable clinical outcome in HPV- associated cancers and, in a preclinical HPV tumor model, predicted the clinical response to anti-PDL-1 antibody.

Material and Methods

Patients

Sixty four newly diagnosed untreated patients with primary histologically proven head and neck squamous cell carcinoma (HNSCC) were included in this study. Patient characteristics

are presented in Table S1. Each patient's disease was staged according to the seventh edition of the International Union Against Cancer/American Joint Committee on Cancer system for head and neck cancer. Treatment modalities consisted of chemoradiotherapy without surgery (organ preservation) or surgery combined or not with radiotherapy and chemotherapy. This study was conducted in accordance with French laws and after approval by the local ethics committee (ID RCB 2007-A01128-45).

Patients were divided in two groups depending on the presence or absence of oncogenic HPV. These two groups were matched for various parameters (gender, primary tumor site, tumor staging, lymph node involvement, presence of metastasis, treatment modalities)(Table S1).

Mice

Six to eight-week-old female C57BL/6 (H-2^b) (B6) mice were purchased from Charles River Laboratories (L'Arbresle, France). All mice were kept under specific pathogen-free conditions at the INSERM U970 animal facility. Experiments were performed according to institutional guidelines after acceptance by the Veterinary School of Maisons-Alfort ethics committee.

Cells

TC-1 cells are transformed murine (H2^b) epithelial cells co-transfected with HPV-16 E6/E7 genes and the activated human Ha-ras (G12V) oncogene DNA. They were obtained from Dr. TC Wu's laboratory (Department of Pathology, School of Medicine, Johns Hopkins University, Baltimore, USA). Cells were grown in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-Glutamine, antibiotics and 0.4 mg/mL G418 at 37°C with 5%CO₂.

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Peptide, vaccine, antibodies

Pool of 15 mer peptides spanning the entire HPV16 E7 protein were designed with a 4 mer overlap between each peptide. They were obtained from PolyPeptide Laboratories (Strasbourg, France), reconstituted in PBS and stored at -20°C.

The STxB-E7₄₃₋₅₇ vaccine was produced by chemical coupling between the N-bromoacetylated E7₄₃₋₅₇ peptide and the sulfhydryl group of STxB-Cys recombinant protein, as previously described (23). After purification, endotoxin concentrations determined by the Limulus assay test (Lonza, Verviers, Belgium) were < 0.5 EU/mg.

The invariant NKT cell ligand α -GalCer (KRN7000) was purchased from Funakoshi (Tokyo, Japan).

CT-011 is a humanized monoclonal anti-PD-1 antibody manufactured by CureTech LTD (Israel). This antibody has already been validated in *ex vivo* functional assays in humans (24).

Anti-mouse PDL-1 and isotype control antibodies were purchased from BioXcell (West Lebanon, U.S.A). Mice received intraperitoneal injections of 200 μ g mAb/mouse as previously described (25).

For immunofluorescence analysis, an anti-PD-1 mAb generated by D Olive's group was selected and used (see Table S2) as previously described (26).

HPV detection

The presence of HPV in head and neck cancer biopsies was detected using the INNO-LIPA genotyping Extra assay (Innogenetics, Ghent, Belgium).

Immunofluorescence staining and flow cytometry analysis

Immunofluorescence staining and flow cytometry analysis were performed as previously described (16) and detailed in supplementary data. The antibodies used for the various immunofluorescence stainings are described in Supplementary Table S2.

Immunization of mice and in vivo tumor protection

C57BL/6 mice were immunized via the intraperitoneal route at day 0 and day 14 with the STxB-E7 vaccine (20 µg) combined with 1 µg of αGalCer during the first immunization.

In a therapeutic setting experiment, 10^5 TC1 tumor cells were injected subcutaneously (s.c.) in the right flank of B6 mice. They were then vaccinated or not at day 9 and day 18 after tumor graft in combination with anti-PD-L1 or isotype control antibodies. Mice were monitored every 48-72h for tumor growth.

Statistical analysis

Survival variables were estimated using the Kaplan-Meier method and compared by the log-rank test for categorical variables and the Cox model and associated Wald χ^2 statistic for quantitative variables.

Overall survival was defined as the time from initial diagnosis until death or until last follow-up (right censored data).

Locoregional control was calculated from the end of treatment and defined as the absence or either persistent or recurrent disease at the primary site or in the cervical lymph nodes. Patients with persistent disease at the end of treatment were considered to have experienced failure at time zero. Patients with no signs of relapse were censored at the time of last-follow up or death. The median follow-up for the overall population was 26 months.

The Mann-Whitney U-test was used to assess whether two samples of observations were derived from the same distribution. The Chi-square test with Yates correction was used to analyze the relationship between tumor cell infiltration and the tumor HPV status.

RESULTS

HPV- associated head and neck cancer has a good prognosis

In a retrospective study, we divided 64 head and neck cancer patients into two groups differing according to presence or absence of oncogenic HPV (30 HPV16, 1 HPV18 and 1 HPV33, 32 HPV negative) in their tumors. These two groups were balanced for gender, primary tumor sites, tumor (T) staging, lymph node involvement and treatment modalities (Table S1). Based on Kaplan-Meier estimates, the overall survival for patients with HPV-positive cancer was significantly better (median survival not reached) than for patients with HPV-negative cancer (median survival : 16 months) ($p = 0.001$)(log rank test)(Fig 1). Patients with HPV-positive tumors also had a significantly better disease-free survival ($p = 0.003$)(log rank test) than patients with HPV-negative cancer (Fig 1).

Intratumoral Foxp3⁺T cells and PD-1⁺T cells differ between HPV positive and HPV-negative head and neck cancers

HPV-positive head and neck cancers were more heavily infiltrated by CD8⁺ cells ($p = 0.01$), PD-1⁺CD4⁺T cells ($p = 0.045$) and the total number of PD-1⁺CD4⁺ and PD-1⁺CD8⁺T cells ($p = 0.045$). A trend was also observed between high levels of total PD-1⁺cell ($p = 0.079$), PD-1⁺CD8⁺ cell ($p = 0.078$) and Foxp3⁺CD4⁺T cell ($p = 0.059$) infiltration and the HPV detection in the tumors (Table 1). PD-1 expression was higher in CD4⁺T cells (median of PD-1⁺CD4⁺ cells = 5.5), than in CD8⁺T cells (median of PD-1⁺CD8⁺ = 1).

Since PD-1⁺CD4⁺ cells and PD-1⁺CD8⁺ cells could also represent macrophages and NK cells respectively, tumor dissociation was performed for 14 tumors and cytometry analysis confirmed that more than 95% of PD-1⁺CD45⁺ cells were CD3⁺T cells (Fig S1).

Thirty Three (51.5%) of the 64 head and neck tumors expressed significant levels of PD-L1 (score ++/+++) with homogeneous staining (Table 1 and Fig S2), but no correlation was observed between PD-L1 expression and HPV status of these head and neck cancer patients (Table 1)

PD-1-positive infiltrating T cells are associated with better overall survival in HPV positive head and neck cancer.

Surprisingly, we showed that HPV positive tumors infiltrated by large numbers of PD-1-positive cells or total number of PD-1⁺CD4 and PD-1⁺CD8 T cells were correlated with better overall survival of these patients. Indeed, patients with PD-1-positive tumor infiltrating T cells above the median value (15 PD-1 cells/fields) had a 93.9% 60-months overall survival, whereas patients with low PD-1-positive cell infiltration had a 63.6% 60-months overall survival (p = 0.025, Hazard ratio 0.13 95% CI, 0.02 to 0.067) (Fig 3A). In line with these results and with the previous demonstration that most PD-1 cells are T cells (Fig S1), we showed that tumors infiltrated by high levels of PD-1⁺CD4⁺ and PD-1⁺CD8⁺ cells had a better survival than tumors with low PD-1⁺T cell infiltration (p = 0.045 Hazard ratio 0.15 95% CI, 0.03 to 0.7). Patients with high infiltration (> median value) by PD-1⁺CD4⁺T cells (p = 0.046) but not by PD-1⁺CD8⁺T cells also had a better overall survival.

A correlation was also demonstrated between the total number of PD-1⁺CD4⁺ and PD-1⁺CD8⁺ infiltrating T cells and 18-month overall survival in the whole population of head and neck patients whatever their HPV status (p = 0.009)(Fig 3B).

In contrast, the levels of infiltration by CD8⁺T cells or CD4⁺T cells or Foxp3 regulatory CD4⁺T cells infiltration were not correlated with overall survival in HPV-positive head and neck tumors (Fig 3A). However, as previously reported by us and other authors (17, 27), the number of Foxp3 regulatory T cells was correlated with better 18-month overall survival in the whole population of head and neck cancer patients ($p = 0.0033$) (Fig S3).

PDL-1 expression was not correlated with overall survival or disease free survival in either the whole head and neck cancer cohort or in HPV-positive patients (Fig S2). In bivariate analysis including PDL-1 and various subpopulation of PD-1 cells each analyzed separately, we showed that only PD1⁺CD4⁺T cells, the total number of PD1⁺CD4⁺ and PD1⁺CD8⁺T cells and the total number of PD-1 cells remained significant (data not shown).

Phenotypic and functional characterization of PD-1⁺T cells infiltrating head and neck cancer.

PD-1⁺T cells infiltrated both the stroma and the nest of tumor cells. As shown in Fig 4A, close contact was demonstrated between PD-L1 tumor cells and PD-1⁺ cells strongly suggesting that a negative signal was delivered to these PD-1⁺tumor-infiltrating T cells. Interestingly, in the whole population, a correlation was observed between PDL-1 expression and the number of tumor infiltrating CD4⁺PD1⁺T cells ($p = 0.029$). A trend was also observed between PDL-1 expression and the number of PD-1⁺ CD4⁺T cells and PD1⁺CD8⁺T cells ($p = 0.07$) and also the total number of PD-1⁺T cells ($p = 0.089$)(Fig S4).

PD-1 is a hallmark of both activated and exhausted T cells (28). We found that PD-1 positive T cells, either CD4 or CD8⁺ T cells, expressed higher levels of HLA-DR and CD38 than PD-1 negative T cells (Fig 4 B and C). No difference in this activation state appeared to occur between HPV- positive or HPV-negative tumors (data not shown). Tim-3 expression was also assessed, as it has been associated with an exhausted phenotype of PD-1⁺T cells (29, 30). One

half of PD-1⁺T cells in head and neck cancer were found to express Tim-3 (Fig 4 D and Fig S5A). The same number of PD-1⁺T cells in HPV-positive and HPV-negative tumors presented hallmarks of exhausted T cells (Fig 4D).

We also showed that PD-1-positive cells did not express the Foxp3 marker of regulatory T cells, despite the fact that, in some cases, PD-1-positive cells and Foxp3 regulatory T cells were in close contact (Fig S5B).

To assess whether the functionality of these T cells could be enhanced, fresh TIL were co-cultured with tumor cells in the presence or absence of anti-PD-1 mAb and a pool of E7 peptides. In 3 (2 HPV negative and 1 HPV positive) out of 4 patients, a significant increase of IFN γ positive T cells was demonstrated by Elispot in the presence of CT-011, an anti-PD-1 mAb (Fig 5). An isotype control antibody had no effect on enhanced IFN γ production (Fig 5). In one HPV positive patient, E7 peptides slightly increased the frequency of CT-011 induced IFN γ secretion (Fig 5).

Vaccination increases PD-1 and PD-1 blockade synergizes with the vaccine anti-tumor effect.

To explain this paradoxical good prognostic value of PD-1⁺T cell infiltration in HPV-associated head and neck cancer, we modeled this phenomenon in a preclinical model.

When mice were grafted with an epithelial tumor cell line (TC1) expressing the E7 protein derived from HPV, tumors were poorly infiltrated by CD8⁺T cells which expressed low levels of PD-1 (6%) (Fig 6 A).

When mice were vaccinated with a non replicative delivery vector targeting dendritic cells, the B subunit of Shiga toxin, coupled to E7, 44% of CD8⁺T cells expressed PD-1 (Fig 6A and B). In addition, 89% of specific anti-E7₄₉₋₅₇ CD8⁺T cells, induced by the vaccine, also expressed PD-1 (Fig 6 A and B). The vaccine therefore appeared to elicit PD-1 expression in

both total and E7₄₉₋₅₇ specific CD8⁺T cells. The vaccine alone had a partial inhibitory effect on the growth of established TC1 tumors (Fig 6D). This effect was significantly enhanced by the addition of anti-PD-L1 (Fig 6D). In contrast, the anti-PD-L1 antibody alone had no effect on tumor growth (Fig 6D), which may be explained by the low (6%) PD-1 expression in infiltrating T cells in the absence of vaccination (Fig 6A). Similar results were obtained, when overall survival or the percentage of tumor-free mice were selected as endpoints (Fig S6)

Discussion

This study shows that HPV-positive head and neck tumors were heavily infiltrated by PD-1⁺ cells, as the number of PD-1⁺CD4⁺ infiltrating T cells and the total number of PD-1⁺CD4⁺ and PD-1⁺CD8⁺T cells were significantly higher in HPV-positive than in HPV-negative head and neck cancers. The relevance of these results is strengthened by the matching between HPV-positive and HPV-negative tumors for various confounding parameters (TNM, treatment modalities, etc).

Surprisingly, we found that high levels of PD-1⁺ cells and the total number of PD-1⁺CD4⁺ and PD-1⁺CD8⁺T cells correlated with better survival compared to low levels of infiltration by these cells in primary HPV-positive head and neck cancers.

These results were unexpected due to the known inhibitory function of PD-1 on T cells and immune cells (20, 31). In addition, in renal cell carcinoma, nasopharyngeal cancer and Hodgkin lymphoma, PD-1 expression on immune cells was correlated with poor prognosis and was associated with shorter survival (32-34). However, Hsu et al showed that the prognostic value of PD-1 differed between CD4⁺ and CD8⁺T cells (33). The other studies did not perform double immunofluorescence staining or cytometry analysis to more precisely characterize PD-1 positive cells that can be expressed by many types of infiltrating cells (NK, B cells, macrophages, etc). In the present study, we demonstrated that most (> 95%) PD-1

positive cells corresponded to T cells (Fig. S1) and that the prognostic value of PD-1 was associated with the total number of CD4⁺ and CD8⁺T cells expressing this marker. It should also be mentioned that in contrast to many tumors, CD8⁺T cells are associated with bad prognosis in renal cell carcinoma and hodgkin lymphoma supporting the fact that the clinical significance of molecules expressed by these cells could also vary depending on the tumor histology (35, 36).

Interestingly, recent studies have reported that follicular lymphomas infiltrated by PD-1⁺T cells were associated with a lower risk of transformation, a higher progression-free survival and a better overall survival than tumors not infiltrated by PD-1⁺cells (37). PD-1 was preferentially expressed by infiltrating CD4⁺T cells in these follicular lymphomas, as also observed in our study. In line with our results, PD-1 mRNA expression determined by RT-PCR in colorectal carcinoma was also associated with a good prognosis (38). It is therefore noteworthy that the three tumors (lymphoma, colorectal cancer and head and neck cancer), in which PD-1 expression was significantly associated with a favorable outcome (37, 38), were also those with an unexpected clinical benefit of Treg infiltration (17, 39, 40). The present study, conducted in an independent series of patients, confirms our previous findings that high levels of Treg in head and neck cancer patients were also positively correlated with a better prognosis (17). These results concerning the good prognostic value of intratumor regulatory T cells in head and neck cancer have subsequently been reproduced by various groups (27, 41). In addition, the frequency of regulatory Foxp3⁺CD4⁺T cells has been shown to be higher in patients with head and neck squamous cell carcinoma with no evidence of disease after oncologic therapy, than in patients with active disease (42). The plasticity of regulatory T cells, transient expression of Foxp3 by activated T cells and a possible role of regulatory T cells in the inhibition of deleterious cancer inflammation could explain the paradoxically good clinical prognosis associated with regulatory T cells in some circumstances (43, 44).

Several hypotheses can also be proposed to explain this paradoxical favorable outcome associated with PD-1 expression on T cells. PD-1 is up-regulated on lymphocytes following TCR activation and remains elevated in the context of persistent antigen-specific immune stimulation (45). The absence of co-expression of Tim-3 and PD-1 in one half of PD-1-positive cells strongly suggests that some PD-1⁺T cells in the tumor microenvironment of HPV-positive head and neck cancers are not exhausted T cells (Fig 4). We did not assess the expression of other inhibitory receptors (Lag-3, CTLA-4...) on PD-1⁺T cells, but Tim-3 is the most frequent co-inhibitory receptors associated with PD-1⁺T cells in tumor and an hallmark of exhausted T cells (29, 46). It has been reported that high PD-1 levels on T cells more closely reflect their “exhausted status” than intermediate PD-1 levels. However in our study PD-1 expression was homogeneous and it was therefore difficult to divide T cells into two groups. Some of our PD-1 infiltrating T cells remained responsive to anti-PD-1 *in vitro* (Fig 5). From these results, PD-1 expression also indicates, that T cell activation has occurred during the development of HPV-associated head and neck cancers and PD-1 could therefore also be viewed as a witness of the anti-tumor immune response.

Indeed, we showed that PD-1-positive T cells expressed higher levels of the HLA-DR and CD38 activation markers, than PD-1-negative T cells (Fig 4). Although this response was not effective to clear the tumor, it may have slowed tumor growth compared to a clinical setting associated with absence of host response to the the tumor. Although the tumor specificity of PD-1 expressing T cells could not be demonstrated due to the small number of cells present in tumor biopsies, it has been previously shown that tumor- reactive CD8⁺T cells generated after *in vitro* culture of dissociated biopsies of melanoma were more frequently derived from PD-1 expressing T cells present at the beginning of culture (47). In preclinical tumor models, the CD137⁺CD8⁺T cells were enriched in anti-tumor effector T cells (48).

PD-1-expressing T cells in HPV-associated oral cancers.

Our preclinical model showed that grafting of the E7-expressing TC1 epithelial cell line, which constitutively expresses PD-L1 led to tumor growth without therapy. Analysis of its tumor microenvironment showed the absence of PD-1 expression by CD8⁺T cells. In contrast, activation of an anti-HPV response by an anti-E7 vaccine, elicited PD-1 expression by CD8⁺T cells and E7-specific T cells, which were associated with partial regression of the tumor. In humans, it has been shown that tumor-infiltrating lymphocytes (TIL) derived from spontaneously regressing melanomas also express substantial levels of PD-1 (49).

Another important result of this study is that the efficacy of anti-PD-L1 mAb depended on the presence of PD-1 on specific T cells induced after vaccination. In preclinical models, immunotherapy approaches which should lead to upregulation of PD-1 following activation of the immune system, such as recombinant cytokines or stimulatory antibodies or transfer of activated adoptive T cells, synergized with blockade of the PD-1-PD-L1 pathway (21, 25, 50). Consistent with these results, a recent report showed that expression of PD-L1, the ligand of PD-1, which was considered to be an immunosuppressive molecule, positively correlated with better survival in melanoma (51). In melanoma, PD-L1 is mainly induced by IFN γ produced by infiltrating T cells and the distribution of T cells within the tumor was correlated with that of PD-L1 (51). In line with these results, in patients treated with anti-PD-1 mAb, tumor cell surface PD-L1 expression appeared to be correlated with the likelihood of response to treatment, possibly due to a reprogramming of infiltrating anti-tumor PD-1⁺T cells (52). PD-1 and PD-L1 expression may therefore represent surrogate markers of endogenous anti-tumor immune response, explaining their unexpected association with good prognosis in some tumors in which PD-L1 expression is not oncogene-driven. Indeed, in glioma, loss of tumor suppressor PTEN function increased PD-L1 (53). In our study, PD-L1 was not associated with a good prognosis in head and neck cancer patients, regardless of HPV status, but low PTEN expression was detected in the majority of these tumors (54).

This study therefore revisits the significance of PD-1-positive tumor-infiltrating T cells in cancer. The inhibitory function linked to PD-1 should not mask the fact that its detection could also reflect a past anti-tumor immune response ready to be reprogrammed by PD-1-PD-L1 blockade.

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Figure and legends

Figure 1: HPV-associated head and neck cancers are associated with a good prognosis.

Relationships between the presence or absence of oncogenic HPV in head and neck tumors and overall survival (A) or locoregional control (B).

Figure 2 : PD-1⁺ and Foxp3⁺ T cells infiltrate HPV- associated head and neck cancer

Tissue derived from biopsies of HPV-positive head and neck cancers were stained with antibodies to human CD4, CD8, Foxp3 and PD-1. Left and 2nd column from the left: results of simple immunofluorescence acquisition with each antibody. 3rd column from the left : Double immunofluorescence staining. Isotype control antibodies were also included in each experiment (right). Arrows indicate colocalization between the markers recognized by the specific antibodies. (Original magnification x 400)

Figure 3: PD-1-positive infiltrating T cells positively correlate with survival in both HPV-positive head and neck cancer and in the overall head and neck cancer population.

A : Tissues derived from biopsies of HPV-positive head and neck cancers were stained with antibodies to human CD8, CD4, PD-1 by simple immunofluorescence analysis. Double immunofluorescence stainings for CD4 and PD-1, CD8 and PD-1, CD4 and Foxp3 were also performed. For overall survival analysis, high and low levels of these various populations were defined using the median of tumor infiltration for these cells as cut-off values.

B: PD-1⁺ cells and total number of PD-1⁺CD4⁺ and PD-1⁺CD8⁺ cells were measured in biopsies derived from all head and neck cancer patients, regardless of their HPV status. The relationship between the total number of these cells selected as a quantitative variable and the

18-months survival is shown. The blue line corresponds to this relationship, whereas the red line represents the upper or lower limits of the 95% CI.

Figure 4: Phenotypic analysis of PD-1 positive T cells in head and neck cancer patients.

A : Head and neck cancer biopsies were stained with anti-PD-1 (2nd image from the right) and anti-PD-L1 antibodies (2nd image from the left). Double immunofluorescence staining for PD-1 and PDL-1 are shown on the right. Isotype control antibodies for anti-PD-L1 (left) and PD-1 (shown in Fig 2) were included in each experiment.

B : Head and neck cancer biopsies were dissociated by DNase and collagenase and cells were stained with CD45, Dye780 and CD3. Live T cells were then stained with CD4, CD8, PD-1, HLA-DR and CD38. After gating on PD-1⁺CD4⁺ or PD-1⁻CD4⁺ or PD-1⁺CD8⁺ or CD8⁺PD-1⁻ cells, expression of the activation markers (HLA-DR and CD38) were compared on the various PD-1-positive and PD-1-negative T cell populations. A representative experiment is shown in B. Mean (\pm standard deviation) expression of HLA-DR and CD38 on CD3⁺T cells, CD4⁺T cells and CD8⁺T cells from 14 patients is shown in C.

D: Twenty PD-1⁺T cells were selected at random from 16 HPV-positive (black square) or HPV-negative (white square) head and neck cancers. The number of cells costained with Tim-3 is shown with the mean indicated for the PD-1⁺Tim-3⁻ and PD-1⁺Tim-3⁺ groups.

Figure 5: Functional analysis of intratumoral PD-1⁺T cells after blockade of the PD-1/PDL-1 pathway.

Head and neck cancer biopsies from 4 head and neck cancer patients (3 HPV-negative and 1 HPV-positive) were dissociated by DNase and collagenase resulting in cell suspensions containing both tumor cells and stromal cells including T cells. These cells ($2 \cdot 10^5$) were

transferred to IFN γ Elispot plates incubated with medium, anti-PD-1 or isotype control antibodies in the presence or absence of a pool of E7 peptides for 48 hours. All tests were performed in triplicate. The number of spots (mean \pm standard deviation) is shown. * indicates $p < 0.05$.

Figure 6: PD-1 induced on CD8⁺T cells after vaccination is required for the efficacy of PD-1-PD-L1 blockade.

A : Mice were subcutaneously grafted with the E7-expressing TC1 epithelial cell line. At day 9, mice were vaccinated twice at a 9-day interval with a vaccine composed of a non replicative delivery vector, the B subunit of Shiga toxin, coupled to an E7 polypeptide (STxB-E7) used at 20 μ g. α GalCer (1 μ g) was mixed with the vaccine during the prime. PD-1 expression on total CD8 or E7₄₉₋₅₇ specific CD8⁺T cells was determined on dissociated cells by cytometry analysis from biopsies of mice tumors (previously vaccinated or not) at a time corresponding to 5-6 days after the second vaccination.

B : PD-1 expression on total CD8⁺T cells and E7₄₉₋₅₇ specific CD8⁺T cells in mice vaccinated or not with STxB-E7. PD-1 expression on E7₄₉₋₅₇ specific CD8⁺T cells from the non vaccinated group is not shown, as we could not detect these cells in this group. This experiment is representative of 3 experiments with 4 mice per group.

C: The epithelia tumor cell line, TC-1, was stained *in vitro* with anti-PD-L1 or isotype control antibodies.

D: Mice were subcutaneously grafted with the E7-expressing TC1 epithelial cell line. Mice were then either vaccinated or not vaccinated at day 9 and day 18 with STxB-E7 mixed during the prime with α GalCer. The vaccine was combined with anti-PD-L1 or isotype control antibodies (200 μ g/injection) which were administered 1, 4 and 9 days following the vaccine injection. A group of mice receiving the anti-PD-L1 mAb without the vaccine was

PD-1-expressing T cells in HPV-associated oral cancers.

also included. Tumor growth was monitored every 3 days. Results shown are representative of 3 experiments with 7-10 mice per group. For statistical analysis, each group was compared to the control group. * $p < 0.05$ ** $p < 0.01$

		HPV-positive head and neck cancer	HPV-negative head and neck cancer	
CD8	High	17	7	p = 0.01
	Low	15	25	
CD4	High	22	16	p = 0.12
	Low	10	16	
PD-1 ⁺ CD4 ⁺	High	20	12	p = 0.045
	Low	12	20	
PD-1 ⁺ CD8 ⁺	High	18	11	p = 0.078
	Low	14	21	
PD-1 ⁺ CD4 ⁺ and PD-1 ⁺ CD8 ⁺	High	20	12	p = 0.045
	Low	12	20	
PD-1	High	19	12	p = 0.079
	Low	13	20	
Foxp3 ⁺ CD4 ⁺	High	20	12	p = 0.059
	Low	12	19	
PD-L1	high	20	13	p = 0.08
	Low	12	19	

Table 1: Correlation between HPV status and T cell infiltration and PD-L1 expression by tumor cells.

For quantitative parameters, low and high levels of each cell type were defined by using the median level of tumor infiltration by these cells as cut-off. For semiquantitative parameters (CD4, CD8, PD-L1), a score of 0 or + corresponds to low levels, while a score of ++/+++ corresponds to high levels.

All parameters were measured on 64 head and neck tumor biopsies (32 HPV-positive and 32 HPV-negative) except for Foxp3⁺CD4⁺ cells, which were counted on 63 biopsies.

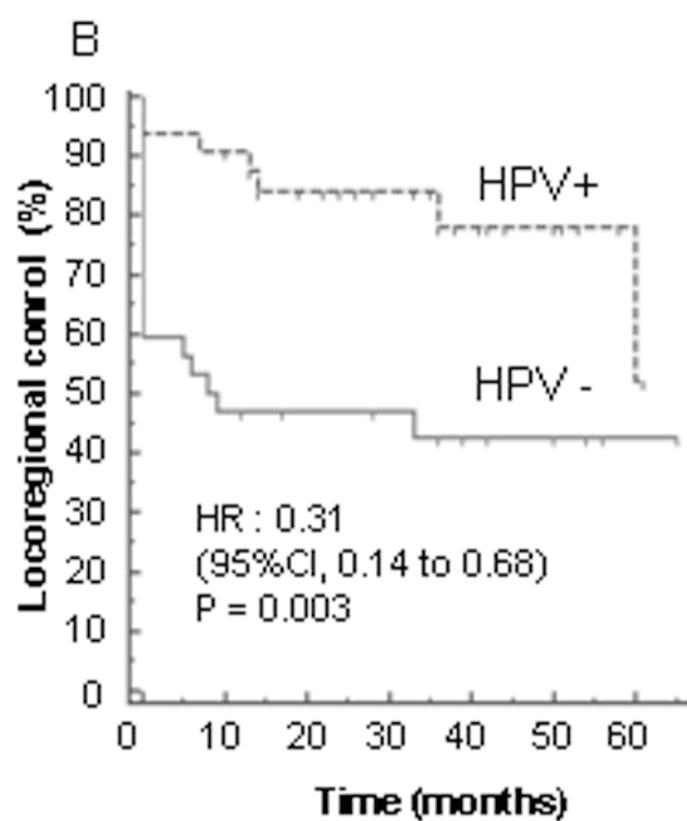
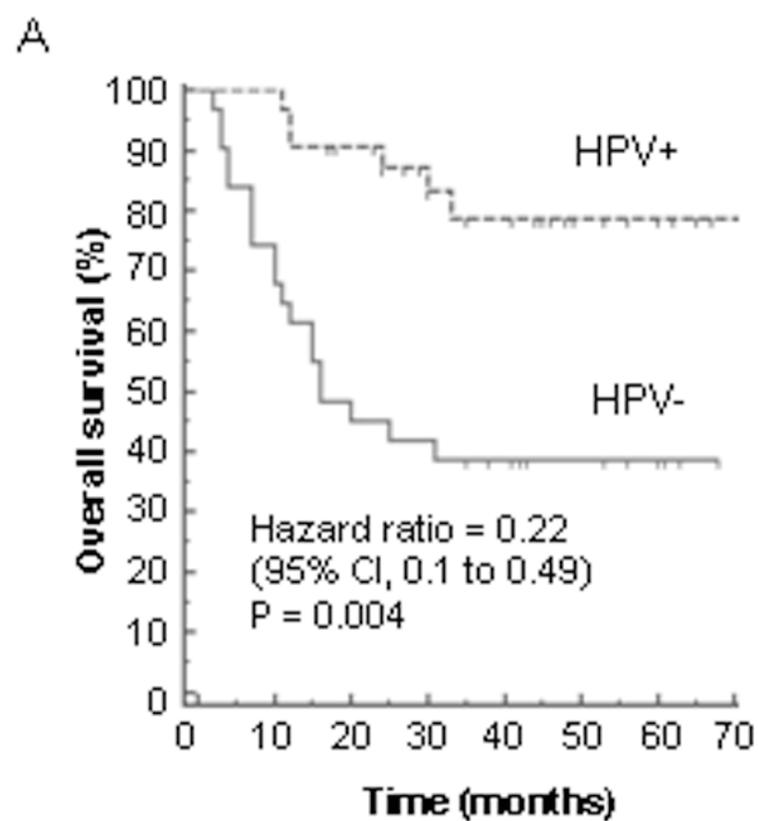


Figure 1

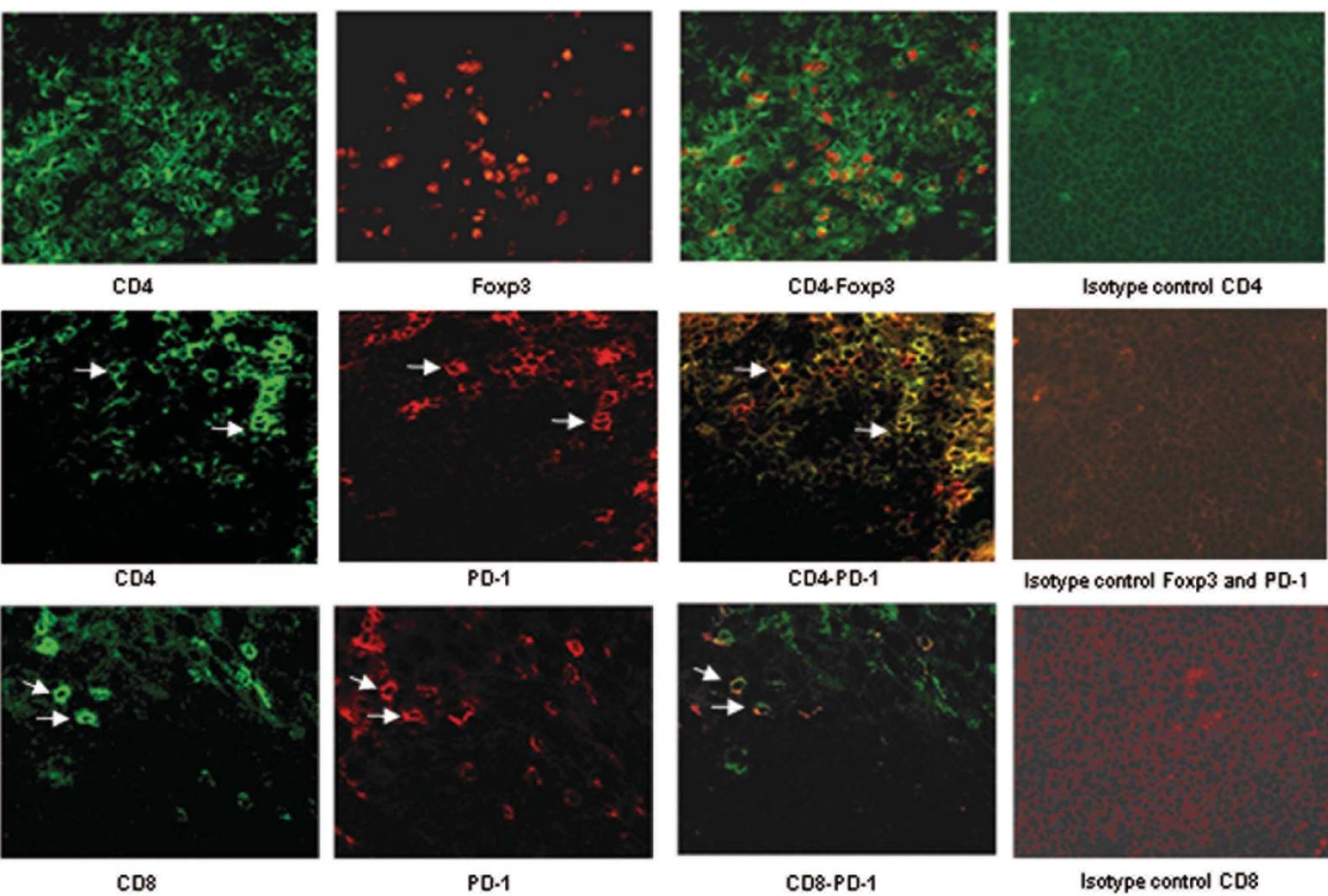
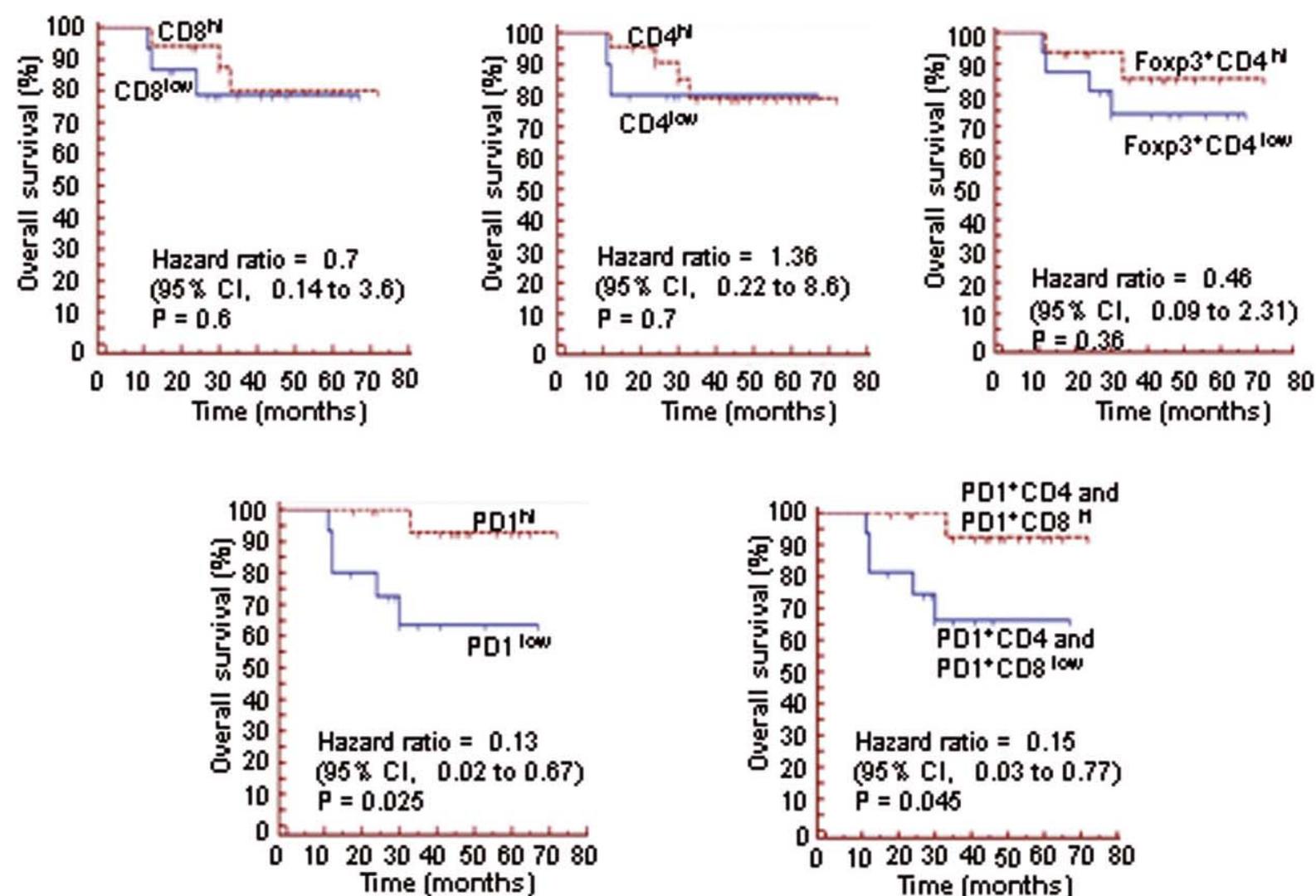
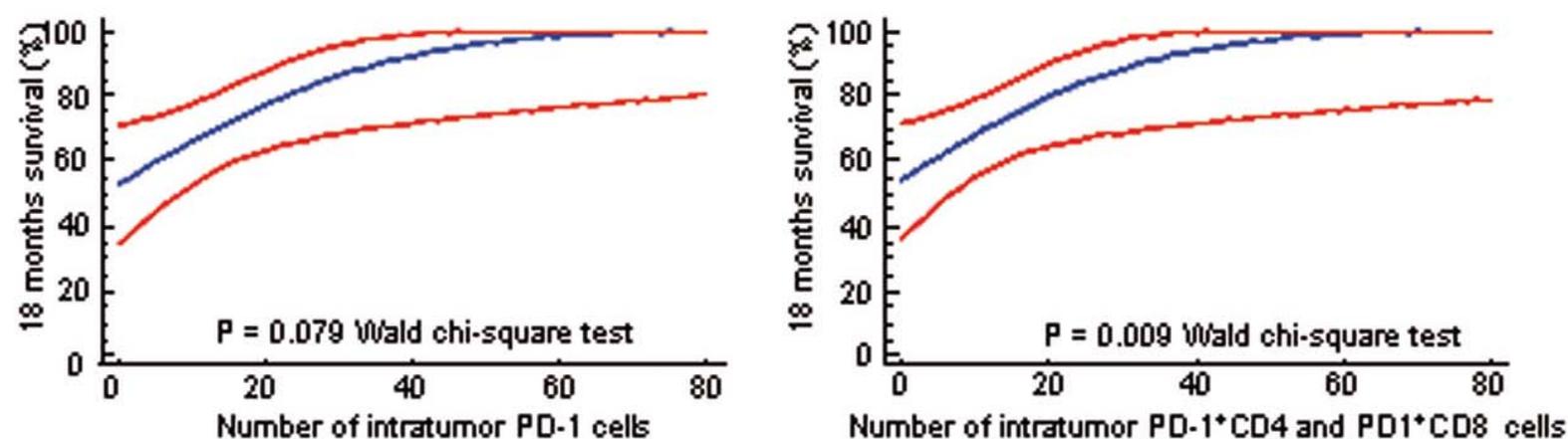


Figure 2

A HPV-positive Head and Neck cancers**B** Total Head and Neck cancers**Figure 3**

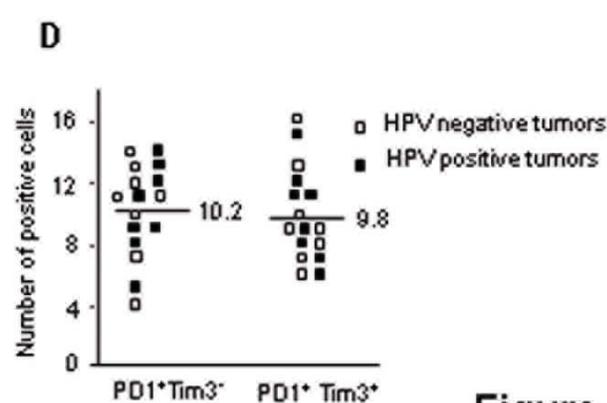
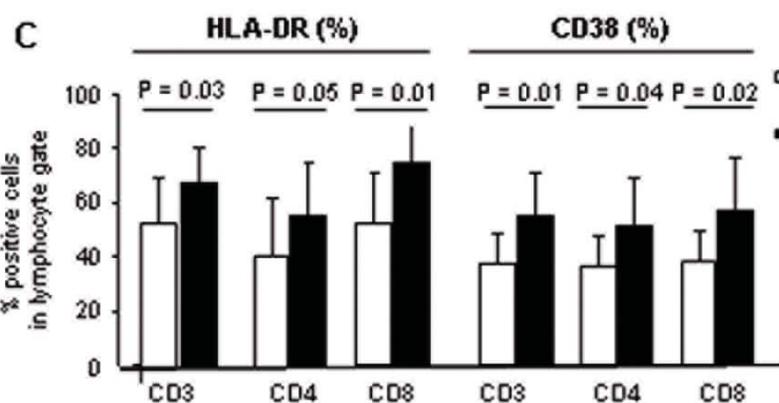
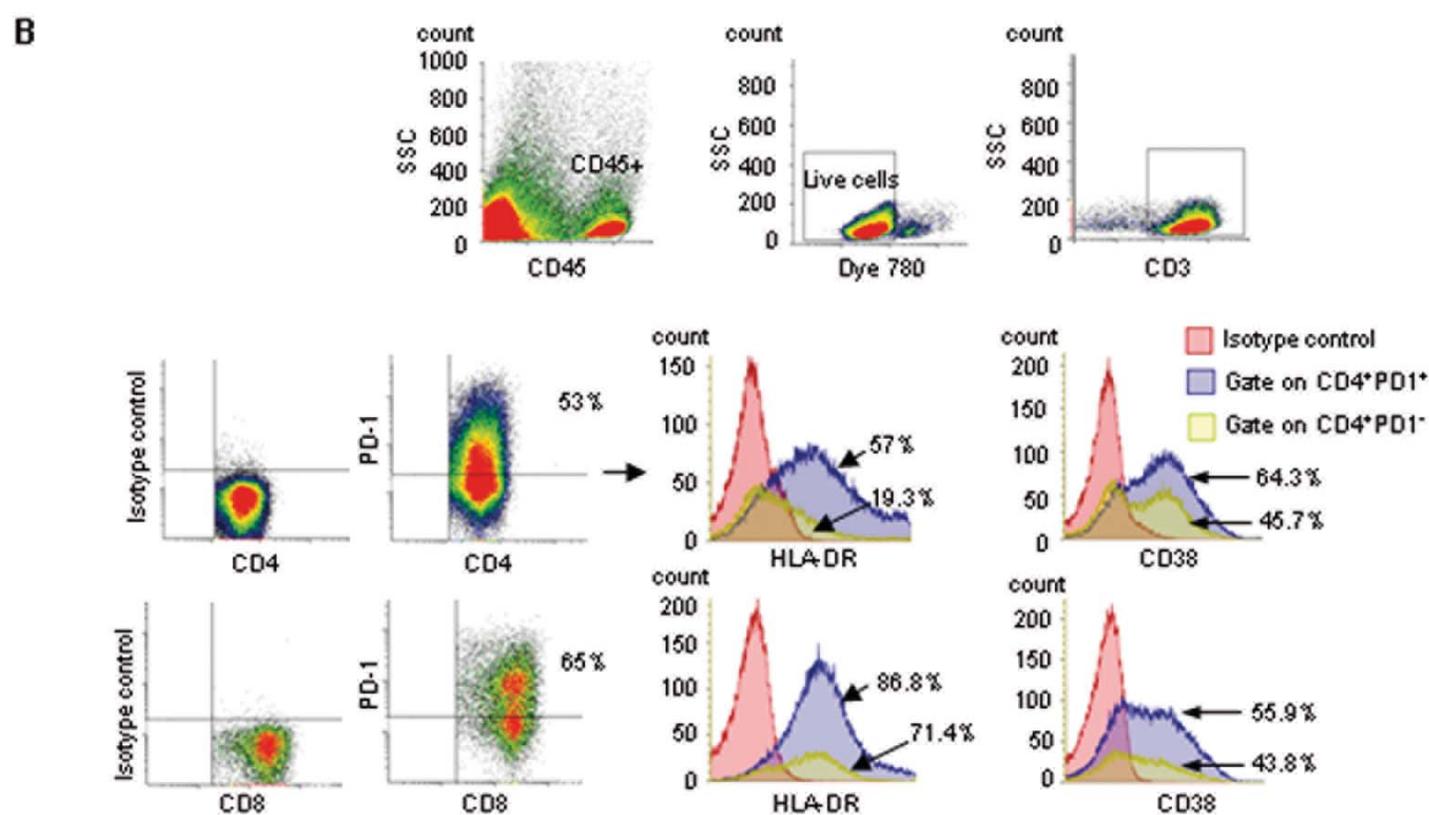
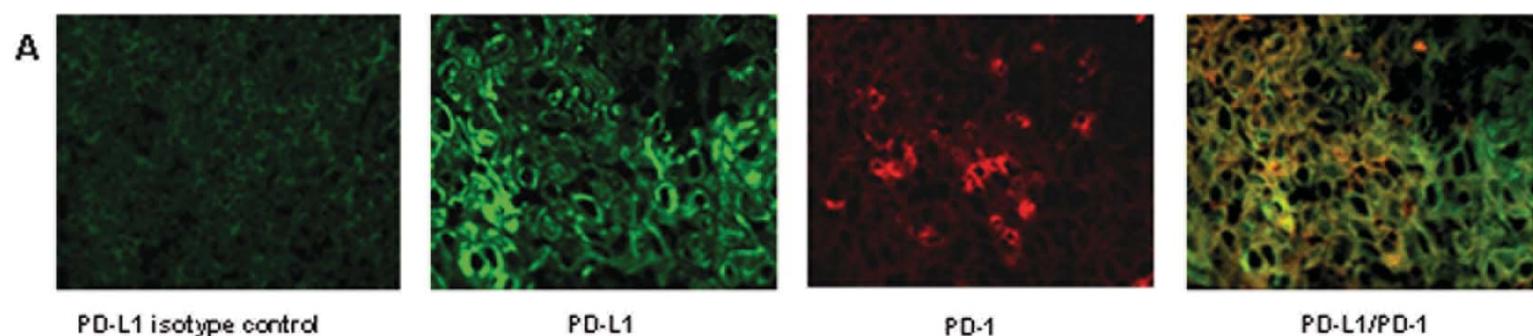


Figure 4

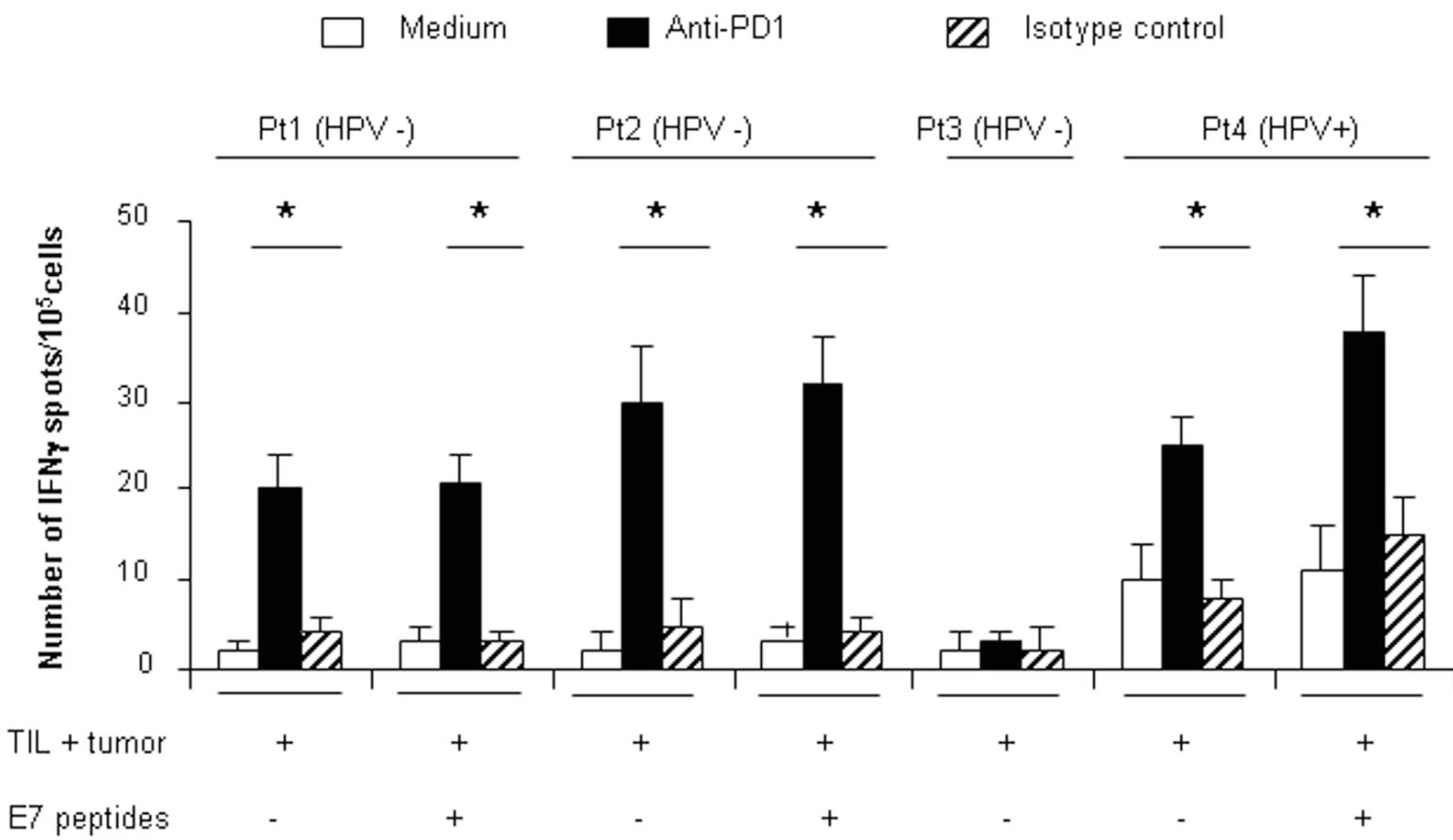
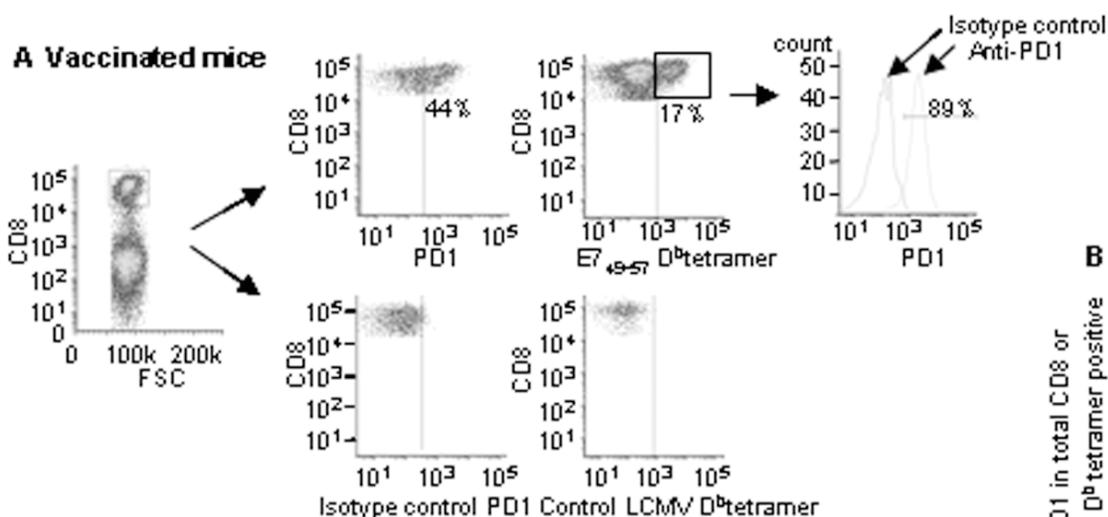
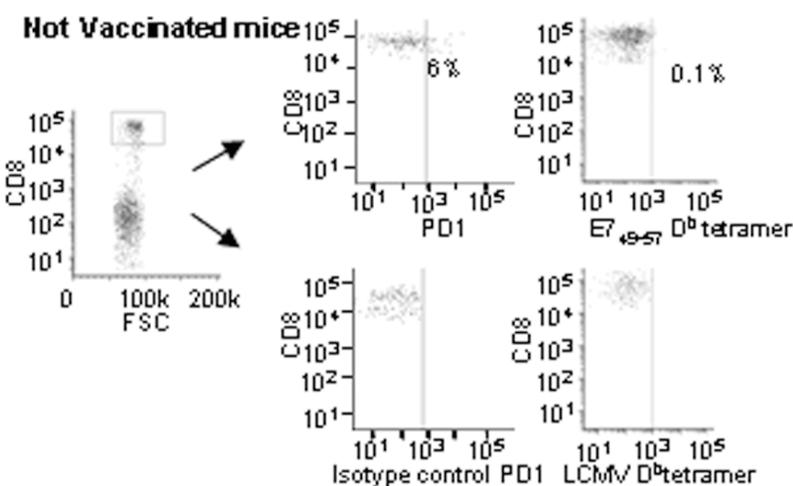


Figure 5

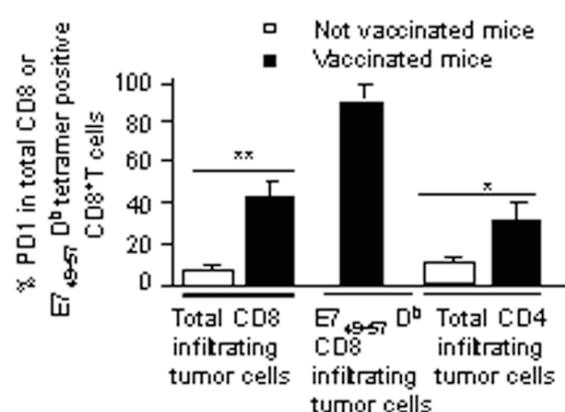
A Vaccinated mice



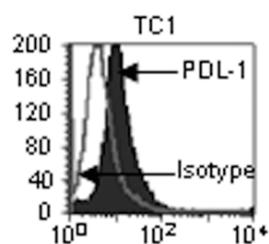
Not Vaccinated mice



B



C



D

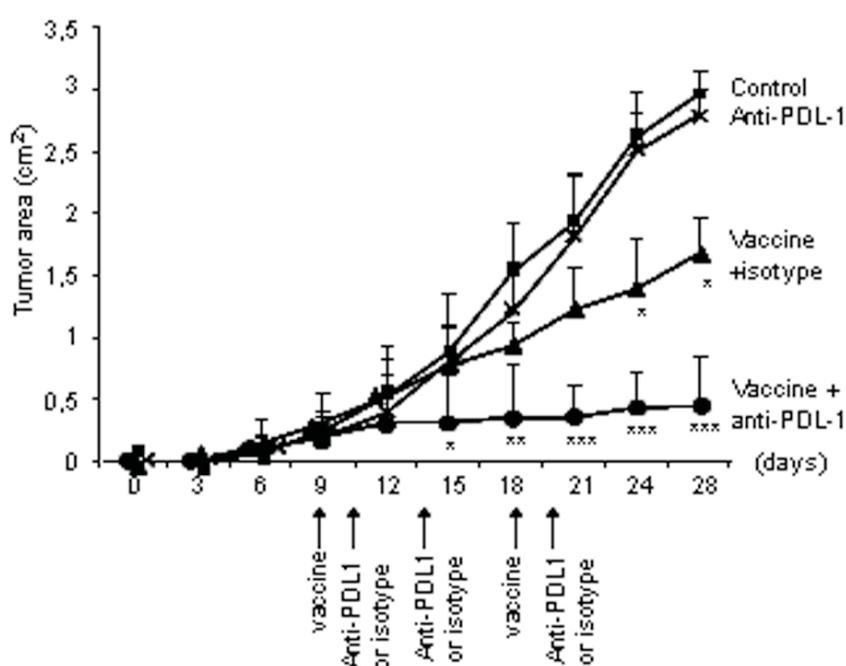


Figure 6