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Vaccine-Induced Effector-Memory CD8\(^{+}\) T Cell Responses Predict Therapeutic Efficacy against Tumors

Suzanne van Duikeren,* Marieke F. Fransen,* Anke Redeker,* Brigitte Wieles,* Gerard Platenburg,† Willem-Jan Krebber,† Ferry Ossendorp,* Cornelis J. M. Melief,*† and Ramon Arens*

CD8\(^{+}\) T cells have the potential to attack and eradicate cancer cells. The efficacy of therapeutic vaccines against cancer, however, lacks defined immune correlates of tumor eradication after (therapeutic) vaccination based on features of Ag-specific T cell responses. In this study, we examined CD8\(^{+}\) T cell responses elicited by various peptide and TLR agonist-based vaccine formulations in nontumor settings and show that the formation of CD62L\(^{-}\)KLRG1\(^{+}\) effector-memory CD8\(^{+}\) T cells producing the effector cytokines IFN-\(\gamma\) and TNF predicts the degree of therapeutic efficacy of these vaccines against established s.c. tumors. Thus, characteristics of vaccine-induced CD8\(^{+}\) T cell responses instill a predictive determinant for the efficacy of vaccines during tumor therapy. *The Journal of Immunology, 2012, 189: 000–000."

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D8\(^{+}\) T cells play a central role in the control of and protection against intracellular pathogens and malignant cells (1, 2). The differentiation of naive CD8\(^{+}\) T cells into effector and memory cell populations is accompanied by substantial plasticity regarding phenotype and functional capacity (3, 4), emphasizing the multifaceted and important role of this T cell subset. Such heterogeneity among T cells might be beneficial in defining correlates of immune protection after vaccination and could expedite better prediction of effective vaccine formulations.

Although certain immunotherapeutic strategies of cancers have clinical efficacy, successful therapeutic vaccines are not yet available for the treatment of most tumors (5). Better characterization of therapeutic efficacy that reckons with the heterogeneity of T cell responses, including more precise charting of function, phenotype, and differentiation, can help to define immune correlates of tumor eradication induced by (therapeutic) vaccination. To date, such correlates of vaccine-induced therapeutic activity against tumors are lacking. Studies in the field of microbial immunity have shown that profiles of Ag-specific T cell responses can be correlated with disease activity and/or protection (6–9). Therefore, immune correlation studies incorporating the characteristics of vaccine-induced anti-tumor T cell responses can be crucial for the design and development of therapeutic vaccines against cancer.

In this study, we assessed whether CD8\(^{+}\) T cell responses elicited by various vaccine formulations in nontumor settings could be useful in defining immune correlates for tumor eradication. These vaccine formulations include the use of TLR agonists, known to be important initiators of innate and adaptive immune responses. Our results show that vaccine-induced effector-memory T cell responses in a tumor-free setting, defined by a CD62L\(^{-}\)KLRG1\(^{+}\) phenotype and simultaneous secretion of IFN-\(\gamma\) and TNF, predicts best the degree of therapeutic vaccine efficacy against established s.c. tumors. The premise that the characteristics of vaccine-induced T cell responses in tumor-free animals can forecast the therapeutic efficacy during tumor therapy implies that characterization of CD8\(^{+}\) T cell responses is critical for rational design of (therapeutic) vaccines against cancer.

Materials and Methods

Mice

Female C57BL/6 (H-2\(^{b}\)) and congenic B6.SJL (CD45.1, Ly5.1) mice were purchased from Charles River (L’Arbresle, France) and maintained in the central animal facility of Leiden University Medical Center. All mice were housed in specific pathogen-free conditions and used at 8–10 wk of age. All animal experiments were approved by the Animal Experiments Committee of the Leiden University Medical Center and performed according to the guide to animal experimentation set by the Leiden University Medical Center and according to the Dutch Experiments on Animals Act, which serves the implementation of “Guidelines on the protection of experimental animals” by the Council of Europe.

Vaccination

The long human papillomavirus 16 (HPV16) E7\(^{43–77}\) peptide (QGAEP-DRAHYNIVTFCKCDSTLRLCVQSTHVDIR), covering both the CTL epitope (indicated in bold letters) and the T helper epitope (underlined), was used with or without addition of an adjuvant and dissolved in PBS or emulsiﬁed in a 1:1 ratio with Montanide ISA 51 (Seppic). For each mouse, 150 \(\mu\)g of this 35-mer long peptide was s.c. injected in a total volume of 200 \(\mu\)l. The following TLR agonist adjuvants were used: CpG (ODN1826, type B; 20 \(\mu\)g per mouse; purchased from InvivoGen), polyinosinic-polycytidylic acid stabilized by lysine and carboxymethylcellulose (poly-ICLC; Hiltonol; 50 \(\mu\)g per mouse; kindly provided by Oncovir), LpxL1-LPS (20 \(\mu\)g per mouse), LpxL1-LPS and L3-LPS were received via the National Institute of Public Health and the Environment (Bilthoven, The Netherlands). All vaccinations were prepared on the day of injection.

Tumor regression experiment

TC-1 tumor cells are derived from primary lung epithelial cells of C57BL/6 mice and cotransformed with HPV16 E6 and E7 and c-Ha-Ras oncogenes (10). These cells were cultured at 37 \(^\circ\)C with 5% CO\(_2\) in IMDM containing 8\% FCS (Greiner), 2 mM glutamine, and 100 IU/ml penicillin in the
presence of 400 μg/ml Geneticin (G418; Life Technologies), nonessential amino acids (Life Technologies), and 1 mM sodium pyruvate (Life Technologies).

On day 0, mice were challenged by s.c. injection in the flank with $1 \times 10^5$ TC-1 tumor cells in a total volume of 200 μl PBS. On day 10 after tumor challenge, when tumors were palpable (~9 mm²), mice were split into groups with similar tumor size and vaccinated as described earlier in the contralateral flank. As a control, naive mice were injected with tumor cells only. Twice a week, the tumor sizes were measured two-dimensionally with calipers to a maximum of 150 mm² after which the mice were sacrificed for ethical reasons.

**In vivo T cell depletion**

For in vivo CD4+ and CD8+ T cell depletion, we injected mice i.p. with 100 μg of the mAbs GK1.5 and 2.43, respectively, on days 0, 7, 11, and 14 after vaccination. mAbs were prepared and purified as described (11).

**Flow cytometry**

Cell surface staining was performed on freshly prepared PBMCs and splenocytes after RBC lysis. Cells were surface stained for 30 min with allophycocyanin-labeled H-2Db E749–57 tetramer [produced as described (12)] and fluorescently labeled Abs specific for mouse CD8, CD127 (IL-7Ra), CD62L, and killer cell lectin-like receptor G1 (KLRG1) (purchased from BD Biosciences and eBioscience) in staining buffer (PBS containing 1% FCS and 0.05% sodium azide). 7-Aminoactinomycin D was used for dead cell exclusion. Flow cytometric intracellular cytokine analysis of PBMCs and splenocytes was performed after 5-h stimulation with the HPV16 E749–57 peptide (5 μg/ml) in presence of brefeldin A (2 μg/ml). After cell surface staining with fluorescently labeled Abs to mouse CD8, cells were fixed with Cytofix/Cytoperm solution (BD Biosciences) and permeabilized with Perm/Wash buffer. Subsequently, cells were stained for 30 min at 4°C with fluorescently labeled Abs against IFN-γ, TNF-α, and IL-2. Samples were acquired with a BD LSR II flow cytometer, and results were analyzed using FlowJo software (Tree Star).

**In vivo cytotoxicity**

Target splenocytes from naive congenic CD45.1+ mice were counted and split into two equal parts. One part was pulsed for 1 h with the HPV16 E749–57 (RAHYNIVTF) peptide and one part with control peptide (adenovirus type 5 E1A234–243). After washing the cells, E749–57 peptide-loaded cells were fluorescently labeled with 5 μM CFSE (CFSElo), whereas control peptide-loaded cells were labeled with 0.5 μM CFSE (CFSEhi). Target cells were adoptively transferred i.v. in 200 μl PBS in a 1:1 ratio in recipient C57BL/6 mice, which were naive or vaccinated previously with long HPV16 E743–77 peptide in combination with different adjuvants (i.e., CpG, poly-ICLC, and LpxL1-LPS) and dissolved in PBS. One day after the adoptive transfer, spleens were harvested, and single-cell suspensions were analyzed by flow cytometry. Specific killing was calculated according to the following formula: \(\left\{1 - \frac{\text{CFSE}_{\text{hi}}/\text{CFSE}_{\text{lo}}^{\text{vaccinated}}}{\text{CFSE}_{\text{hi}}/\text{CFSE}_{\text{lo}}^{\text{naive}}}\right\} \times 100\).

**Statistical analyses**

We assessed the significance of differences in the magnitude and phenotypical and functional properties of CD8+ T cells by two-tailed Student
Results

Vaccine-mediated tumor regression depends on induction of CD8+ T cell responses

To define immune correlates of vaccine-mediated protection against tumors in vivo, we tested multiple therapeutic vaccine formulations in a preclinical model of HPV16-induced cervical cancer (13). We examined vaccine formulations containing the long peptide E743–77 mixed with the adjuvants CpG (TLR9 ligand), poly-ICLC (TLR3 ligand), or different forms of LPS (TLR4 ligand) [i.e., Neisseria meningitidis wild-type L3-LPS and mutant penta-acylated LpxL1-LPS (14)] for their efficacy to restrain the outgrowth of established transplanted HPV16+ TC-1 tumors expressing the E7 oncogene. These vaccine formulations were dissolved in either PBS or Montanide, a clinically approved mineral oil that causes a gradual release of Ag (15–17). On day 10 after TC-1 tumor inoculation, when tumors were palpable (∼9 mm²), mice were vaccinated twice with the different vaccine formulations with a 2-wk interval.

All nonvaccinated mice developed tumors of >100 mm² within 26 d after tumor inoculation (Fig. 1). Vaccination with peptide in PBS had no inhibitory effect on tumor outgrowth, whereas peptide in Montanide delayed or inhibited outgrowth of TC-1 tumors, indicating that the depot function of Montanide considerably improves tumor eradication. Notably, the addition of either CpG or poly-ICLC in PBS as well as in Montanide induced substantial tumor regression compared with peptide–PBS vaccination, suggesting that a depot effect is less important when TLR3 or TLR9 stimulation is provided. In contrast, LpxL1-LPS– and L3-LPS–containing formulations induced only a marginal inhibitory effect on tumor growth compared with CpG and poly-ICLC (Fig. 1). Addition of CpG without peptide had no inhibitory effect on tumor outgrowth (Ref. 13 and S. van Duikeren, unpublished observations). The requirement for CD8+ and/or CD4+ T cells in mediating tumor growth inhibition was assessed experimentally by depletion of these subsets at the time of peptide–CpG vaccination. The lack of CD8+ T cells but not of CD4+ T cells abolished the vaccine-induced tumor regression (Fig. 1).

Together, these results indicate a differential impact of TLR agonists containing vaccine formulations on tumor progression in a CD8+ T cell-dependent fashion.

FIGURE 2. Vaccine formulations inducing high frequencies of Ag-specific CD8+ T cells correlate with their therapeutic efficacy. Naïve C57BL/6 mice (non-tumor-bearing) were vaccinated twice (on day 0 and day 14) with different vaccine formulations containing HPV16 E743–77 long peptide with or without TLR agonists (CpG, poly-ICLC, LpxL1-LPS, or L3-LPS) dissolved in either PBS or Montanide. (A) Graphs show the percentage of E749–57-specific CD8+ T cells (±SEM) determined longitudinally (until day 50 postvaccination) in the blood by MHC class I tetramers. Triangles indicate injection time points (day 0 and 14). (B) Bar graphs show the mean percentage of E7-specific CD8+ T cells (+SEM) on day 21 and day 50 after the first vaccination (day 7 and day 36 after second vaccination). Each vaccinated group represents 5–10 mice. Experiments were performed twice with similar results. *p < 0.05, ***p < 0.0005 (compared with no TLR adjuvant; Student t test).
FIGURE 3. Vaccine formulations inducing formation of CD62L^-KLRG1^ effector-memory CD8^+ T cells correlate with their therapeutic efficacy. Naive C57BL/6 mice (non-tumor-bearing) were vaccinated twice (on day 0 and day 14) with different vaccine formulations containing HPV16 E743–77 long peptide with or without TLR ligands (CpG, poly-ICLC, LpxL1-LPS, or L3-LPS) dissolved in either PBS or Montanide. (A) Shown are representative fluorescent intensity plots of E749–57-specific CD8^+ T cell responses in blood of vaccinated mice at day 21 after the first vaccination (day 7 after the second vaccination) with the indicated vaccine formulations. Background staining of E749–57 tetramer in control peptide (E1A234–243) vaccinated mice is shown. Numbers indicate the percentage of E749–57-specific CD8^+ T cells within the total CD8^+ T cell population. Within the population of E749–57-specific CD8^+ T cells, the cell surface expression of (B) CD62L versus KLRG1 and (C) CD127 versus KLRG1 is depicted. Numbers in each quadrant represent the percentage of E749–57-specific CD8^+ T cells that are positive or negative for the indicated cell surface markers. (D) Bar graphs indicate the mean percentage of CD62L^-KLRG1^ cells (±SEM) within the E749–57-specific CD8^+ T cell population at day 21 and day 50 after the first vaccination. Each bar represents four to five mice. At day 50, the E749–57-specific CD8^+ T cell response in mice vaccinated with peptide and L3-LPS in PBS was (Figure legend continues)
Vaccine formulations inducing high frequencies of Ag-specific CD8+ T cells correlate with their therapeutic efficacy

On the basis of the importance of CD8+ T cells for eradication of s.c. tumors, we decided to test whether vaccinations in a tumor-free setting could predict the efficacy of these vaccines in therapeutic situations by determining the characteristics of the CD8+ T cell responses such as magnitude and quality. To this end, wild-type (non-tumor-bearing) mice were vaccinated with the aforementioned vaccine formulations and schedules. The magnitude of the peptide-specific CD8+ T cell response was monitored longitudinally in blood with H-2D8 E749–57 tetramers (Fig. 2A). One week after the first vaccination, the mixture of peptide in Montanide but not in PBS elicited detectable E7-specific CD8+ T cell responses (~1%). Addition of the adjuvant CpG increased the E7-specific response when used in a mixture with PBS (~1%), but similar percentages as peptide alone were found with Montanide. The adjuvants poly-ICLC, LpxL1-LPS, or L3-LPS elicited no substantial increase in E7-specific cells, either dissolved in PBS or Montanide (Fig. 2A, 2B). Seven days after the second (boost) vaccination (day 21 after the first vaccination), the E7-specific T cell responses were significantly higher in mice that received peptide vaccines containing CpG, dissolved in either PBS or Montanide, compared with those in mice that received peptide-only vaccines (Fig. 2A, 2B and Fig. 3A). Also, vaccine formulations with poly-ICLC, dissolved in PBS and Montanide, elicited high frequencies of E7-specific CD8+ T cells. Importantly, these CpG-containing and poly-ICLC–containing vaccine formulations had profound efficacy against established TC-1 tumors (Fig. 1). In contrast, presence of the adjuvants LpxL1-LPS or L3-LPS, which were not effective in reducing tumor outgrowth, did not markedly increase the frequency of Ag-specific CD8+ T cells after the booster vaccination (Fig. 2A, 2B and Fig. 3A). Several weeks later (day 50 after the first immunization), the percentage of E7-specific CD8+ T cells elicited by CpG and poly-ICLC dissolved in PBS but not in Montanide was still significantly higher in the vaccinated group. These results indicate that the frequency of vaccine-specific CD8+ T cells after booster vaccination in a tumor-free setting correlates with the therapeutic vaccine efficacy against established tumors.

Vaccine formulations inducing formation of CD62L+KLRG1+ effector-memory CD8+ T cells correlate with their therapeutic efficacy

Distinct subsets of CD8+ T cells expand upon Ag re-encounter (18, 19). Effector T cells dominate the expansion phase, migrate to peripheral organs, and display immediate effector function. Effector-memory T cells preferentially home to peripheral tissues and respond to Ag encounter with immediate effector functions, whereas central-memory T cells home to lymphoid organs and can strongly expand upon Ag re-encounter (18, 19). In this study, we determined by polychromatic flow cytometry (20) the formation of effector and memory T cell populations within the vaccine-induced E7-specific CD8+ T cell population based on the cell-surface expression of lymphocyte homing receptor CD62L, KLRG1, and IL-7 receptor α (CD127) (21, 22). After the booster vaccination, the percentages of effector/effector-memory cells, defined by CD62L+KLRG1+, were significantly higher in the effective adjuvant groups (i.e., containing CpG and poly-ICLC) (Fig. 3B, 3D). Expression of CD127 was, albeit less pronounced than CD62L, downregulated on the cell surface of Ag-specific KLRG1+ CD8+ T cells (Fig. 3C). Thus, like the frequency of vaccine-specific T cells, the cell surface phenotype (i.e., CD62L−KLRG1+) of these cells after vaccination in tumor-free mice is predictive for therapeutic vaccine efficacy against established tumors. Moreover, because a positive correlation (correlation coefficient \( R^2 = 0.7537 \) exists between the magnitude of the E7-specific T cell response and the percentage of CD62L+KLRG1+ cells within this population (Fig. 3E), either the percentage of Ag-specific CD8+ T cells or the CD62L+KLRG1− phenotype of these cells can be used as a predictor for effective therapeutic tumor vaccines. At later time points postvaccination, the CD62L+KLRG1− cell surface expression was also significantly higher on Ag-specific CD8+ T cells elicited by vaccine formulations containing CpG (dissolved in either PBS or Montanide) and poly-ICLC in PBS but not in Montanide (Fig. 3D). Together, these results indicate that at early times after vaccine boosting, both the frequency and phenotype of vaccine-induced CD8+ T cells can predict effective vaccine formulations, but at later time points postbooster the predictive authority of T cell frequency and phenotype induced by vaccine formulations dissolved in Montanide wanes.

Simultaneous and high production of IFN-γ and TNF is a functional characteristic of effective effector-memory CD8+ T cells

To determine the functional killing capacity of the vaccine-induced effector/effector-memory CD8+ T cell pools, we performed in vivo cytotoxicity assays (Fig. 4A). Vaccination with either CpG or poly-ICLC resulted in strong cytolytic activity (i.e., ~70%), whereas the killing of target cells in mice immunized with LpxL1-LPS or peptide alone was weak (<30%) (Fig. 4A). Besides cytolytic activity, the capacity to produce effector cytokines such as IFN-γ is a key mechanism for tumor eradication by CD8+ T cells (23). At day 50 after vaccination, we measured cytokine production by performing polychromatic intracellular cytokine staining (Fig. 4B, 4C). In mice immunized with the long E743–77 peptide in PBS, the majority of the IFN-γ− cells (~50%) were single producers, which are known to have a poor capacity to develop into memory cells (24). Addition of the adjuvants CpG and poly-ICLC to the long E743–77 peptide vaccine resulted in a relative decrease of single producers and a related increase in the frequency of IFN-γ+TNF− double-producing cells (Fig. 4B, 4C). Immunization with the CpG-containing and poly-ICLC–containing vaccines in Montanide resulted in an increase in double producers and a concomitant decrease in triple (IFN-γ+TNF+IL-2+)–producing CD8+ T cells compared with the Montanide-only–containing vaccine (Fig. 4B, 4C). Both the LpxL1-LPS and L3-LPS adjuvants, either dissolved in PBS or Montanide, did not significantly induce increased percentages of double-producers (Fig. 4B, 4C). Thus, the production of simultaneous IFN-γ−TNF+ but not of triple IFN-γ−TNF+IL-2+ or single IFN-γ+ is a functional capacity of effective vaccine-induced CD8+ T cells.

Distinct with the hierarchy of the vaccine efficacy of the formulations dissolved in PBS, the IFN-γ mean fluorescence intensity (MFI), reflecting the production of this cytokine on a per cell basis, within the triple (IFN-γ+TNF+IL-2+) and double (IFN-γ+TNF+) positive populations of the E7-specific CD8+ T cells was the highest for the CpG-containing and poly-ICLC–containing...
vaccines followed by LpxL1-LPS and L3-LPS (Fig. 4D). Of the vaccine formulations dissolved in Montanide, the CpG-containing vaccine elicited a slightly elevated IFN-γ MFI. All the vaccine formulations dissolved in either PBS or Montanide showed a lower IFN-γ MFI of the single IFN-γ-producing CD8+ T cells compared with the IFN-γ MFI of the triple and double producers (Fig. 4D). Together, these results indicate that increased production of IFN-γ on a per cell basis is a functional characteristic of the effector-memory CD8+ T cells that are elicited by the effective vaccine formulations in therapeutic settings.

**Discussion**

In this study, we have delineated immune correlates for tumor eradication based on the frequency and heterogeneity of Ag-specific CD8+ T cell responses elicited by various vaccine formulations in tumor-free conditions. Vaccines containing the TLR ligands CpG (TLR9) and poly-ICLC (TLR3) elicited increased frequencies of effector-memory cells (CD62L−KLRG1+IFN-γ+TNF+) after vaccination in nontumor settings, which correlated with a superior effect of these vaccines on inhibiting tumor outgrowth when used as therapeutic vaccines. Furthermore, our study...
indicates that induction of effector-memory CD8+ T cells is preferred over central-memory cells in case of effective regression of s.c. tumors. This is consistent with the fact that effector-memory T cells can migrate to or are already present in extralymphoid sites. In addition, effector-memory cells have immediate effector function compared with central-memory cells (18, 19). Consistent with our findings are recent reports showing that vaccines eliciting persistent effector-memory T cell responses have an overall superior protective capacity against mucosal infection by chronic pathogenic viruses in comparison with vaccines that induce central-memory T cell responses (25, 26). Certain systemic infections, however, are better controlled by central-memory T cells than by effector-memory T cells (27), presumably due to their better capacity to expand and to their preference for homing in spleen and lymph nodes.

Although the peptide vaccines containing TLR3/9 agonists induced regression of established tumors, immune evasion mechanisms (28, 29) such as class I downmodulation can occur (Ref. 13 and S. van Duikeren, unpublished observations). Strategies that specifically counterattack such immune evasion and tumor cell apoptosis-inducing therapies (e.g., chemotherapy and/or radiotherapy) (30, 31) may therefore be synergistic with peptide-based vaccines for tumor eradication. In addition, tumor-specific T cells may eventually display an exhausted phenotype, and overcoming this (e.g., by PD-L1 blockade) can also improve therapeutic results (32, 33).

In conclusion, our findings show that the efficacy of therapeutic vaccine formulations against cancer can be predicted based on characteristics of the Ag-specific T cell response that is elicited in healthy (non-tumor-bearing) individuals. Further understanding of the mechanisms that influence the expansion and development of heterogeneous CD8+ T cell populations can be used to advance vaccine design. Our findings could therefore be valuable for improving and expediting the design of therapeutic vaccines against cancer.

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Disclosures
R.A. and W.-J.K. declare competing financial interests in the form of a pending patent application on the topic described in this article. The other authors have no financial conflicts of interest.

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