

Therapeutic Peptide Vaccine-Induced CD8 T Cells Strongly Modulate Intratumoral Macrophages Required for Tumor Regression

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Abstract

Abundant macrophage infiltration of solid cancers commonly correlates with poor prognosis. Tumor-promoting functions of macrophages include angiogenesis, metastasis formation, and suppression of Th1-type immune responses. Here, we show that successful treatment of cervical carcinoma in mouse models with synthetic long peptide (SLP) vaccines induced influx of cytokine-producing CD8 T cells that strongly altered the numbers and phenotype of intratumoral macrophages. On the basis of the expression of CD11b, CD11c, F4/80, Ly6C, Ly6G, and MHC II, we identified four myeloid subpopulations that increased in numbers from 2.0-fold to 8.7-fold in regressing tumors. These changes of the intratumoral myeloid composition coincided with macrophage recruitment by chemokines, including CCL2 and CCL5, and were completely dependent on a vaccine-induced influx of tumor-specific CD8 T cells. CD4 T cells were dispensable. In-

duction of tumor cells with T cell-derived IFN γ and TNF α recapitulated the chemokine profile observed *in vivo*, confirming the capacity of antitumor CD8 T cells to mediate macrophage infiltration of tumors. Strikingly, complete regressions of large established tumors depended on the tumor-infiltrating macrophages that were induced by this immunotherapy, because a small-molecule drug inhibitor targeting CSF-1R diminished the number of intratumoral macrophages and abrogated the complete remissions. Survival rates after therapeutic SLP vaccination deteriorated in the presence of CSF-1R blockers. Together, these results show that therapeutic peptide vaccination could induce cytokine-producing T cells with strong macrophage-skewing capacity necessary for tumor shrinkage, and suggest that the development of macrophage-polarizing, rather than macrophage-depleting, agents is warranted. *Cancer Immunol Res*; 3(9); 1042–51. ©2015 AACR.

Introduction

Cervical cancer is strongly associated with human papillomavirus (HPV) infection (1, 2). The two HPV oncogenes E6 and E7 are critical for maintenance of cellular transformation; they are constitutively expressed by HPV-associated tumors and are therefore ideal targets for therapeutic vaccination (3). We have shown that overlapping synthetic long overlapping peptides (SLP) comprising these proteins are capable of inducing potent T-cell responses in mice and humans (4–7). In patients with premalignant lesions, vaccine-induced T-cell responses correlate with complete clinical responses. However, in patients with advanced or recurrent HPV16-induced gynecologic carcinoma, there is no apparent effect on

survival (8, 9), suggesting that the induction of a measurable T-cell response alone is not sufficient for a therapeutic effect.

Preclinical data from mouse models of HPV16-induced malignancies suggest that tumor infiltration of macrophages [tumor-associated macrophages (TAM)] causes suppression of antitumor T-cell responses, thereby facilitating tumor growth (10). In fact, abundant infiltration of TAMs correlates with poor prognosis in most human cancers, and these macrophages are characterized by a "tissue-repair" function, via production of epithelial growth factors and stimulation of angiogenesis, tissue remodeling, and immunoregulatory functions. This M2-like differentiation program also includes suppression of Th1-mediated cytotoxic immune responses in order to minimize tissue damage (11, 12). Therefore, TAM functions are often compared with those in wound-healing responses in which cytotoxic immunity is dampened. This role of myeloid cells in the local tumor microenvironment (TME) is the subject of extensive studies in mouse tumor models and human cancer (11, 12).

The myeloid compartment is very heterogeneous, and circulating monocytes can differentiate toward M1-type macrophages that support Th1-polarized immunity and mediate killing of bacteria (13). Skewing TAMs toward this "classically" activated phenotype can result in antitumor activity and clearance of established tumors, indicating that tumor-associated myeloid cells might hold the key to switching the local immune system to a tumor-rejection response (14, 15). Silencing of the NF- κ B pathway through targeting of IKK β in macrophages was shown to result in such a desirable differentiation (14, 15). We demonstrated that a combination of IFN γ and CD40L signals prevents

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Supplementary data for this article are available at Cancer Immunology Research Online (<http://cancerimmunolres.aacrjournals.org/>).

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doi: 10.1158/2326-6066.CIR-15-0052

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cancer-induced M2-type differentiation (16). Interestingly, low-dose local radiotherapy and anthracyclin-type chemotherapies were shown to program macrophage differentiation to support intratumoral recruitment of CD8 T cells (17, 18). Therapy-induced inflammatory macrophages were responsible for uptake and presentation of tumor antigens to T cells, thereby leading to effective antitumor immunity.

Long peptide cancer vaccines are strong immunogens and are capable of inducing regressions in experimental mouse tumors and premalignant lesions in patients (4, 6). We use two models of HPV-induced malignancies and show that only when therapeutic vaccination with SLP induces influx of cytokine-producing CD8 T cells, the tumor cells produce myeloid-cell attracting and skewing chemokines. Indeed, TC-1 tumors of vaccinated mice are massively infiltrated by myeloid cells with a preponderance of inflammatory macrophages, whereas identical therapeutic vaccination in mice with C3 tumors has no effect. Importantly, we found that macrophages critically contributed to vaccine-induced tumor regressions. Complete tumor shrinkage induced by this CD8 T cell–based immunotherapy was lost when macrophages were targeted by CSF-1R inhibitors. Together, our data indicate an essential role for intratumoral macrophages during immunotherapy and argue for macrophage-skewing, rather than macrophage-depleting, strategies for future clinical development.

Materials and Methods

Mice

C57BL/6jico female mice were purchased from Charles River Laboratories and housed in the animal facility of the Leiden University Medical Center (Leiden, the Netherlands). All mice were maintained under specific pathogen-free conditions and used at 8 weeks of age. Experiments on mice were approved by the local university committee for the care of laboratory animals, in line with guidelines of the European Committee.

Tumor cell lines

The tumor cell line TC-1 (a gift from T.C. Wu, John Hopkins University, Baltimore, MD) was produced and maintained as previously described (6). The tumor cell line C3 was generated by transfection of B6 mouse embryonic cells with the complete HPV16 genome and maintained as previously described (19). Both cell lines were tested by PCR for rodent viruses, most recently in January 2012, with negative results. Authentication was determined by the presence of HPV E7 protein using specific T cells. All cell lines used were not cultured for longer than 1 month. The immature splenic dendritic cell (DC) line D1 was provided by P. Ricciardi-Castagnoli (University of Milano-Bicocca, Milan, Italy). The immature DC phenotype was tested twice monthly by flow cytometry. *Mycoplasma* testing for both cell lines was performed every 6 months by PCR.

In vivo T-cell depletion, PLX3397, and PLX5622

For *in vivo* CD8 T-cell depletion, mice were injected i.p. with 100 μ g of the CD8-depleting monoclonal antibody (mAb) 2.43 on day 7 and then every 6 days onward. mAbs were prepared and purified as described previously (20). Only mice that had a >99% depletion of CD8 T cell, as measured by flow cytometry, were included in the experiment. The CSF-1R/KIT kinase inhibitor PLX3397 was incorporated into chow at 290 mg/kg chow (resulting in a daily delivery of approximately 45 mg/kg), the CSF-1R–

specific kinase inhibitor PLX5622 was incorporated into chow at 1,200 mg/kg chow. Both inhibitors were provided along with control chow by Plexxikon Inc. (21, 22).

Tumor experiments

On day 0, mice were subcutaneously inoculated with 1×10^5 TC-1 tumor cells. From day 5 and onward, mice received the M-CSF receptor kinase inhibitor PLX3397, PLX5622, or vehicle control in chow. On day 8, when a palpable tumor was present, mice were split into groups with similar tumor size and were treated with SLP HPV16 E7_{43–77} (GQAEPDRA-HYNIIVTFCKCDSTLRLCVCQSTHVDIR) emulsified at a 1:1 ratio with Incomplete Freund's Adjuvant (IFA; Difco) in the contralateral flank. Tumor sizes were measured three-dimensionally twice weekly to a maximum of 2,000 mm³, after which the mice were sacrificed for ethical reasons.

Analysis of tumor-infiltrating immune populations

To analyze the presence of antigen-specific T cells, blood of vaccinated mice was stained for CD8[a] and CD3 combined with HPV16 E7_{49–57} (RAHYNIIVTF) MHC class I (H-2D^b) tetramer. For analysis of tumor-infiltrating populations, mice were sacrificed 10 days after SLP, transcardially perfused, and tumors of similar size were harvested, disrupted in small pieces, and incubated with Liberase (Roche) in Iscove's modified Dulbecco's medium (IMDM) for 15 minutes at 37°C. Single-cell suspensions were prepared by mincing the tumors through a 70- μ m cell strainer (BD Biosciences). Cells were resuspended in staining buffer (PBS + 2% FSC + 0.05% sodium azide) and incubated with antibodies to Ly6C (FITC), Ly6G (Alexa 700), F4/80 (PE), CD11c (BV605), CD11b (eFluor 450), allophycocyanin (APC)-labeled H-2D^b tetramers containing HPV16 E7_{49–57} peptide, 7-aminoactinomycin D (for dead-cell exclusion; Invitrogen), CD8a (A700), CD3 (Pacific Blue), CD45.2 (FITC/Alexa 780), and Class II (V500), for 30 minutes at 4°C. Fluorescent-conjugated antibodies were purchased from BD Biosciences, BioLegend, and eBioscience. To analyze the intracellular cytokine production of circulating CD8 T cells, blood was incubated for 5 hours with HPV16 E7_{49–57} peptide. For analysis of cytokine production by T cells, single-cell suspensions of tumor (-infiltrate) were incubated for 5 hours with 40,000 D1 cells loaded with the HPV16 E7_{49–57} (2 μ g/mL) peptide in the presence of Brefeldin A (2 μ g/mL). After cell-surface staining with fluorescently labeled antibodies to mouse CD45, CD8, and CD3, overnight fixation with 0.5% paraformaldehyde solution, and permeabilization with Perm/Wash buffer (BD Biosciences), cells were stained at 4°C with fluorescently labeled antibodies against IFN γ (APC) and TNF α (FITC). Samples were analyzed with a BDLSR II flow cytometer, and results were analyzed using the FlowJo software (TreeStar).

Quantitative real-time PCR

Tumor material. Tumors from perfused mice were embedded in Tissue-Tek and immediately frozen in cooled 2-methylbutane. RNA was isolated from 20 sections of 20 μ m.

***In vitro* chemokine expression levels.** TC-1 tumor cells were harvested and seeded at 1.2×10^5 cells per well and C3 at 1.6×10^5 cells per well (6-well plate). After 24 hours, TC-1 cells were incubated with 20 ng TNF α and/or 200 ng IFN γ and C3 with 10 ng TNF α and/or 40 ng IFN γ . Twenty-two hours later, tumor

cells were harvested, washed in PBS, and snap-frozen in liquid nitrogen.

RNA from *in vitro* and *in vivo* samples was isolated using an RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. cDNA synthesis was performed using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). Reactions were performed using iQ SybrGreen supermix (Bio-Rad) on the CFX384 Real-Time PCR Detection System (Bio-Rad) with an annealing temperature of 60°C. Cycle threshold (C_T) values were normalized to the expression levels of the GAPDH and β -actin genes (ΔC_T). The difference between the individual ΔC_T and the average expression was calculated for each gene and expressed as fold change of the average and displayed in a heatmap. All oligonucleotide primers are compiled in Supplementary Table S1.

Microscopy

IHC for the F4/80 macrophage marker was performed as previously described (23).

Statistical analysis

Survival for differentially treated mice was compared using the Kaplan–Meier method and the log-rank (Mantel–Cox) test. Additional statistical methods are stated in the figure legends. All $P < 0.05$ were considered statistically significant.

Results

Infiltration of tumor-specific CD8 T cells coincides with influx of high numbers of macrophages into the tumors

Established TC-1 mouse tumors, which are transformed by the E6 and E7 oncogenes of HPV16, respond to prophylactic and

therapeutic vaccination with an SLP, comprising the immunodominant E7 peptide emulsified in the mineral oil-based adjuvant IFA. Ten days after the start of therapy, the tumor-specific CD8 T-cell response is sufficiently elevated to mediate a robust regression of tumors with sizes up to 600 mm³. However, the independently derived HPV16 tumor line C3 responds to prophylactic but not to therapeutic vaccination with the same SLP vaccine modality (5, 19). To better understand these differences, we analyzed tumor-infiltrating leukocytes 10 days after peptide vaccination, when TC-1 tumors just start to regress but C3 tumors continue to grow. It became apparent that peptide vaccination induced an abundant infiltration of E7-specific, IFN γ - and TNF α -producing CD8 T cells in TC-1 tumor (Fig. 1A–C) but not in C3 tumors (Fig. 1D–F). Interestingly, SLP vaccination also induced an extensive increase of the F4/80⁺/CD11b⁺ macrophage population in the TC-1 model (3.3-fold enhanced compared with untreated) but not in the C3 tumor model (Fig. 1G vs. H). Because CD8 T-cell depletion in vaccinated TC-1 tumor-bearing mice fully abrogated the SLP vaccine-induced macrophage increase, we concluded that CD8 T cells are essential for an enhanced myeloid tumor infiltration. This conclusion was confirmed by our observation that CD4 depletion had no effect on vaccine-induced changes (Fig. 1G) and that mice that were injected with an empty IFA depot had a comparable myeloid cell tumor infiltration as untreated tumors (Supplementary Fig. S1A).

Inflammatory macrophages are the dominant cells in peptide-treated TC-1 tumors

Characterization of the intratumoral myeloid population (CD45⁺CD3⁻CD11b⁺) by flow cytometry allowed the

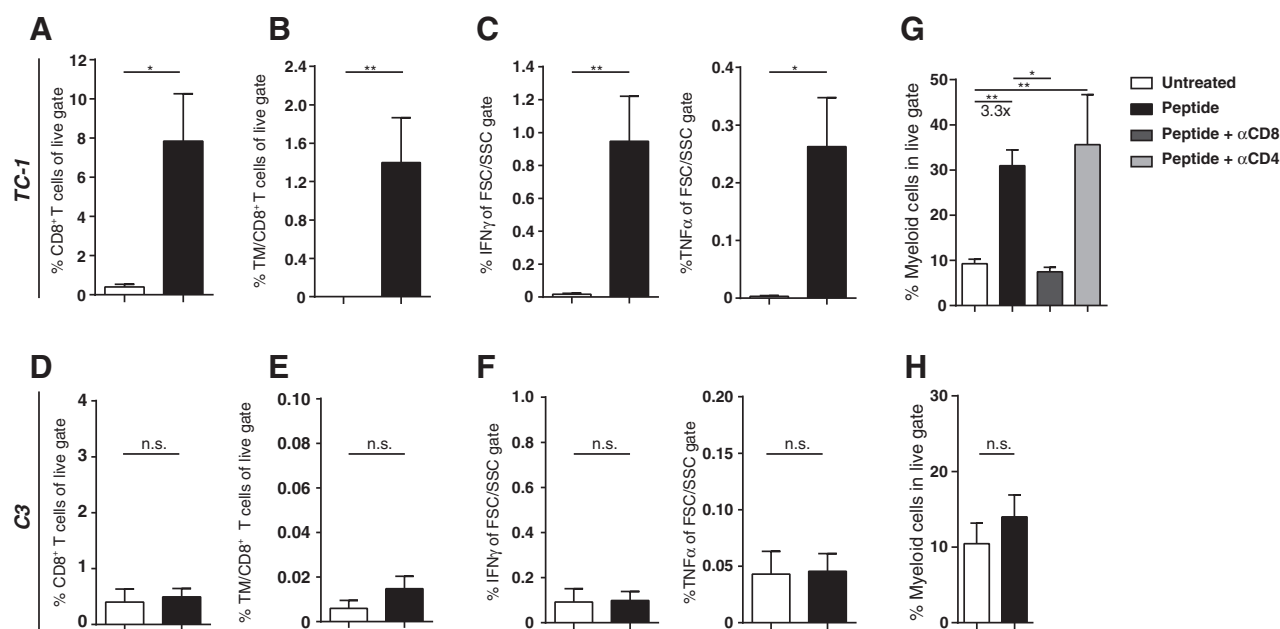


Figure 1.

An increased CD8 T-cell infiltration leads to enhanced numbers of myeloid cells. TC-1 and C3 tumors were resected 10 days after vaccination and single-cell suspensions of infiltrating cells were analyzed by multicolor flow cytometry. Percentages of intratumoral CD8 T cells in TC-1 tumors (A–C) and C3 tumors (D–F) are depicted. A and D, frequency of all CD8 T cells, (B and E) HPV16 E7-specific tetramer (Tm)⁺ CD8 T cells, and (C and F) cytokine-producing CD8 T cells out of live gate. G–H, shown are the percentages of intratumoral myeloid cells (CD45⁺CD11b⁺) in the live gate of TC-1 tumors (G) and C3 tumors (H). Data shown for TC-1 are representative for three independent experiments with 5 animals per group. Data shown for C3 are from one experiment with 4 to 5 mice per group. All data are expressed as mean with standard error of the mean (SEM; Student *t* test; *, $P < 0.05$; **, $P < 0.01$).

identification of distinct subsets, based on differential expression of the macrophage marker F4/80 and the activation marker Ly6C (Fig. 2A). To gain insight into the identity of these distinct myeloid subsets in the two models, we examined the expression levels of CD11c, MHC-II, and Ly6G (Fig. 2B and C). We could delineate four subsets: "inflammatory macrophages" (F4/80^{hi}Ly6C^{hi}) with intermediate levels of CD11c and MHC-II (population 1); "tissue-resident macrophages" (F4/80^{int}Ly6C^{int}; population 2); DC-like macrophages (F4/80^{hi}Ly6C^{int}) with the highest expression of CD11c and MHC-II (population 3); and granulocytic myeloid cells that were Ly6C⁺ (F4/80^{low}Ly6C^{hi}; population 4). The myeloid profile of untreated TC-1 tumors strongly differed from that of TC-1 tumors from SLP-vaccinated mice, even though tumor sizes were comparable at the time of analysis (Supplementary Fig. S1B). Peptide vaccination enhanced the presence of all myeloid subsets between 2-fold and 8-fold (Fig. 2D), but inflammatory macrophages increased the most, around 8-fold. Tissue-resident macrophages (population 2) was the most prominent subset in untreated tumors, whereas inflammatory macrophages (population 1), expressing high levels of Ly6C, dominated the SLP-vaccinated tumors (Fig. 2C). The observation that infiltration of the inflammatory macrophages was significantly correlated to CD8 T-cell infiltration confirmed our observation that CD8 T cells are essential for myeloid cell infiltration. In C3 tumors of SLP-treated mice, there were no differences in the frequencies of these subsets. Furthermore, in TC-1 tumors, but not in C3 tumors, peptide vaccination resulted in increased CD11c expression on all myeloid population, a more uniform expression of class II on population 2 and a decreased Ly6G expression on population 4 (Fig. 2B and D), indicating that only successful therapeutic vaccination altered the activation status of the different myeloid subsets. Together, these data demonstrated that a vaccine-induced tumor regression phase is precluded by a strong recruitment of CD11b⁺F4/80⁺Ly6C⁺ macrophages into tumors. Because these differences appeared only in tumors with a dense CD8 T-cell infiltration, we concluded that vaccine-induced CD8 T cells are essential for this myeloid modulation.

Infiltration of tumor-specific CD8 T cells enhances the levels of macrophage-attracting chemokines

Next, we tested how CD8 T cells would modulate myeloid cell infiltration. Because various chemokines have been linked to the attraction of inflammatory macrophages, we analyzed chemokine gene expression on whole-tumor material and detected increased *CCL2* levels in tumors of SLP-vaccinated mice compared with that of untreated tumors (Fig. 3A). The levels of monocyte-attracting chemokines *CCL5* and *CCL12* as well as the T cell-attracting chemokines *CXCL9*, *CXCL10*, and *CXCL11* clearly increased upon peptide vaccination (Fig. 3A). When TC-1 tumor cells were co-cultured *in vitro* with TNF α and IFN γ , two cytokines produced by SLP vaccination-induced intratumoral T cells (Fig. 1; ref. 5), a striking increase in *CCL2*, *CCL5*, and *CXCL10* gene expression was observed (Fig. 3B). *CXCL9* and *CXCL11* were undetectable in untreated and single-treated samples but clearly evident upon combined incubation with both IFN γ and TNF α (data not shown). Interestingly, similar chemokine levels were induced by IFN γ -treated and/or TNF α -treated C3 tumor cells (Fig. 3C), indicating that the capacity of C3 tumor cells to produce myeloid- and T cell-attracting chemokines did not differ from that of TC-1 tumor cells. Analysis of whole TC-1 tumors revealed that, although *Arginase-1* and *CXCL2* levels were similar between

control and treated groups, tumors from vaccinated mice showed a tendency toward enhanced levels of the myeloid marker *CD11b*, confirming that vaccination enhanced myeloid infiltration. Moreover, the enhanced expression of *CD74*, the invariant chain chaperone of MHC class II, and "Fc γ ", the common gamma chain of Fc receptors for IgG, confirmed the activated phenotype of infiltrated myeloid subsets. Together, these data indicated that stimulated tumor cells express enhanced levels of monocyte- and T cell-attracting chemokines. Because the attraction of T cells appeared crucial for the observed myeloid changes (Figs. 1 and 2), this strongly suggests that the levels of T cell-produced cytokines within the tumor bed are crucial for myeloid attraction to and alterations within the tumor.

PLX3397 selectively removes tissue-resident macrophages and DC-like myeloid cells

Next, we dissected the role of intratumoral macrophages and their function in vaccine-induced tumor rejection responses as observed in the TC-1 tumor model. To this end, we used the tyrosine kinase inhibitor PLX3397. TC-1 tumor cells did not express the PLX3397-targeted receptors CSF-1R and c-Kit (data not shown), precluding direct effects of the compound on tumor cells. In concordance with previous studies, PLX3397 primarily deleted intratumoral macrophages (Supplementary Fig. S2), as the numbers of liver and spleen macrophages appeared unaffected (Supplementary Fig. S3; refs. 21, 22).

F4/80⁺ cells were scarcely present in tissue sections of TC-1 tumors after PLX3397 application as determined by IHC (Supplementary Fig. S2) and flow cytometry (Fig. 4A). In SLP-vaccinated mice, PLX3397 treatment also clearly reduced the total number of macrophages, although a small proportion remained. Together, these data demonstrate a strong reduction of intratumoral macrophages by the small-molecule inhibitor PLX3397.

Importantly, treatment with PLX3397 did not change the total number, the antigen specificity, or cytokine production by systemic (Fig. 4B and C) or intratumoral CD8 T cells (Fig. 4D–F). Furthermore, no changes in the vaccine-induced specificity of CD8 T cells were detected in the lymph nodes and blood of non-tumor-bearing mice (Supplementary Fig. S4). We concluded that PLX3397 did not affect vaccine-mediated T-cell priming, effector functions, or homing, but strongly reduced the numbers of intratumoral macrophages.

Tumor regression depends on macrophages

Next, we examined the effect of PLX3397 on vaccine-induced tumor-rejection responses. Mice bearing TC-1 tumors were treated in the presence or absence of PLX3397. SLP vaccination on day 10 after tumor inoculation resulted in strong regressions of large established tumors (Fig. 5A). Depletion of CD8⁺ T cells completely abolished tumor control, indicating that CD8⁺ T cells are crucial for peptide-induced antitumor responses (Fig. 5A). Accordingly, control vaccination had no effect on tumor growth, supporting the fact that tumor-specific T cells are indispensable for tumor regressions (data not shown).

Strikingly, PLX3397 application during peptide vaccination abrogated the typical complete tumor regressions (Fig. 5A and B). After initial reduction in tumor sizes, progressive tumor growth was observed (Fig. 5A and B). Tumor sizes initially decreased in vaccine-treated animals, but these regressions were not sustained in the absence of macrophages. Application of the

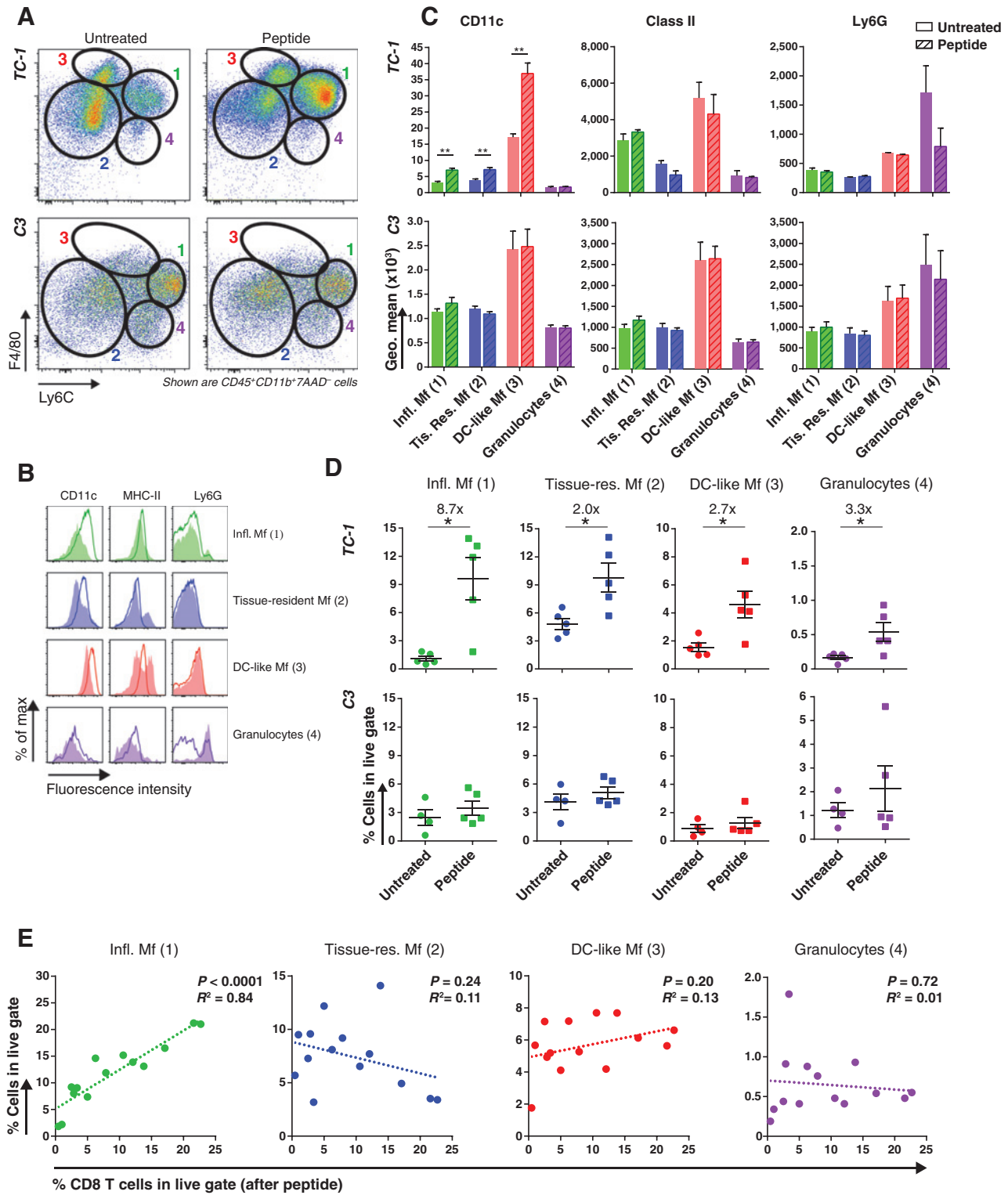


Figure 2. Vaccination with SLP massively enhances numbers of macrophage subsets. TC-1 tumors and C3 tumors were resected 10 days after vaccination and single-cell suspensions of infiltrating cells were analyzed by multicolor flow cytometry. A, flow cytometry plots are shown for F4/80 and Ly6C staining of tumor-infiltrating myeloid cells in untreated (left) and peptide-treated (right) tumors. Four different subpopulations were distinguished. B, the expression of CD11c, MHC-II, and Ly6G on each subpopulation of untreated TC-1 tumors (filled histograms) and TC-1 tumors treated with SLP vaccine (solid lines). C, geometric means of CD11c (left), MHC II (middle), and Ly6G (right) on all four tumor-infiltrating subsets are shown. D, percentages of the four different myeloid cell subsets were determined for both tumor types. Data shown for TC-1 are representative of three independent experiments with 5 animals per group. (Continued on the following page.)

CSF-1R inhibitor alone did not lead to any change in tumor outgrowth. Depletion of intratumoral macrophages by PLX3397 resulted in survival curves that were comparable with those of T cell-depleted animals (Fig. 5C). Additionally, the more specific but weaker CSF-1R inhibitor PLX5622 (24) abrogated the peptide-induced tumor regression (Supplementary Fig. S5). Together, our data illustrate how SLP vaccination causes substantial changes in the myeloid cell composition of tumors and, moreover, indicate that the induction of this major inflammatory population of macrophages is required for successful and more sustained tumor-regression responses.

Discussion

Successful tumor-rejection responses evoked by long-peptide vaccines depend on tumor-specific CD8 T cells and, as we show here, also on intratumoral macrophages. The typical steep shrinkage of tumors that starts 10 days after SLP vaccination was abrogated in the presence of PLX3397, an inhibitor of the myeloid growth receptors CSF-1R and c-Kit (21, 22), and in the presence of PLX5622, a weaker but selective CSF-1R inhibitor (24). Upon inhibition of intratumoral macrophages an initial stabilization of tumor sizes was followed by rapid regrowth, instead of shrinkage. Because only those tumors with a clear infiltration of IFN γ - and TNF α -producing, tumor-specific CD8 T cells displayed altered myeloid subsets (Figs. 1 and 2), we conclude that cytotoxic CD8 T cells have the capacity to deploy a tumor-rejection response by local attraction and skewing of macrophages in the TME. In addition to the well-known CD4 T cell-macrophage axis revealed in intracellular bacterial infections and tumor progression (25, 26), we demonstrate that intratumoral CD8 T cells can also instruct macrophages to exert effector functions. Our data show that a strong CD8 T-cell response induces a DC-like macrophage population, expressing not only high levels of F4/80 but also the classical DC markers MHC-II and CD11c. A phenotypically similar population has been found in the MMTV-PyMT mammary tumor model (27). Characterization of this cell population by transcriptional profiling revealed that DC lineage-specific transcription factors and receptors were absent in this population, indicating that these cells represent macrophages with a DC-like phenotype (27). However, the most prominent population that is present after vaccination and the only population that corresponds to the density of CD8 T-cell infiltration are the inflammatory macrophages, expressing high levels of Ly6C. At this moment, we are investigating the precise biologic activities of these instructed macrophages that are apparently important for tumor regressions.

Interestingly, comparable strong shifts in intratumoral macrophage subsets were recently described for anthracycline-based chemotherapy and local radiotherapy (17, 18). Successful cancer therapy leading to tumor regressions apparently coincides with strong changes in myeloid cell subsets in the tumor. Slight differences in macrophage compositions between these models might be based on the preexisting macrophages of these tumor models or on unique skewing capacities of the treatments.

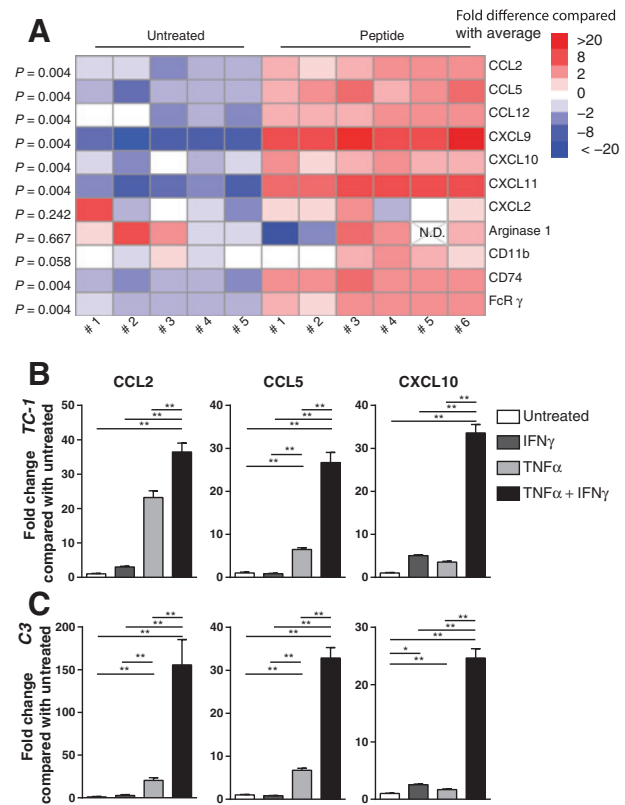


Figure 3. Induced expression of chemokines in tumors after therapeutic peptide vaccination. A, mice were inoculated with TC-1 tumors and treated with SLP vaccination. Gene expression profiling by qPCR was performed on tumors isolated on day 10 after vaccination. Each sample was tested in quadruplicates and 5 to 6 mice per group were analyzed and displayed in a heatmap (Mann-Whitney test, P values are indicated on the left). B and C, gene expression of the chemokines *CCL2*, *CCL5*, and *CXCL10* in TC-1 tumor cells (B) and C3 tumor cells (C) 22 hours after exposure to TNF α and/or IFN γ , represented as fold change over untreated tumor cells. Data presented are representative of two independent experiments. Mean and standard deviations are depicted (one-way ANOVA; *, $P < 0.05$; **, $P < 0.01$).

Because peptide vaccination can induce a dense infiltration of TNF α - and IFN γ -producing CD8 T cells, it is likely that these cytokines are involved in the modulation of intratumoral myeloid cells in our model. Previously, we and others have shown that IFN γ can overcome cancer-induced M2-type differentiation (16, 28). Here, we show that coincubation of two HPV16-transformed tumor cell lines with TNF α and IFN γ enhances the expression of various chemokines that are known to mediate the recruitment of (inflammatory) macrophages to the tumor. Furthermore, C3 tumors and CD8 T cell-depleted TC-1 tumors showed no altered myeloid cell infiltration. Only tumors infiltrated with cytokine-producing T cells and a large number of inflammatory macrophages, and a similar chemokine profile as found in tumor cells incubated with IFN γ and TNF α . The link

(Continued.) Data shown for C3 are from one experiment with 4 to 5 mice per group. All data are expressed as mean with standard error of the mean (SEM; Student t test; *, $P < 0.05$; **, $P < 0.01$). E, correlation between the percentages of CD8 T cells (horizontal axis) and the four subsets (vertical axis) within resected tumors of vaccinated mice. Each dot represents data from an individual mouse; data shown are pooled from three independent experiments. The data were analyzed by linear regression; R^2 and P values are indicated in the figure.

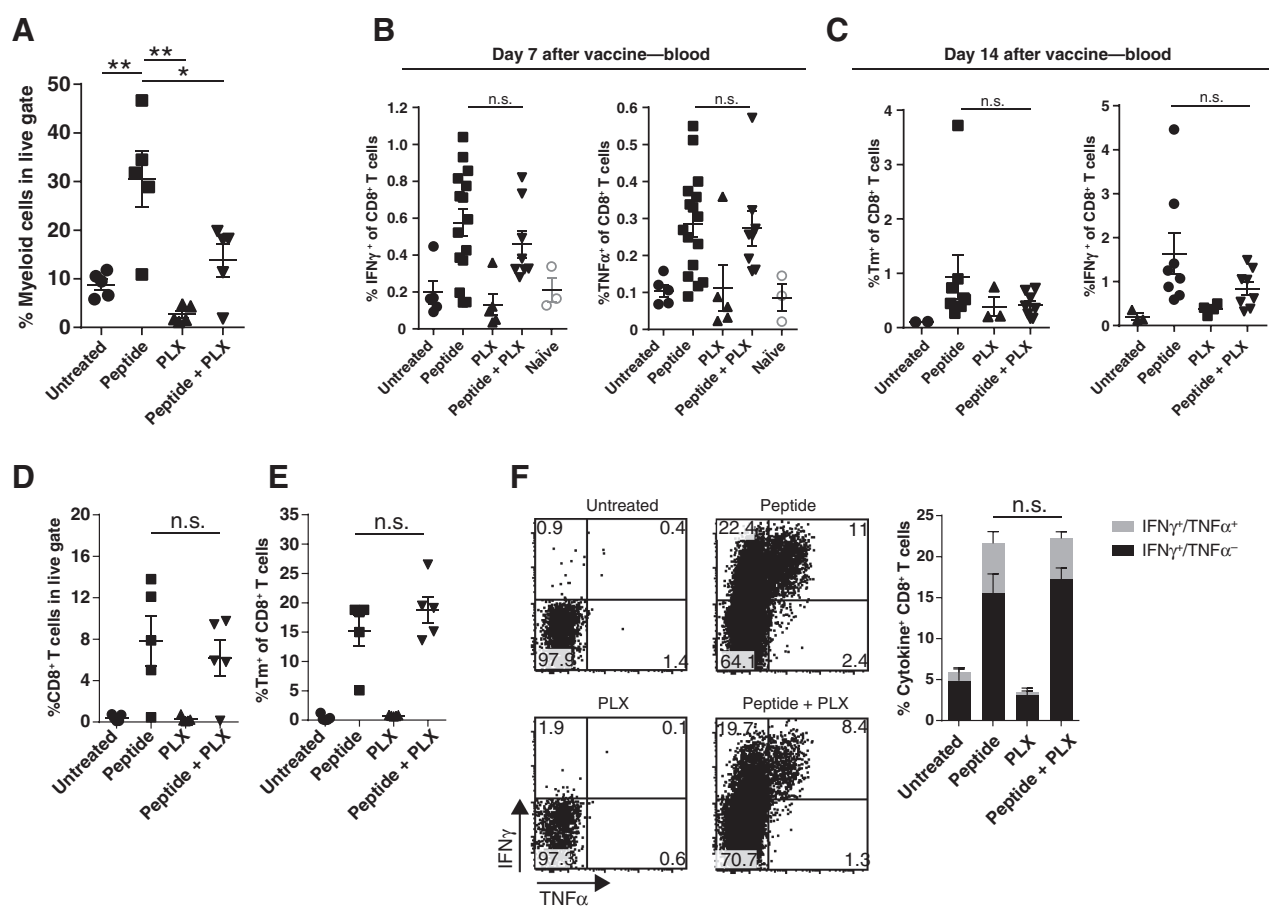


Figure 4. CSF-1R kinase inhibitor PLX33397 reduces myeloid cells but does not affect CD8 T cells. Mice were inoculated with TC-1 tumors and treated with SLP vaccines in the presence or absence of PLX33397. A, 10 days after vaccination, tumors were resected and percentages of intratumoral myeloid cells were determined by flow cytometry. B and C, analysis of antigen-specific CD8 T cells in the blood of tumor-bearing mice on (B) day 7 after vaccination and (C) day 14 after vaccination. D–F, SLP vaccine-induced HPV16 E7-specific CD8 T cells were monitored in tumors 10 days after vaccination. D, frequencies of CD8⁺ T cells in flow cytometry in live gate and (E) frequencies of tetramer⁺ CD8 T cells in total CD8 T-cell pool are shown. F, examples of *ex vivo* analysis of cytokine-secreting intratumoral CD8 T cells by intracellular flow cytometry staining (left) and their quantifications (right). Data shown are representative of two independent experiments with 5 animals per group and expressed as mean with SEM (two-way ANOVA; *, $P < 0.05$; **, $P < 0.01$).

between CCL2 and monocyte attraction has been reported (29). In fact, in 1983, CCL2 was already described as a tumor-derived chemotactic factor (30) and has since been linked to the abundance of TAMs in many tumors (31). Furthermore, Popivanova and colleagues (32) showed that TNF α -induced CCL2 enhanced infiltration of macrophages in colon tumors. CCL5 was strongly induced by the combination of IFN γ and TNF α (Fig. 3; refs. 33, 34) and has been implicated in the attraction and survival of macrophages in adipose tissue (35) and various tumors (36, 37). Together, these data show that cytokines can directly or indirectly skew and attract myeloid cells. The ability of cytokine-producing T cells to skew myeloid subsets is emphasized by the observation that the absence of CD8 T cells in skin wounds also decreases macrophage infiltration (38). At the same time, modified macrophages within the tumor might boost an antitumor type I inflammatory response (39) and previous studies showed that CSF-1R-triggering enhanced the tumoricidal capacity of macrophages (40). Importantly, these studies together reveal the large plasticity of the intratumoral myeloid population and indicate that

conventional and immune-based treatments can polarize the tumor-promoting function of macrophages into tumor-rejection responses.

Targeting of macrophages with CSF-1R inhibitors paradoxically enhanced overall survival in various mouse tumor models, especially when combined with other forms of immunotherapy, chemotherapy, or with radiotherapy (21, 22, 38, 41–43). We hypothesize that the critical factor to explain this discrepancy is the capacity of the treatment to functionally polarize macrophages toward a tumor-rejection activity. Our SLP vaccination clearly induced high levels of IFN γ and TNF α in the local TME and thereby inducing the polarization of the resident tumor-promoting macrophages into M1-type of cells. If the antitumor treatment is not capable of such strong polarization, the treatment efficacy can be enhanced by removing macrophages via CSF-1R inhibition. Moreover, most mouse tumor models evaluate growth delay or control, but not actual regressions, which might depend on other macrophage subsets. Combination therapies with CSF-1R inhibitors, such as adoptive T-cell

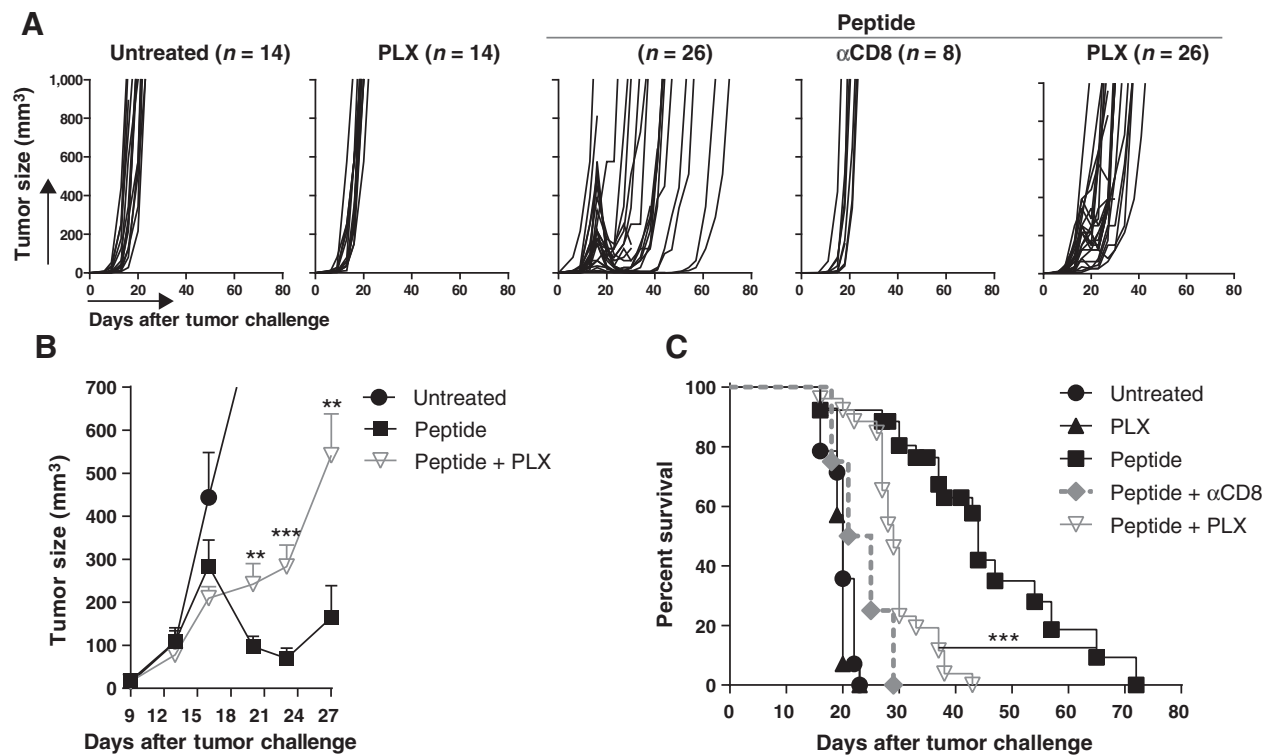


Figure 5. Intratumoral macrophages are required for SLP vaccine-induced tumor regression. A, TC-1 tumor outgrowth of individual mice. B, mean tumor sizes of living animals are depicted per group together with standard error of the mean. Shown are three groups: untreated ($n = 14$), SLP vaccinated (peptide; $n = 26$), and SLP vaccination in the presence of PLX3397 (peptide + PLX; $n = 26$). Statistically significant differences are observed at day 21 and onward (Student t test; *, $P < 0.01$; ***, $P < 0.001$). C, the Kaplan-Meier survival curves for different treatment regimens; shown are pooled data from three experiments (log-rank analysis ***, $P < 0.001$).

therapy, usually include systemic inflammatory factors, such as total body irradiation or strong adjuvants such as the TLR-7 agonist imiquimod (22, 42, 44). These inflammatory factors might be capable of skewing the phenotypes of macrophages, but they might not have such a strong impact on the local TME as infiltrating CD8 T cells. Therefore, a positive versus negative contribution of TAMs to tumor eradication might depend on the size and quality of the natural or vaccine-induced T-cell response and the unique abilities of the adjuvants provided along with the therapies.

We therefore conclude that harnessing macrophage targeting for cancer therapy needs careful reevaluation. Deciphering the exact molecular cues responsible for macrophage polarization during treatment seems critical for effective therapy. Recently, we have demonstrated a positive correlation of a dense infiltration of M1-type macrophages with patient survival in a cohort of cervical cancer patients (45), supporting the notion that macrophage skewing toward antitumor activity, rather than macrophage depletion, might be the optimal strategy. Our study illustrates that immunogenic cancer vaccines can be instrumental to induce such a repolarization of intratumoral macrophages.

Disclosure of Potential Conflicts of Interest

C.J.M. Melief reports receiving a commercial research grant from ISA Pharmaceuticals. S.H. van der Burg is an Advisor Immunomonitoring at Dutch Cancer Institute and is a consultant/advisory board member for ISA Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors thank W. Benckhuijsen and J.W. Drijfhout for providing peptides, K. Franken for providing tetramers, and K.A. Marijt for his help in figure preparation of the heatmap.

Grant Support

This work was supported by a grant from the Dutch Cancer Society (2009-4400, to C.J.M. Melief and S.H. van der Burg).

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Received February 24, 2015; revised April 3, 2015; accepted April 6, 2015; published OnlineFirst April 17, 2015.

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