CD27 Agonism Plus PD-1 Blockade Recapitulates CD4+ T-cell Help in Therapeutic Anticancer Vaccination

Tomasz Ahrends1, Nikolina Babala1, Yanling Xiao1, Hideo Yagita2, Hans van Eenennaam3, and Jannie Borst1

Abstract

While showing promise, vaccination strategies to treat cancer require further optimization. Likely barriers to efficacy involve cancer-associated immunosuppression and peripheral tolerance, which limit the generation of effective vaccine-specific cytotoxic T lymphocytes (CTL). Because CD4+ T cells improve CTL responsiveness, next-generation vaccines include helper epitopes. Here, we demonstrate in mice how CD4+ T-cell help optimizes the CTL response to a clinically relevant DNA vaccine engineered to combat human papillomavirus–expressing tumors. Inclusion of tumor-unrelated helper epitopes greatly increased CTL priming, effector, and memory T-cell programming. CD4+ T-cell help optimized the CTL response in all these aspects via CD27/CD70 costimulation. Notably, administration of an agonistic CD27 antibody could largely replace helper epitopes in promoting primary and memory CTL responses, acting directly on CD8+ T cells. CD27 agonism improved efficacy of the vaccine without helper epitopes, more so than combined PD-1 and CTLA-4 blockade. Combining CD27 agonism with CTLA-4 blockade improved vaccine-induced CTL priming and tumor infiltration, but only combination with PD-1 blockade was effective at eradicating tumors, thereby fully recapitulating the effect of CD4+ T-cell help on vaccine efficacy. PD-1 blockade alone did not affect CTL priming or tumor infiltration, so these results implied that it cooperated with CD4+ T-cell help by alleviating immune suppression against CTL in the tumor. Helper epitope inclusion or CD27 agonism did not stimulate regulatory T cells, and vaccine efficacy was also improved by CD27 agonism in the presence of CD4+ T-cell help. Our findings provide a preclinical rationale to apply CD27 agonist antibodies, either alone or combined with PD-1 blockade, to improve the therapeutic efficacy of cancer vaccines and immunotherapy generally. Cancer Res; 76(10); 2921–31. ©2016 AACR.

Introduction

Therapeutic vaccination with protein or DNA, aimed at raising a cytotoxic T lymphocyte (CTL) response to pre-existing cancer, is an attractive form of immunotherapy. It is easy to apply, potentially cheap, and can be designed to avoid autoimmune responses (1). Up to now, however, therapeutic vaccines have not been very successful, not even against immunogenic virus-induced cancers (1, 2). In therapeutic vaccination, dendritic cells (DC) are a key target for antigen delivery (3). DCs are expert at cross-presenting antigens, i.e., loading peptides derived from the extracellular vaccine into MHC Class I molecules for presentation to CD8+ T cells. Moreover, activated DCs are optimal for T-cell priming, because they produce the relevant costimulatory molecules and cytokines. Resting DCs, however, maintain peripheral tolerance to self-antigens, with the aid of natural (n)regulatory T cells (Treg) and their co-inhibitory receptor CTLA-4 (4, 5).

A key goal in therapeutic vaccination is therefore to activate DCs. This can be done with adjuvants that bind to pattern-recognition receptors (PRR; ref. 6). CD4+ T cells can also activate DCs and thus compensate for deficient “danger” signaling (7, 8). Therefore, vaccines can be optimized by inclusion of MHC Class II–binding epitopes (1). Upon priming with an MHC Class I–binding epitope only, CD8+ T cells are nonresponsive or even tolerant (9). However, when (a) helper epitope(s) is included, CD4+ T cells will optimize (“license”) DCs for CTL priming. In this way, the DC translates instructions from the CD4+ T cell to the CD8+ T cell. CD4+ T-cell help not only promotes the primary CTL response, but also instills memory capacity into responder CTLs (7, 8). Helper epitope inclusion was successful in therapeutic vaccination against premalignant human papillomavirus (HPV)–induced disease (10), but it has not yet delivered its promise in cancer patients (1, 11).

Costimulation is important in the delivery of CD4+ T-cell help. When the CD4+ T cell recognizes peptide/MHC Class II complex on the DC, it upregulates CD40 ligand (L). This triggers CD40 on the DC, which “licenses” the DC to induce a CTL response (12–14). It is not known how exactly the DC changes as a result of CD40 triggering and how this subsequently optimizes priming, effector, and memory differentiation of the CD8+ T cell. However, it is clear that the DC acquires costimulatory capacity and thereby promotes the CD8+ T-cell response. Upon PRR- or CD40 stimulation, the DC upregulates CD80 and CD86, the ligands of the T-cell costimulatory...
Ahrends et al.

receptor CD28. The DC also starts to express CD70, the ligand of the costimulatory receptor CD27. DC activation thus allows costimulation via CD28 and CD4^+ T-cell responses by complementary mechanisms (15). CD27 is a member of the TNF receptor family that also includes CD134 (OX40), CD137 (4-1BB), and CD357 (GTR). All these receptors support the T-cell response—in a large part by survival signaling (16). Agonistic mAbs to all these receptors are in clinical trials in cancer patients (17), in the expectation that they may improve results achieved by current CTLA-4 and PD-1 blockade (18).

Thus, changing the vaccine formulation and/or adding immunomodulatory antibodies to overcome deficient DC activation could improve CTL priming. The CTLs raised must furthermore overcome suppression in the tumor microenvironment, as imposed by tumor cells, regulatory Tregs, and other cell types. Blocking PD-1 with therapeutic mAb can alleviate one of these suppressive mechanisms (18, 19). It has not been investigated whether CD4^+ T-cell help also addresses this bottleneck in the CTL response. Here, we examined by which mechanisms CD4^+ T-cell help improves the CTL response, using a protocol of intraepidermal DNA vaccination. This protocol proved superior to intramuscular vaccination in raising a CTL response to human immunodeficiency virus in monkeys and is under clinical development (20, 21). We identified CD27 costimulation of CD8^+ T cells as the key effector pathway of CD4^+ T-cell help for the CTL response. We demonstrate that antibody-based CD27 agonism is a very powerful combination to optimize therapeutic vaccination that acts synergistically with PD-1 blockade. Our study provides the guidelines for rational optimization of cancer immunotherapy with immunomodulatory antibodies alone or in combination with therapeutic vaccines.

Materials and Methods

Mice

Gender matched, 7- to 8-week-old C57BL/6j mice from The Jackson Laboratory were used in accordance with national guidelines and as approved by the institutional Experimental Animal Care Committee (DEC) and maintained in HEPA-filtered isolators in a cGMP pathogen-free facility.

Vaccination

HELP-E7SH and E7SH DNA vaccines were generated as described (22). The amino acid sequences they encode are provided in Supplementary Fig. S1. For DNA “tattoo” vaccination, the hair on a hind leg was removed using depilating cream (Veet; Reckitt Benkiser) on day −1. On days 0, 3, and 6, mice were anesthetized, and 15 μL of a 2 mg/mL DNA solution in 10 mmol/L Tris and 1 mmol/L EDTA, pH 8.0, was applied to the hairless skin with a Permanent Make Up tattoo machine (MT Derma GmbH), using a sterile disposable 9-needle bar with a needle depth of 1 mm and oscillating at a frequency of 100 Hz for 45 seconds (20, 23). For rechallenge, the hair removal step was repeated at day −1, and mice received a single DNA tattoo and an i.p. injection with 5 μg lipopolysaccharide (LPS) from E. coli O55:B5 (Sigma) in L Hank’s Buffered Salt Solution (HBSS) on day 0.

Antibody treatments

Blocking mAbs to CD70 (FR70; ref. 24), CTLA-4 (9D9), and PD-1 (RMPI1-14) or agonistic mAb to CD27 (RM27-3E5; ref. 25) were injected i.p. at 100 μg per mouse in 100 μL HBSS on each day of primary vaccination and in case of FR70 mAb also at day 9. Control mice were injected with equal amounts of rat IgG2b (LIF-2) or IgG2a (2A3) isotype controls (Bio X Cell). Depleting anti-CD4 mAb (GK1.5; Bio X Cell) was injected i.p. at 200 μg per mouse in 100 μL HBSS on day −2 and on each day of primary vaccination.

In vivo cytotoxicity assay

Splenocytes from naïve mice were labeled ex vivo with 0.1 μmol/L carboxyfluorescein succinimidyl ester (CFSE; Life Technologies) and pulsed with 5 μmol/L E749-57 peptide (specific target), or labeled with 1 μmol/L CFSE and left unpulsed (control). Subsequently, 5 × 10^6 cells of each population were injected in the retro-orbital plexus of the same recipient mouse, and 16 hours later, spleens were analyzed by flow cytometry. Percent killing was calculated as follows: 100 − ([% specific targets in vaccinated recipients/[% control targets in vaccinated recipients])/[% specific targets in control recipients/% control targets in control recipients] × 100).

Tumor challenge

At day 0, mice were injected s.c. with 1 × 10^5 TC-1 tumor cells that express HPV16 E6 and E7 proteins (26). Vaccination was performed on the indicated days after inoculation. Tumor growth was measured by caliper in two dimensions. Tumor volume was calculated as: volume = (width^2 x length)/2. Mice were sacrificed when the tumor diameter reached 15 mm or when the tumor volume exceeded 1,000 mm^3. The TC-1 cell line was received in 2015 from Leiden University Medical Center, and further authentication was not performed by the authors.

Tissue preparation and flow cytometry

Peripheral blood cells were obtained by tail bleeding; spleen, inguinal lymph nodes, and tumors were passed through a 70 μm cell strainer (BD Falcon). After erythrocyte lysis, cell suspensions were selected based on propidium iodide- or near-infra red dye exclusion. Fluorochrome-labeled mAbs employed were as follows: anti-CD8α (53-6.7), anti-CD4 (RM4-5), anti-IFNγ (XMG1.2), anti-TNFα (TN3-19.12), all from BD Biosciences or eBioscience, and anti-GZMB (CLB-G81; Enzo Life Sciences). Prior to cytokine detection with the BD Cytofix/Cytoperm Kit (BD Biosciences), cells were incubated for 16 hours with 1 μg/mL PADRE (AKFVAWTLKAAAAAA, E749-57 (RAHYNIVTVF), or no peptide (control) in IMDM with 8% FCS. In calculating the frequency of responder cells, the signal observed in corresponding nonstimulated control samples was subtracted. Intracellular Foxp3 and granzyme B (GZMB) staining was performed after cell fixation and permeabilization using the Foxp3/ transcription factor staining buffer set (eBioscience). Flow cytometry was performed using the LSRFortessa (BD Biosciences), and data were analyzed with FlowJo software (Tree Star Inc.). Live cells were selected based on propidium iodide- or near-infra red dye (Life Technologies) exclusion.

Statistical analysis

Data were analyzed with GraphPad Prism software using unpaired two-tailed Student t test or a log-rank test. Error bars in figures indicate SD. A P value < 0.05 was considered statistically significant; *, P < 0.05; **, P < 0.005; ***, P < 0.001; and ****, P < 0.0001.
Intraepidermal DNA vaccination reveals the effects of CD4\(^+\) T-cell help for the CD8\(^+\) T-cell response

In our vaccination strategy, naked DNA encoding the antigen of interest is "tattooed" into the skin (20). This results in keratinocyte transfection and delivery of antigen to the draining lymph nodes (dLN; ref. 23). The two DNA vaccines used do or do not contain helper epitopes, which offered us a unique window to read out the effects of CD4\(^+\) T-cell help on the CTL response (Fig. 1A; Supplementary Fig. S1; ref. 22). The vaccines encode a "gene-shuffled" version of HPV-type 16 E7 protein, including the immunodominant H-2Db\(^--\)restricted epitope E749-57. In the "Help" version, the vaccine additionally encodes unrelated epitopes binding to all human MHC Class II alleles (Supplementary Fig. S1), of which the p30 (27) and PADRE (28) epitopes also bind to mouse MHC Class II.

To validate the system, we first examined effects of helper epitope inclusion on the primary CD8\(^+\) T-cell response. "Help" clearly increased the magnitude of the primary H-2Db\(^--\)E749-57 tetramer\(^+\) cells among total CD8\(^+\) T cells (B). On day 10, 3 mice per group were sacrificed, and absolute numbers of H-2Db\(^--\)E749-57 tetramer\(^+\) CD8\(^+\) T cells in dLN, non-dLN, and spleen were enumerated (C). The remaining 7 mice per group were rechallenged once with "No Help" vaccine and injected i.p. with LPS on day 50, and the CD8\(^+\) T-cell response was subsequently followed in blood (D). Representative staining of cells from indicated organs with H-2Db\(^--\)E749-57 tetramer and anti-CD8 mAb at day 10 is depicted in D. Numbers in quadrants indicate the percentage of H-2Db\(^--\)E749-57 tetramer\(^+\) cells within total CD8\(^+\) T cells. Results are representative of three experiments. E, mice (n = 5 per group) were challenged with "Help" or "No Help" vaccine and treated with control mAb or depleting aCD4 mAb. Percentages of H-2Db\(^--\)E749-57 tetramer\(^+\) cells were measured among total CD8\(^+\) T cells in blood over time. Results are representative of two experiments.

Figure 1.

CD4\(^+\)T-cell help optimizes the CD8\(^+\) T-cell response upon intraepidermal DNA vaccination. A, vaccine design: both "Help" and "No Help" vaccines encode a "gene-shuffled" version of HPV-16 E7. The "Help" vaccine additionally encodes human tetanus toxoid p30, PADRE, and HIV NEF epitopes (Supplementary Fig. S1). B–D, mice (n = 10 per group) received "Help" or "No Help" vaccine on days 0, 3, and 6. The CD8\(^+\) T-cell response was followed in time by flow cytometric analysis of peripheral blood with H-2Db/E749-57 tetramers and anti-CD8 mAb and is depicted as percentage of H-2Db/E749-57 tetramer\(^+\) cells among total CD8\(^+\) T cells (B). On day 10, 3 mice per group were sacrificed, and absolute numbers of H-2Db/E749-57 tetramer\(^+\) CD8\(^+\) T cells in dLN, non-dLN, and spleen were enumerated (C). The remaining 7 mice per group were rechallenged once with "No Help" vaccine and injected i.p. with LPS on day 50, and the CD8\(^+\) T-cell response was subsequently followed in blood (D). Representative staining of cells from indicated organs with H-2Db/E749-57 tetramer and anti-CD8 mAb at day 10 is depicted in D. Numbers in quadrants indicate the percentage of H-2Db/E749-57 tetramer\(^+\) cells within total CD8\(^+\) T cells. Results are representative of three experiments. E, mice (n = 5 per group) were challenged with "Help" or "No Help" vaccine and treated with control mAb or depleting aCD4 mAb. Percentages of H-2Db/E749-57 tetramer\(^+\) cells were measured among total CD8\(^+\) T cells in blood over time. Results are representative of two experiments.

Results

Intraepidermal DNA vaccination reveals the effects of CD4\(^+\) T-cell help for the CD8\(^+\) T-cell response

In our vaccination strategy, naked DNA encoding the antigen of interest is "tattooed" into the skin (20). This results in keratinocyte transfection and delivery of antigen to the draining lymph nodes (dLN; ref. 23). The two DNA vaccines used do or do not contain helper epitopes, which offered us a unique window to read out the effects of CD4\(^+\) T-cell help on the CTL response (Fig. 1A; Supplementary Fig. S1; ref. 22). The vaccines encode a "gene-shuffled" version of the HPV type 16 E7 protein, including the immunodominant H-2Db\(^--\)E749-57 epitope (Supplementary Fig. S1). In the "Help" version, the vaccine additionally encodes unrelated epitopes binding to all human MHC Class II alleles (Supplementary Fig. S1), of which the p30 (27) and PADRE (28) epitopes also bind to mouse MHC Class II.

To validate the system, we first examined effects of helper epitope inclusion on the primary CD8\(^+\) T-cell response. "Help" clearly increased the magnitude of the primary H-2Db\(^--\)E749-57-specific CD8\(^+\) T-cell response, as measured in blood up to day 50 (Fig. 1B and D). At the peak of the response at day 10, "Help" had also significantly increased the total numbers of E7-specific CD8\(^+\) T cells in dLN, non-dLN, and spleen (Fig. 1C and D). Thus, CD4\(^+\) T-cell help benefitted the priming of E7-specific CD8\(^+\) T cells and their systemic dissemination.

To test whether the "Help" vaccine could program memory function into CD8\(^+\) T cells, mice that had been primed with "Help" or "No Help" vaccine were rechallenged with "No Help" vaccine. In this setting, mice primed with "Help" vaccine had a much higher secondary E7-specific CD8\(^+\) T-cell response than mice primed with "No Help" vaccine, even though neither received help during rechallenge (Fig. 1B). Thus, the model revealed the effect of CD4\(^+\) T-cell help delivered during priming.
Ahrends et al.

in the form of memory programming. Note that we needed to inject LP5 i.p. as "danger signal" in conjunction with the secondary "No Help" vaccine to reveal the effect of CD4⁺ T-cell help on the memory CD8⁺ T-cell response.

Next, we confirmed that increased CTL responsiveness to the "Help" vaccine was due to engagement of CD4⁺ T cells. Depletion of CD4⁺ cells by infusion of anti-CD4 mAb nullified the effects of helper epitope inclusion on the CTL response, while it did not affect the response to the "No Help" vaccine (Fig. 1E). We conclude that the intraepidermal DNA vaccination model robustly reveals the effects of CD4⁺ T-cell help in terms of magnitude of the primary and memory CD8⁺ T-cell response.

CD4⁺ T-cell help increases the magnitude of the CD8⁺ T-cell response via the CD27/CD70 pathway

We next determined the importance of CD27/CD70 costimulation as downstream effector of CD4⁺ T-cell help. To test the requirement of CD27/CD70 costimulation for delivery of CD4⁺ T-cell help, the CD27/CD70 pathway was inhibited. Mice were given 'Help' vaccine combined with either control mAb or an anti-CD70 mAb that blocks interaction with CD27 (24). At day 10 after vaccination, when the primary response in the "Help" control situation was at its peak, anti-CD70 mAb treatment after vaccination, when the primary response in the "Help" control situation was at its peak, anti-CD70 mAb treatment lowered the magnitude of the response to the level in the "No Help" situation (Fig. 2A). At later time points, the effect was less pronounced, but peak response levels remained significantly lower than in the "Help" control situation. CD70 blocking also significantly reduced the E7-specific CD8⁺ T-cell response in dLNs, splenocytes, and blood at day 10 (Fig. 2B; primary flow cytometric data in Supplementary Fig. S2). Interestingly, blocking the closely related TNF ligands 4-1BBL or OX40L did not or only marginally impede the effects of CD4⁺ T-cell help for the CTL response (Supplementary Fig. S3).

Next, we tested whether deliberate CD27 costimulation could overcome the requirement for CD4⁺ T-cell help for the CD8⁺ T-cell response. For this purpose, mice were given "No Help" vaccine combined with either a control mAb or a CD27 agonist mAb that stimulates CD27 function in the absence of CD70 binding (25). CD27 agonism significantly increased the magnitude of the CD8⁺ T-cell response to "No Help" vaccination as monitored in the blood, throughout the entire kinetics of the primary response (Fig. 2C). At the peak of the response on day 10, E7-specific CD8⁺ T-cell numbers in dLN and spleen were also greatly increased as a result of CD27 agonism (Fig. 2D). After CD4⁺ depletion with specific mAb, CD27 agonism elevated the CD8⁺ T-cell response to the "No help" vaccine even more (Fig. 2E). We conclude therefore that deliberate CD27 costimulation largely bypasses the requirement of CD4⁺ T-cell help for CD8⁺ T-cell priming.

To determine the effect of CD27/CD70 costimulation on memory programming, mice were rechallenged at day 50 with "No Help" vaccine. In the mice that had been primed with "Help" vaccine and treated with blocking mAb to CD70, the memory response did not exceed the response to "No Help" vaccine, even at its peak. Thus, "memory programming" as a result of CD4⁺ T-cell help greatly depends on CD27/CD70 costimulation (Fig. 2F). Strikingly, in mice that had been primed with "No Help" vaccine and treated with agonist mAb to CD27, the memory response was comparable in magnitude with that in mice primed with "Help" vaccine (Fig. 2F). The combined data indicate that CD27/CD70 costimulation is the key effector pathway of CD4⁺ T-cell help for the CD8⁺ T-cell response.

The CD27/CD70 pathway dictates the generation of CTL and Th1 cell responses

Next, we assessed the contribution of CD4⁺ T-cell help and CD27/CD70 pathway to the quality of the primary T-cell response. To monitor CTL differentiation, the cytolytic effector molecule GZMB was detected in CD8⁺ T cells in blood, dLN, and spleen at day 10 after vaccination. Comparison of "Help" versus "No Help" vaccination indicated that CD4⁺ T-cell help greatly increased the frequency of GZMB⁺ CD8⁺ T cells raised (Fig. 3A and E). CD70 blocking reduced the CTL response to "Help" vaccination to the level seen after "No Help" vaccination. Moreover, CD27 agonism strongly promoted the CTL response to 'No Help' vaccination (Fig. 3A and E). The frequency of CD8⁺ T cells coexpressing IFNγ and TNFα was also greatly increased as a result of CD4⁺ T-cell help, reduced by CD70 blocking after 'Help' vaccination and increased by CD27 agonism after "No Help" vaccination (Fig. 3B and F).

IFNγ and TNFα expressions were also assessed in CD4⁺ T cells. The "Help" vaccine raised a Th1 response that was dependent on CD27/CD70 costimulation (Fig. 3C). CD27 agonism did not engage a CD4⁺ T-cell response after "No Help" vaccination, as assessed by either single IFNγ⁺ or TNFα⁺ CD4⁺ T cells (results not shown) or double IFNγ⁺ TNFα⁺ CD4⁺ T cells, although we observed a slight increase of IFNγ⁺ TNFα⁺ CD4⁺ T cells in the spleen (Fig. 3C). The inclusion of helper epitopes in the vaccine did not elicit the expansion of CD4⁺ Tregs, as identified by Foxp3 expression, nor did deliberate CD27 costimulation (Fig. 3D). Taken together, the data validate the effects of helper epitope inclusion on the Th1 and CTL response and show that these responses are greatly dependent on the CD27/CD70 pathway.

CD4⁺ T-cell help and the CD27/CD70 pathway dictate antitumor efficacy of therapeutic vaccination

CTL function was tested by in vivo cytotoxicity assays. In the first assay, we injected syngeneic splenocytes as target cells that had been loaded with control or E749-57 peptide and labeled with either a high or low dose of CFSE (29). This assay clearly revealed the effect of CD4⁺ T-cell help for the CTL response: About 90% of the E749-57 peptide-loaded target cells were killed in the group that had received "Help" vaccine, whereas less than 10% of the target cells were killed in mice that had received "No Help" vaccine (Fig. 4A and B). Furthermore, CD70 blockade upon "Help" vaccination reduced CTL activity to the levels seen in the "No Help" control group, whereas CD27 agonism upon "No Help" vaccination raised CTL activity to the levels seen in the "Help" control group (Fig. 4A and B). These data prove that the CD27/CD70 costimulatory pathway is the key effector pathway of CD4⁺ T-cell help for the CTL response.

We next examined the contribution of CD4⁺ T-cell help and the CD27/CD70 pathway to the antitumor effect of the "Help" vaccine. For this purpose, TC-1 tumor cells expressing HPV E7 protein (26) were implanted 5 days prior to vaccination. The tumor reached a palpable size while the response to vaccination was ongoing, which allowed us to evaluate tumor rejection (Fig. 4C). In this setting, all mice that had received the "Help" vaccine experienced complete tumor regression (Fig. 4C) and survived long-term (Fig. 4D), whereas all mice that had received
Figure 2. 

CD4⁺ T-cell help promotes the magnitude of primary and memory CD8⁺ T-cell responses largely via CD27/CD70 costimulation. A–D, mice (n = 7 per group) received “Help” or “No Help” vaccine on days 0, 3, and 6 in combination with isotype control, blocking aCD70 (A and B), or agonistic aCD27 mAb (C and D). On day 10, 3 mice per group were sacrificed for analysis, and the response of the remaining 4 mice per group was followed in blood until day 50. Percentage of H-2Db/E749-57 tetramer⁺ cells among total CD8⁺ T cells in blood over time is depicted in A and C. Absolute numbers of H-2Db/E749-57 tetramer⁺ CD8⁺ T cells in dLN, non-dLN, and spleen at day 10 are depicted in B and D. E, mice (n = 5 per group) were challenged with “No Help” vaccine and treated with control mAb or depleting aCD4 mAb alone or in combination with aCD27 mAb. Percentages of H-2Db/E749-57 tetramer⁺ cells were measured among total CD8⁺ T cells in blood over time. F, mice (n = 7 per group) received “Help” or “No Help” vaccine and were treated with control, aCD70, or aCD27 mAb as outlined above. On day 50, mice were rechallenged with “No Help” vaccine and i.p. injected with LPS. Depicted is the percentage of H-2Db/E749-57 tetramer⁺ cells among total CD8⁺ T cells in blood at the indicated days after rechallenge. All results are representative of at least two experiments.
Ahrends et al.

the "No Help" vaccine had to be sacri-
ficed due to tumor outgrowth (Fig. 4C and D). CD70 blockade almost abolished the therapeutic effect of the "Help" vaccine. Conversely, the "No Help" vaccine was rendered more curative upon combination with agonistic CD27 mAb treatment: improved tumor control resulted in long-term survival of 60% of the animals (Fig. 4C and D).

Thus, helper epitope addition converts the vaccine from being nonfunctional to therapeutically effective, leading to 100% curative effect of tumor-bearing mice in this experimental setting. This curative effect greatly depends on CD27/CD70 costimulation, as demonstrated by CD70 blockade. Engagement of CD27 with agonistic mAb renders the vaccine that lacks helper epitopes therapeutically effective, albeit less so than the vaccine with helper epitopes.

Combined CD27 agonism and PD-1 blockage recapitulates the therapeutic effects of CD4+ T-cell help

Combined CTLA-4 and PD-1 blockage constitutes the current breakthrough in cancer immunotherapy (18). Therefore, we examined whether CTLA-4 and/or PD-1 blockage could bypass the effects of CD4+ T-cell help on antitumor efficacy. Treatment with αCTLA-4 or αPD-1 mAb alone did not improve tumor control by "No Help" vaccination, whereas combined treatment had a modest beneficial effect that was less significant than that of CD27 agonism alone (Fig. 5A and B). These data suggested that CD27 costimulation was unique in its effects on CTL priming.

To examine this, we tested the effects of CTLA-4 or PD-1 blockade alone or in combination with CD27 agonism on CTL priming in response to "No help" vaccination. As shown before, CD4+ T-cell help and CD27 agonism promoted CTL priming (Fig. 6A and B). In contrast, CTLA-4 or PD-1 blockage did not. Interestingly though, combined CTLA-4 blockage and CD27 agonism improved CTL priming, even to a greater magnitude than did CD4+ T-cell help, whereas PD-1 blockage did not add to the effect of CD27 agonism (Fig. 6A and B). Despite the superior CTL response in case of combined CTLA-4 blockade and CD27 agonism, CTLA-4 blockade did not improve tumor control as compared with CD27 agonism alone, whereas combined PD-1 blockade and CD27 agonism was 100% curative (Fig. 6C and D). This was remarkable, because combined CTLA-4 blockade and

Figure 3.

Generation of effector CD4+ T cells and CD8+ T cells depends on CD27/CD70 costimulation. Mice (n = 3 per group) received "Help" or "No Help" vaccine on days 0, 3, and 6 in combination with isotype control, blocking αCD70, or agonistic αCD27 mAb. On day 10, flow cytometric analyses were done in cells from blood, dLN, and spleen. GZMB and Foxp3 expressions were determined directly ex vivo. IFNγ and TNFα expressions in CD8+ or CD4+ T cells were determined after 16-hour in vitro stimulation of total blood, LN, or spleen cells with MHC Class I or MHC Class II restricted peptides or no peptide (control). A, frequencies of GZMB+ cells within total CD8+ T cells. B, frequencies of IFNγ+TNFα+ cells within total CD8+ T cells. C, frequencies of IFNγ+TNFα+ cells within total CD4+ T cells. D, frequencies of Foxp3+ cells within total CD4+ T cells. E and F, representative flow cytometric analyses. Results are representative of two experiments.
CD27 agonism was also superior in regards to numbers of tumor-specific CTLs in the tumor as compared with combined PD-1 blockade and CD27 agonism (Fig. 6E and F). We conclude that CD27 agonism and PD-1 blockade together recapitulate the effects of CD4⁺ T-cell help on the CTL response against the tumor.

To test the effect of CD27 agonism on tumor control upon "Help" vaccination, we created a therapeutic window by vaccinating 12 instead of 5 days after tumor inoculation. CD27 agonism significantly increased the generation of HPV E7-specific and effector phenotype CD8⁺ T cells as compared with "Help" vaccination only and did not induce Treg expansion (Fig. 7A–D). In this setting, the "Help" vaccine was not curative, whereas combined CD27 agonism reduced tumor outgrowth and significantly improved survival (Fig. 7E and F). Thus, CD27 agonism also improved the therapeutic efficacy of the vaccine with helper epitopes.

**Discussion**

The intraepidermal DNA "tattoo" strategy is very potent as compared with intramuscular vaccination (21). The vaccine DNA is expressed in keratinocytes (22). How antigen is subsequently delivered to antigen-presenting cells (APC) is not known, but most likely part of the antigen passively drains to the lymph node and many APCs that receive antigen are not activated for lack of contact with "danger" signal. This follows from the fact that helper epitope inclusion and consequent CD4⁺ T-cell help robustly promotes the CTL response to the vaccine. We here make use of
Ahrends et al.

Figure 5.
Effects of CTLA-4, PD-1, or combined blockade on antitumor immunity. Mice were injected s.c. with 1 × 10^7 TC-1 tumor cells on day 0 and subsequently challenged with "Help" or "No Help" vaccine. The "No Help" vaccinated mice were treated with isotype control antibody or with aPD-1 mAb alone, aCTLA-4 alone, or in combination on days 5, 8, and 11. Tumor sizes (A) and overall survival of the mice (B) were monitored. Cumulative data of two experiments with n = 5 and n = 6 per group are shown.

exogenous helper epitopes, and the CD4+ T cells raised can therefore not recognize the tumor, whereas they can deliver help for CTL priming and memory programming.

Intravitral imaging of T cells and APCs in the lymph node is elucidating how CD4+ T-cell help for the CTL response is orchestrated. T-cell priming involves successive interactions between T cells and different APCs in the lymph node, as guided by chemokines (30). In a mouse model of virus infection, CD4+ and CD8+ T cells were seen to independently contact XCR1+ DCs. However, they next contacted the same XCR1+ DCs. This second priming event promoted memory precursor formation and recall capacity of CD8+ T cells and thus proved to be the platform for delivery of CD4+ T-cell help (31).

We have identified CD27/CD70 costimulation of CD8+ T cells as a key effector pathway of CD4+ T-cell help. CD70 blocking strongly reduced the effects of CD4+ T-cell help, whereas 4-1BBL or OX40L blocking had none or only modest effects. Moreover, CD27 agonism could largely replace CD4+ T-cell help in the generation and memory responsiveness of CTL. Findings in mouse and human have already indicated the importance of CD27 signaling for primary and memory CTL responses (32). In CD8+ T-cell priming, CD27 signaling promotes clonal expansion by antiapoptotic and prometabolic effects (33). It broadens the T cell receptor repertoire of the effector T-cell pool, by maintaining low-affinity clones (34). CD70 can be induced on DCs by PRR stimulation (35, 36), so that both CD4+ and CD8+ T cells in the dLN may profit from CD27/CD70 costimulation. Notably, CD8+ T cells make XCL1 upon CD27 costimulation, which may promote their subsequent interaction with XCR1+ DCs (37). XCR1+ DCs may upregulate CD70 expression when they contact CD4+ T cells, since CD70 expression is under control of CD40 signaling (35, 36). In this way, ‘licensed’ DCs can exert CD27 costimulation of CD8+ T cells (36, 38).

CD27/CD70 costimulation is also important for CD4+ T cells. It promotes Th1 differentiation in mouse and human (32, 39, 40). In our model, CD27 agonism did not induce a Treg expansion. This is in agreement with a study in human where transient CD70 costimulation of Tregs inhibited their suppressive function and converted them into Th1 cells (39). Tregs can respond to therapeutic vaccination, as has been observed upon vaccination with long peptides in human patients (41). However, we did not observe any effect of exogenous helper epitope inclusion on Treg expansion after vaccination. CD4-cell depletion improved the effect of CD27 agonism, suggesting that natural Tregs did restrain CD8+ T-cell priming to the ‘No help’ vaccine. Using tumor-intrinsic helper epitopes in vaccines has the advantage that the effector CD4+ T cells raised can recognize the tumor and directly act upon the tumor or indirectly by promoting CTL function (42). However, exogenous helper epitopes can be useful when tumorspecific helper epitopes are unknown.

Strikingly, the CTL response raised upon combined CD27 agonism and CTLA-4 blockade was greater in magnitude to the one raised after CD4+ T-cell help, also in the tumor, but this did not improve tumor control. In contrast, combined CD27 agonism and PD-1 blockade recapitulated the effects of CD4+ T-cell help on tumor control, even though the combination did not significantly increase CTL priming or tumor infiltration as compared with CD27 agonism alone. PD-1 blockade most likely overruled suppression exerted on the CTLs in the tumor site, so that they could kill their targets. This agrees with clinical data, where the
patient’s response to PD-1 blockade correlates with PD-L1 expression in the tumor (43). If this is indeed the case, we conclude that CD4+ T-cell help also aids in overriding tumor-associated immune suppression. CD27 agonism could also enhance the efficacy of vaccine that contained helper epitopes. This can easily be understood, because not all APCs that present the vaccine

Figure 6.
Combined effects of CD27 agonism and CTLA-4 or PD-1 blockade on the CTL response and antitumor immunity. A and B, mice (n = 6 per group) were challenged with “Help” or “No Help” vaccine on days 0, 3, and 6. The “Help” vaccinated mice were treated with isotype control antibody. The “No Help” vaccinated mice were treated with isotype control, agonistic αCD27, or blocking αCTLA-4 or αPD-1 mAb alone or with agonistic αCD27 mAb in combination with αCTLA-4 or αPD-1 mAb. A, percentage of H-2Dβ/E749-57 tetramer+ cells among total CD8+ T cells in blood over time. B, frequencies of GZMB+ CD8+ T cells in blood at day 10 after vaccination. C and D, in independent experiments, mice (n = 6 to 10 per group) were injected s.c. with 1 × 10⁶ TC-1 tumor cells on day 0 and subsequently challenged with “Help” vaccine and isotype control mAb, with “No Help” vaccine and isotype control mAb, or agonistic αCD27 mAb alone, or in combination with αCTLA-4 or αPD-1 mAb on days 5, 8, and 11. C, tumor sizes. D, overall survival. E and F, mice (n = 4 per group) were injected s.c. with 1 × 10⁶ TC-1 tumor cells on day 0 and subsequently vaccinated and treated with mAb as indicated in C and D. E and F, frequencies of H-2Dβ/E749-57 tetramer+ (E) and GZMB+ (F) cells among total CD8+ T cells in tumors at day 15 after tumor inoculation. Results are representative of two independent experiments.
antigen may have benefited from CD4<sup>+</sup> T-cell help. CD4<sup>+</sup> T-cell help or antibody-based immunomodulation did not alter the low NK-cell numbers in the tumor (results not shown), but we do not exclude that NK-cell activity may have contributed to the observed treatment effects.

Our data highlight that CD27 agonism, CTLA-4 blockade, and PD-1 blockade all optimize the CTL response by distinct, nonredundant mechanisms. Combined CTLA-4 and PD-1 blockade was previously shown to improve the efficacy of cellular vaccines against s.c. implanted melanoma and colon cancer cell lines (44, 45). However, in our setting, this combination was much less potent than combined CD27 agonism and PD-1 blockade.

We have used a setting of overtly immunogenic cancer with viral epitopes. However, the same principles apply in case of cancers that do not have pathogen-derived antigens, but to which central tolerance is incomplete, such as cancers bearing neoantigens or immunogenic cancer-testis antigens. For example, CD27 agonism can stimulate a CTL response to endogenous antigens in mouse melanoma and T-cell lymphoma (46, 47). We believe that exploiting the division of labor between key costimulatory and coinhibitory receptors provides a rational guideline to improve the efficacy of cancer therapy. This includes therapeutic vaccination, but also radiation- or drug-based tumor cell destruction, wherein the tumor acts as endogenous vaccine. A CD27 agonist mAb (48) is currently in clinical trials, and it will be of interest to observe how it performs, especially in combination with PD-1 blockade.

 Disclosure of Potential Conflicts of Interest

H. van Eenennaam and J. Borst are inventors on a patent claiming CD27 agonist antibodies. H. van Eenennaam is shareholder of Aduro Inc. No potential conflicts of interest were disclosed by the other authors.

 Authors' Contributions

Conception and design: T. Ahrends, J. Borst

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Ahrends, N. Bąbała, Y. Xiao

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Ahrends, N. Bąbała, Y. Xiao, J. Borst

Writing, review, and/or revision of the manuscript: T. Ahrends, H. Yagita, H. van Eenennaam, J. Borst

Acknowledgments

The authors thank Drs. K. Oosterhuis and A. Bins for advice on the vaccination model, professors T.N. Schumacher and J.B.A.G. Haanen for provision of the DNA vaccines, and M. Toebes for assistance with MHC tetramer production.

Grant Support

This work was supported by grant NKI 2012-5397 of the Dutch Cancer Society (J. Borst).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 20, 2015; revised March 7, 2016; accepted March 11, 2016; published OnlineFirst March 28, 2016.


Bullock TN, Yagita H. Induction of CD70 on dendritic cells through CD40 or TLR stimulation contributes to the development of CD8+ T cell responses and contributes to survival signaling in primed CD8+ T cells upon CD27 costimulation. J Immunol 2010;185:6670–8.


Yagita H. Induction of CD70 on dendritic cells through CD40 or TLR stimulation contributes to the development of CD8+ T cell responses and contributes to survival signaling in primed CD8+ T cells upon CD27 costimulation. J Immunol 2010;185:6670–8.


CD27 Agonism Plus PD-1 Blockade Recapitulates CD4+ T-cell Help in Therapeutic Anticancer Vaccination

Tomasz Ahrends, Nikolina Babala, Yanling Xiao, et al.


Updated version

Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-15-3130

Supplementary Material

Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2016/03/26/0008-5472.CAN-15-3130.DC1

Cited articles

This article cites 48 articles, 17 of which you can access for free at:
http://cancerres.aacrjournals.org/content/76/10/2921.full#ref-list-1

Citing articles

This article has been cited by 23 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/76/10/2921.full#related-urls

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/76/10/2921.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.