TLR Stimulation during T-cell Activation Lowers PD-1 Expression on CD8\(^+\) T Cells

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Abstract

Expression of T-cell checkpoint receptors can compromise antitumor immunity. Blockade of these receptors, notably PD-1 and LAG-3, which become expressed during T-cell activation with vaccination, can improve antitumor immunity. We evaluated whether T-cell checkpoint expression could be separated from T-cell activation in the context of innate immune stimulation with TLR agonists. We found that ligands for TLR1/2, TLR7, and TLR9 led to a decrease in expression of PD-1 on antigen-activated CD8\(^+\) T cells. These effects were mediated by IL12 released by professional antigen-presenting cells. In two separate tumor models, treatment with antitumor vaccines combined with TLR1/2 or TLR7 ligands induced antigen-specific CD8\(^+\) T cells with lower PD-1 expression and improved antitumor immunity. These findings highlight the role of innate immune activation during effector T-cell development and suggest that at least one mechanism by which specific TLR agonists can be strategically used as vaccine adjuvants is by modulating the expression of PD-1 during CD8\(^+\) T-cell activation.

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Introduction

The expression of T-cell checkpoint molecules, notably programmed death 1 (PD-1), lymphocyte activation gene 3 (LAG-3), and T-cell immunoglobulin and mucin-domain containing 3 (TIM-3), was first described on T cells specific for viruses and associated with cells with a reduced functional phenotype (1, 2). They were thus initially described as markers of T-cell exhaustion (3). It has become increasingly recognized, however, that expression of these molecules occurs normally with T-cell activation, and cells expressing these molecules can have functional activity (4). This activation-induced upregulation is also consistent with the role of T-cell checkpoint molecules as safeguards against autoimmunity. In the case of PD-1, signaling mediated by binding to one of its ligands leads to reduced effector function (5). Thus, the expression of PD-1 sustains peripheral tolerance, as normal tissues express the ligand for PD-1 (PD-L1) to abrogate autoimmune toxicity by CD8\(^+\) T cells. It has been demonstrated in multiple models that PD-L1 expression by tumors is a mechanism of immune evasion and that blocking PD-1 and PD-L1 interaction can restore T-cell function and enhance antitumor responses (6). The demonstration of antitumor activity using PD-1 or PD-L1 blockade alone has led to multiple new FDA approvals over the last 5 years, underscoring the power of this single mechanism of immune regulation.

The notion that T-cell checkpoint receptors are purely markers of T-cell exhaustion has been contested (7). We have found that T cells activated with a strong antigen signal have increased PD-1 expression, and this expression can persist over time (8). CD8\(^+\) T cells stimulated with a lower affinity epitope became activated, but with lower, more transient, PD-1 expression. Furthermore, activated CD8\(^+\) T cells with reduced PD-1 expression mediated greater antitumor activity in vivo. Similarly, blocking PD-1 signaling at the time of activation with vaccination led to greater antitumor activity (8, 9). We have demonstrated that this mechanism of resistance is critical for successful human immunotherapy, as blockade of PD-1/PD-L1 interaction at the time of T-cell activation with vaccination led to greater antitumor response than blockade alone in patients with metastatic, castration-resistant prostate cancer (mCRPC; ref. 10). These findings suggest that if the expression of T-cell checkpoint inhibitors such as PD-1 could be reduced following CD8\(^+\) T-cell activation, these cells would have greater antitumor activity.

Certain bacterial and viral pathogens can elicit robust and rapid immune responses. This response serves as the basis for using specific pathogens as viral delivery vehicles and using bacterial components as vaccine adjuvants. The molecular basis of these rapid "innate" immune responses is partially due to recognition of pathogenic characteristics by Toll-like receptors (TLR; ref. 11). TLR ligands are being increasingly explored as vaccine adjuvants. However, although studies suggest that innate immune stimulation at the time of T-cell activation leads to a superior adaptive effector and cytotoxic T-cell response, the precise mechanisms for this have not been elucidated (12).

Other groups have demonstrated that innate immune activation with vaccination can lead to autoreactive immunity. In addition to molecular mimicry of pathogens encoding self-antigens, TLR activation is thought to be required to develop autoreactive T cells (13). Such findings suggest that TLR stimulation might have effects on T-cell function by bypassing normal
immune tolerance mechanisms, perhaps via changes to the expression of T-cell checkpoint molecules. Such a finding might then be strategically used to improve the efficacy of antitumor vaccines by using specific TLR agonists as molecular adjuvants. Consequently, we sought to determine if TLR stimulation, at the time of T-cell activation, leads to changes in CD8+ T-cell checkpoint expression and whether TLR stimulation might be used to improve the efficacy of antitumor vaccines. We used two model systems, the OT-1 model in which transgenic CD8+ T cells express the same T-cell receptor, and a native model targeting a dominant epitope derived from the synovial sarcoma X breakpoint 2 (SSX2) tumor antigen. In each model, we activated CD8+ T cells with peptide ligands in the presence or absence of TLR agonists. Using in vitro and murine models, we evaluated the effect of TLR stimulation on T-cell activation and antitumor activity. Our findings provide insight into the role of TLR stimulation at the time of T-cell activation in eliciting an effective adaptive antitumor T-cell response. Our data support the use of defined TLR agonists as vaccine adjuvants because of their ability to modulate expression of a specific T-cell checkpoint molecule at the time of CD8+ T-cell activation.

Materials and Methods

Mice

HLA-A2.01/HLA-DR1-expressing (HHDII-DR1) mice were obtained from Charles River Laboratories courtesy of Dr. François Lemmonier (14). OT-1 (Stock No: 003831) and C57BL/6J (B6, Stock No: 00664) were purchased from The Jackson Laboratory. All experiments were conducted under an IACUC-approved protocol conforming to NIH guidelines.

Cell lines

E.G7-OVA (CRL-2113) cells were obtained from ATCC in 2015. E.G7-OVA cells were lentivirally transduced to express PD-L1, as previously described (8). E.G7-OVA cells were not used beyond 1 week post lentiviral transduction. E.G7-OVA cells were not used beyond 1 week post lentiviral transduction. E.G7-OVA (CRL-2113) cells were obtained from ATCC in 2015. E.G7-OVA cells were lentivirally transduced to express PD-L1, as previously described (8). E.G7-OVA cells were not used beyond 1 week post lentiviral transduction.

Peptides

Peptides encoding the H2Kb-restricted epitope from chicken ovalbumin (SIINFEKL, OVA), or variants with lower affinity (SIINFEKEL, SIINFEEKP), and the dominant HLA-A2.01/HLA-DR1-expressing (HHDII-DR1) mice were obtained from Charles River Laboratories courtesy of Dr. François Lemmonier (14). OT-1 (Stock No: 003831) and C57BL/6J (B6, Stock No: 00664) were purchased from The Jackson Laboratory. All experiments were conducted under an IACUC-approved protocol conforming to NIH guidelines.

In vitro T-cell stimulation

Splenocytes were isolated from OT-1 mice after red blood cell osmotic lysis (0.15 mol/L NH4Cl, 10 mmol/L KHC03, 0.1 mmol/L EDTA). Splenocytes were cultured at 2 × 10^6/mL in RPMI 1640 medium supplemented with L-glutamine, 10% fetal calf serum (FCS), 200 U/mL penicillin/streptomycin, 1% sodium-pyruvate, 1% HEPES, 50 μmol/L β-MeOH, and 2 μg/mL of the designated peptide. TLR agonists were purchased from InvivoGen and added 1 hour before peptides at the following concentrations: 300 ng/mL Pam3CSK4, 10 μg/mL Poly(I:C) HMW, 10 μg/mL MPLAs, 3 μg/mL Gardiquimod, 10 μg/mL R848, 5 μmol/L ODN 1826. At the time points indicated, cells were stained, fixed, and frozen. Cells were then washed, and resuspended in PBS + 3% FCS + 1 mmol/L EDTA for flow cytometry. Cells were stained for 30 minutes at 4°C in a 1:4 dilution of brilliant stain buffer (BD 563794) in PBS + 3% FCS + 1 mmol/L EDTA. Intracellular cytokine staining was performed using standard procedures (8). In order to assess costimulation, blocking antibodies for CD80 (0.6 μg/mL), CD86 (0.25 μg/mL), or OX40L (0.6 μg/mL) were added 1 hour prior to antigen.

RNA preparation and sequencing

Cells were stimulated in vitro as above, and at the times indicated CD8+ T cells were isolated via immunomagnetic negative selection (Stemcell Technologies; 19853). RNA was collected as per the manufacturer’s instruction (Direct-zol RNA MiniPrep Plus w/ TRI Reagent, Zymo Research), treated with DNase (TURBO DNA-free Kit, Invitrogen), and stored at −80°C until analysis at the UW-Madison Biotechnology Center. Total RNA was verified via Agilent 2100 Bioanalyzer. Samples were prepared using the Illumina TruSeq Stranded mRNA kits (Illumina Inc.). For each library, mRNA was purified from 1,000 ng total RNA. Each poly A−enriched sample was fragmented, synthesized into double-stranded cDNA, incubated with Klenow DNA Polymerase and DNA fragments, ligated to Illumina adapters, and purified by paramagnetic beads. Adapter ligated DNA was amplified in a Linker Mediated PCR reaction (LM-PCR) for 11 cycles and then purified by paramagnetic beads. Libraries were standardized to 2 μmol/L. Cluster generation was performed using standard Cluster Kit (v3) and the Illumina Cluster Station. Single 100 bp sequencing was performed, using standard SBS chemistry (v4) on an Illumina HiSeq2500 sequencer. Images were analyzed using the standard Illumina Pipeline, version 1.8.2.

RNA-seq data analysis

Illumina sequencing reads were adapter and quality trimmed using the Skewer trimming program. Quality reads were subsequently aligned to the annotated reference genome using the STAR aligner (16). Quantification of expression for each gene was calculated by RSEM (17), the expected read counts from RSEM were filtered for low/empty values and used for differential gene-expression analysis using DESeq2 (18). All remaining genes from the pairwise comparisons were analyzed using Gene Set Enrichment Analysis (GSEA; refs. 19, 20) to identify pathways based on GO terms (<3-all.v6) from the Molecular Signatures Database (MsigDB; ref. 21) as well as custom gene sets involved in CD8+ T-cell activation. GSEA was performed by calculating a ranked vector as log2 fold change. Analysis was performed using R 3.3.1 and Biocductor 2.32.0 (22). Venn diagrams were generated using the Venn Diagram package. Data (accession number GSE110144) are publicly available at (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE110144).

In vitro coculture experiments

APC subsets were enriched from splenocytes using PE-labeled antibodies specific for either CD19 or CD1c (Stemcell Technologies). CD8+ T cells were isolated as above. Primary dendritic cells (DC) were harvested as previously described (23). Each APC subset, and a subset of T cells with no APCs present, were cultured at 2 × 10^6 cells/mL in PBS in the presence of 2 μg/mL peptide, with or without TLR agonists for 1 hour. Cells were rinsed and transferred to fresh medium. naïve T cells were added at a 1:1 ratio and incubated for 3 days, after which CD8+ T cells were analyzed by
flow cytometry. To assess the effects of secreted factors, cells were incubated as described for 4 hours, rinsed, and incubated for 24 hours in fresh media. These conditioned media were then added to a well containing untreated APCs and O1-1 T cells, isolated as above, and incubated for an additional 24 hours. Where indicated, 1.0 ng/mL recombinant IL12 (R&D Systems; 419-ML-010) was added in place of the TLR agonists or 0.06 μg/mL blocking antibody (R&D Systems, AF-419-SP) was added in addition to the agonists. Where indicated, IL12 concentrations in culture supernatant were determined by ELISA according to the manufacturer’s recommendations (R&D Systems, kit M1270).

Adaptive transfer and immunization of mice

OT-1 splenocytes were harvested, and CD8⁺ T cells were isolated as described. OT-1 T cells (2 × 10⁶) were adoptively transferred into 6- to 10-week-old female B6 mice intraperitoneally. The following day, mice were immunized subcutaneously with 100 μg SIINFEKL (OVA) peptide in PBS. Alternatively, HHDII-DR1 mice were immunized directly with 100 μg of the dominant HLA-A2-restricted epitope from SSX2 (FLQGISPKI) in PBS. TLR agonists were coinfected with vaccine: 20 μg/mouse Pam3CSK4, 100 μg/mouse Gardiquimod, 50 μg/mouse ODN 1826, or vehicle. Mice were euthanized at the times indicated. Spleens were collected, processed as described, and T-cell populations were analyzed by flow cytometry. Tetramers specific for FLQGISPKI (NIH Tetramer Core Facility, Emory University, Atlanta, GA) were used as previously described (15). Data collected on different days were normalized using rainbow beads (Spherotech, RFP-30-5A).

Ovalbumin tumor treatment studies

Ovalbumin-expressing E.G7 PD-L1high cells (10⁶) were injected subcutaneously into 6- to 10-week-old female B6 mice. When tumors were palpable and similarly sized (~0.2 cm²), 2 × 10⁶ naïve OT-1 CD8⁺ T cells were adoptively transferred to each mouse as described above. The following day, mice were immunized subcutaneously with 100 μg of an individual peptide with or without TLR agonists as described. In mice receiving PD-1 blocking antibody, 100 μg of antibody (clone G4) was injected intraperitoneally on the day following vaccination. Tumor volume was measured using calipers and calculated in cubic centimeters according to the formula: (π/6) × (long axis) × (short axis)². Tumors obtained at necropsy were digested in collagenase and DNase1 for 1 to 2 hours at 37°C, and passed through a 100-μm screen and analyzed by flow cytometry.

SSX2 tumor treatment studies

Six- to 8-week-old HHDII-DR1 mice were inoculated with 10⁵ A2/Sarc-SSX2 cells administered subcutaneously and then immunized intradermally with DNA vaccines weekly beginning 1 day after tumor implantation (9). TLR agonists or vehicle were coadministered with the vaccine intradermally, and tumor volumes were measured as described above.

Statistical analyses

Comparison of group means was performed using GraphPad Prism software, v5.01. Analysis of variance (ANOVA; one- or two-sided depending on the nature of the data) statistical modeling was used to analyze data sets, followed by the Bonferroni multiple-comparison post hoc procedure to compare individual group means. For all comparisons, P values ≤ 0.05 (or 0.01 where indicated) were considered statistically significant.

Results

TLR stimulation at the time of CD8⁺ T-cell activation in vitro lowers expression of PD-1

We have previously found that immunization with peptides with high MHC-I affinity, or DNA immunization encoding epitopes with high MHC-I affinity, led to increased expression of PD-1 on cognate CD8⁺ T cells (8, 9). Others have reported that immunization with a TLR9 agonist led to decreased expression of PD-1 on antigen-specific CD8⁺ T cells (24, 25). These observations suggest that innate signals at the time of T-cell activation might affect the expression of checkpoint molecules on activated CD8⁺ T cells. To evaluate this directly, we used the well-characterized ovalbumin model to question whether TLR stimulation could similarly affect expression of different T-cell checkpoints. OT-1 splenocytes were stimulated in vitro with SIINFEKL peptide alone or in the presence of different TLR ligands: TLR 1/2 (Pam3CSK4), TLR 3 (Poly I:C), TLR 4 (MPLAs), TLR 7 (Gardiquimod), TLR 7/8 (R848), ODN 1826, or vehicle (Supplementary Fig. S1; ref. 8). Effects on T-cell checkpoint expression appeared to be limited to PD-1, as we did not detect decreases in expression of LAG-3, TIM-3, CTLA-4, VISTA, CD244, TIGIT, or CD160 following activation of OT-1 cells with OVA peptide (SIINFEKL) were activated, as measured by 4-1BB expression, and activation was not affected over 4 days by the presence of different TLR ligands (Fig. 1). Similarly, LAG-3 expression increased by day 3, but was not affected by the different TLR ligands. However, PD-1 expression was reduced in the presence of TLR1/2, TLR7, TLR7/8, and TLR9 ligands. This reduction in PD-1 expression in the presence of these TLR ligands was also observed following activation with lower affinity epitopes (Supplementary Fig. S1; ref. 8). Effects on T-cell checkpoint expression appeared to be limited to PD-1, as we did not detect decreases in expression of LAG-3, TIM-3, CTLA-4, VISTA, CD244, TIGIT, or CD160 following activation of OT-1 cells with OVA peptide (SIINFEKL) were activated, as measured by 4-1BB expression, and activation was not affected over 4 days by the presence of different TLR ligands (Fig. 1).

To determine whether CD8⁺ T cells activated in the presence of PD-1 ligands were functional, or whether the decrease in PD-1 was due to decreased activation, and to determine whether the effects of TLR1/2 and TLR7 activation were redundant, OT-1 splenocytes were activated in the presence of SIINFEKL peptide alone or with TLR1/2 (Pam) or TLR7 (Gardiquimod) ligands. CD8⁺ OT-1 T cells were then purified and evaluated for gene-expression changes by mRNA sequencing (RNA-seq). OT-1 CD8⁺ T cells activated in the presence of either TLR1/2 (Pam3CSK4) or TLR7 (Gardiquimod) ligands showed similar but distinct changes in enriched GO-term expression when compared with cells activated by peptide antigen alone (Fig. 3A). Both TLR ligands increased expression of genes associated with Th1-biased effector function (IFNγ, TNFα, granzymes A–C, perforin, and T-Bet), reduced EOMES expression, and reduced expression of genes associated with T-cell regulation (including PD-1 and IL10) when compared with cells stimulated with peptide alone (Fig. 3B). Hence, the reduced PD-1 expression we observed corresponded to increased expression of molecules associated with Th1-biased effector CD8⁺ T cells.

TLR1/2 or TLR7 ligands induce expression of genes associated with effector T-cell function

To determine whether CD8⁺ T cells activated in the presence of PD-1 ligands were functional, or whether the decrease in PD-1 was due to decreased activation, and to determine whether the effects of TLR1/2 and TLR7 activation were redundant, OT-1 splenocytes were activated in the presence of SIINFEKL peptide alone or with TLR1/2 (Pam) or TLR7 (Gardiquimod) ligands. CD8⁺ OT-1 T cells were then purified and evaluated for gene-expression changes by mRNA sequencing (RNA-seq). OT-1 CD8⁺ T cells activated in the presence of either TLR1/2 (Pam3CSK4) or TLR7 (Gardiquimod) ligands showed similar but distinct changes in enriched GO-term expression when compared with cells activated by peptide antigen alone (Fig. 3A). Both TLR ligands increased expression of genes associated with Th1-biased effector function (IFNγ, TNFα, granzymes A–C, perforin, and T-Bet), reduced EOMES expression, and reduced expression of genes associated with T-cell regulation (including PD-1 and IL10) when compared with cells stimulated with peptide alone (Fig. 3B). Hence, the reduced PD-1 expression we observed corresponded to increased expression of molecules associated with Th1-biased effector CD8⁺ T cells.
Changes in PD-1 expression following TLR stimulation driven by professional APC

TLRs are expressed by both T cells and professional APCs. To determine if the effects on T-cell checkpoint expression were mediated by direct effects on T cells or APCs, purified OT-1 CD8^+ T cells were activated with SIINFEKL peptide alone or in the presence of TLR1/2 or TLR7 agonist, or with purified DCs or B cells pretreated with TLR agonists and peptide. After 24 hours, cells were evaluated for the expression of 4-1BB, PD-1, and LAG-3. Cells were activated in the presence or absence of professional APCs, as measured by 4-1BB expression (Fig. 4A). The expression of LAG-3 was not affected by the APC type or the presence of TLR ligand. PD-1 expression required presentation by professional APCs and was reduced in the presence of either TLR ligand. Blockade of CD80, CD86, or OX40L did not affect PD-1 expression, suggesting that APC signals other than these costimulatory molecules were required (Supplementary Fig. S3). To determine if these APC signals were due to secreted factors, conditioned medium from DCs incubated with or without TLR ligands was used in place of the TLR agonist during T-cell stimulation. OT-1 CD8^+ T cells activated by DCs in the presence of TLR-conditioned media showed a reduction in PD-1 similar to that observed with TLR agonist treatment directly, suggesting the decrease of PD-1

Figure 2.
The effect of TLR1/2, TLR7, and TLR9 agonists is greatest on PD-1 following T-cell activation. OT-1 splenocytes were stimulated with either the SIINFEKL (OVA) peptide or a nonspecific peptide control (NS), alone or in combination with the designated TLR agonists, for 3 days. The expression of PD-1 and intracellular cytokines, as a percentage of total CD8^+ T cells, were assessed by flow cytometry (A). Representative flow cytometry plots showing IFNγ and TNFα expression with each stimulation condition are shown in B. *P < 0.01, with each comparison made to stimulation with OVA peptide alone. Beads = stimulation of OT-1 cells with beads coated with anti-CD3 and anti-CD28. Results are from one experiment, with samples assessed in triplicate, and are representative of two similar experiments.
expression was due to release of a secreted factor by TLR-stimulated DCs (Fig. 4B).

Changes in PD-1 expression following TLR stimulation can be mediated by IL12
CpG treatment of mice leads to CD8+ T cells with lower expression of PD-1, an effect dependent on IL12 signaling (26). Given this, and the Th1 effector bias of T cells identified in our gene-expression studies, we tested whether treatment of APCs with TLR ligands elicited production of IL12. Treatment of DCs with TLR1/2 and TLR7 agonists led to the greatest secretion of IL12 (Fig. 5A). No significant secretion of IL12 was detected following treatment with TLR3 or TLR4 agonists. Moreover, OT-1 cells activated with peptide and DCs in the presence of IL12 had lower PD-1 expression, and the reduction of PD-1 expression with TLR stimulation was decreased in the presence of IL12 blockade (Fig. 5B). Finally, when beads coated with anti-CD3 and anti-CD28 were used in place of APCs, IL12 alone reduced PD-1 expression (Fig. 5C). Together, these findings demonstrate that TLR stimulation leads to secretion of IL12 from DCs, and IL12 acting directly on T cells can lead to reduced PD-1 expression during T-cell activation.

TLR stimulation at the time of T-cell activation reduced expression of PD-1 in vivo
To determine whether TLR stimulation in vivo at the time of T-cell activation with vaccination elicited a similar reduction in PD-1, OT-1 cells were adoptively transferred to naïve C57Bl/6 mice as above, and immunized once with OVA peptide (or nonspecific peptide) 1 day later in the presence or absence of a TLR 1/2, TLR7,
or TLR9 ligand. Splenocytes were collected up to 14 days after immunization and evaluated for expression of 4-1BB, PD-1, and LAG-3. 4-1BB and LAG-3 expression increased after immunization, and their expression was not affected by concurrent delivery of one of the TLR agonists (Fig. 6A). PD-1 expression, however, was significantly reduced within days of vaccination when vaccine was codelivered with a TLR ligand. Similar results were found following immunization of HLA-A2 transgenic mice with a separate high-affinity HLA-A2–restricted peptide derived from the SSX2 tumor antigen (8) in the presence or absence of TLR stimulation (Fig. 6B).

Immunization in the presence of TLR1/2 or TLR7 ligands improved antitumor immunity

To determine whether the reduced expression of PD-1 on T cells activated in the presence of TLR stimulation could mediate greater antitumor response, ovalbumin- and PD-L1–expressing E.G7 cells were implanted in C57BL/6 mice and permitted to grow until palpable (14 days). OT-1 T cells were then adoptively transferred and the following day mice were immunized with SIINFEKL (OVA) peptide alone or with TLR1/2 or TLR7 agonist. Other groups of mice received immunization with 100 μg anti–PD-1 delivered the day following. In this model, PD-1 blockade alone had limited antitumor efficacy in the absence of T-cell activation, as we have previously reported (8). Immunization with OVA peptide in the presence of TLR1/2 or TLR7 agonist produced a greater antitumor effect than with peptide alone (Fig. 7A). The combination of vaccine with TLR agonists was at least as effective as combination with PD-1 blockade. This antitumor effect was not observed with TLR agonist treatment alone (Supplementary Fig. S4). As shown in Fig. 7B, immunization with TLR1/2 (Pam3CSK4) or TLR 7 (Gardiquimod) agonists elicited a greater number of CD8 Tumor infiltration of lymphocytes (TIL). These CD8 TILs had similar expression of 4-1BB, but reduced
expression of PD-1 (Fig. 7C). Similar findings were observed in a separate tumor model using a DNA vaccine. We have previously reported that a DNA vaccine encoding the SSX2 antigen expressing epitopes with high affinity for HLA-A2 (SSX2 opt) elicited antigen-specific CD8+ T cells expressing higher PD-1 and an inferior antitumor response (9). HLA-A2–expressing transgenic mice were implanted with SSX2-expressing tumor cells. After 2 days, mice were immunized weekly with control vector (pTVG4) or DNA encoding high-affinity HLA-A2 epitopes (pTVG-SSX2 opt) in the presence or absence of Pam3CSK4 (TLR1/2) or Gardiquimod (TLR7) agonist. Although TLR1/2 or TLR7 agonists alone (with vector DNA) had modest effects on tumor growth, in combination with antigen-specific DNA vaccine, they significantly increased antitumor efficacy (Fig. 7D).

Discussion

In this article, we investigated whether expression of PD-1, and other T-cell immune-checkpoint molecules, at the time of T-cell activation can be reduced in the context of innate immune stimulation using TLR ligands. We found that specific TLR ligands, notably ligands for TLR1/2, TLR7, and TLR9, led to lower expression of PD-1 on activated CD8+ T cells, with less effect on other checkpoint molecules. This was mediated by professional antigen-presenting cells, but not through the direct interaction with CD80, CD86, or OX40L on APCs. Rather, IL12 release from APCs following TLR stimulation was found to occur, and IL12 alone could reduce expression of PD-1 on CD8+ T cells during activation. In vivo use of TLR ligands at the time of T-cell activation with vaccines led to superior antitumor immunity in two different murine tumor systems. TLR agonists have been previously explored as vaccine adjuvants. Our findings demonstrate that at least one mechanism by which they can act as adjuvants is by their effects on PD-1 expression on vaccine-elicited CD8+ T cells. Our results also show that PD-1 expression can be separated from T-cell activation, a finding that has implications for T-cell therapies for cancer. Finally, gene-expression results suggested that TLR stimulation may modulate development of effector and memory

Figure 5.

Changes in PD-1 expression following TLR stimulation can be mediated by IL12: A, Purified DCs were stimulated in the presence of TLR1/2 agonist (Pam3CSK4), TLR3 agonist (HMV PolyC), TLR4 agonist (MPLA), TLR7 agonist (Gardiquimod), TLR 7/8 agonist (RB48, resiquimod), TLR9 agonist (CDN 1826), or media only (no TLR) for 24 hours. Culture supernatant was then evaluated for the presence of IL12 p70 by ELISA. *, significant (P < 0.05) IL12 release compared with media alone. B, Purified DCs were stimulated in the presence of TLR1/2 agonist, TLR7 agonist, or media alone. After 24 hours, media were collected and used for culture of purified OT-1 CD8+ T cells with naive DCs in the presence of SIINFEKL peptide (OVA) and, where indicated, 1.0 ng/mL recombinant IL12 or 0.06 μg/mL anti-IL12 blocking antibody. After 24 hours, CD8+ T cells were assessed for the expression of the indicated surface receptors (4-1BB or PD-1) by flow cytometry. Shown is mean and standard error of the MFI for each marker, with samples assessed in triplicate, and are representative of two independent experiments. *, P < 0.01. C, Purified OT-1 CD8+ T cells were stimulated with anti-CD3- and anti-CD28-coated latex beads and in the presence or absence of recombinant IL12, or left untreated (NS). After 24 hours, cells were assessed for the expression of Th1 cytokines or PD-1 by flow cytometry. Results shown are from one experiment and are representative of two similar experiments.
function in CD8⁺ T cells, leading to more effective antitumor responses.

Many investigators have evaluated TLR agonists as vaccine adjuvants. Indeed, CpG-rich DNA and double-stranded RNA were found to promote Th1-biased immune responses and were suggested to be part of the mechanism of plasmid DNA immunization (27). This effect was mediated by activation of TLR9. TLR9 agonists continue to be explored as antitumor therapies alone or in combination with other therapies in clinical trials (28). TLR9-deficient mice have impaired priming of CD8⁺ T cells (29). Similarly, TLR9 activation using CpG-rich oligonucleotides had an adjuvant effect promoting Th1-biased immunity, augmented Th1 memory responses, and induced protective immunity when injected into tumors (30–32). TLR9 agonists can stimulate antigen-presenting cell function and promote the lytic activity of NK cells (33). The specific effects of TLR9 agonists on CD8⁺ T cells have not been entirely elucidated, although TLR9 activation could lead to a decrease in PD-1 expression on activated CD8⁺ T cells (27). Our findings support and expand upon these findings with other TLR agonists and further demonstrate that changes in T-cell checkpoint molecule expression are specific for PD-1 compared with other immunomodulatory molecules expressed with T-cell activation.

The effect of TLR1/2 activation on T-cell checkpoint expression has not been specifically explored, although TLR2 activation has been evaluated as a means of circumventing T-cell exhaustion (34): with naïve T cells stimulated with anti-CD3 and anti-CD28, stimulation in the presence of the TLR1/2 agonist Pam2Cys led to persistent expression of Th1 cytokines and decreased expression of PD-1 and LAG-3. These results are consistent with our finding that TLR1/2 activation at the time of T-cell activation led to a decrease in PD-1 expression; however, we did not observe changes in LAG-3 expression.

TLR7 agonists have been explored as antitumor treatments alone, and as vaccine adjuvants. In particular, imiquimod is a topical TLR7 agonist approved for the treatment of basal cell carcinoma (35). Many studies have used imiquimod and other TLR7 agonists as vaccine, and specifically antitumor vaccine, adjuvants (36, 37). Although TLR7 agonists have been demonstrated to increase the activity of tumor-specific CD8⁺ T cells, this mechanism of action has not been explored. However, consistent with our findings, TLR7 knockout mice infected with lymphocytic choriomeningitis virus (LCMV) developed LCMV-specific CD8⁺ T cells that expressed higher PD-1 than similarly treated wild-type mice (38).

Our results provide evidence that PD-1 expression can be separated from CD8⁺ T-cell activation. In the context of antitumor immunity, this can have important consequences, because we have demonstrated that even a short-term increase in PD-1 expression following T-cell activation by vaccination can impair antitumor immunity (8). We demonstrate here that using specific TLR stimulation to decrease PD-1 expression following CD8⁺ T-cell activation with vaccines can lead to antitumor responses at least as effective as those following use of vaccines combined with PD-1 blockade directly. Our results further suggest that PD-1 expression on CD8⁺ T cells was affected at the time of activation with TLR stimulation, and this was not altered in tumor-bearing mice; however, this needs to be confirmed in other tumor systems.
Our studies focused on antigen-specific CD8⁺ T cells, and did not address PD-1 expression on CD4⁺ T cells. Insights into the impact of TLR or IL12 stimulation on activated antigen-specific CD4⁺ T cells will benefit from further research.

Our gene-expression analysis suggests that TLR1/2 and TLR7 stimulation activates similar T-cell transcriptional profiles; hence, their effects on PD-1 expression may be redundant. Our studies demonstrate that this reduction of PD-1 expression can be mediated by IL12; however, other soluble factors released by APCs may also play a role. Although certain TLR agonists (notably TLR 7, but not TLR3 or TLR4) can lead to IL12 production by DC, our findings here link IL2 production by DCs to expression of PD-1 on activated T cells (39–41). Our findings are consistent with observations in which TLR9 activation led to decreased PD-1 on CD8⁺ T cells that was abrogated in IL12 receptor knockout mice (26). Likewise, treatment of mice with IL12 led to increased CD8⁺

Figure 7.
Immunization in the presence of TLR1/2 or TLR7 ligands, codelivered as vaccine adjuvants, elicits greater antitumor immunity in vivo: A, Ovalbumin-expressing E.G7 cells were implanted in C57BL/6 mice and permitted to grow until tumors were palpable (~14 days). OT-1 T cells were then adoptively transferred and mice were immunized subcutaneously the following day with SIINFEKL (OVA) peptide alone, or in combination with TLR1/2 or TLR7 agonist, or with 100 μg anti–PD-1 delivered the day following immunization. Shown are the tumor growth curves (median ± standard error, n = 5 animals per group). Results are from one experiment and are representative of two independent experiments. B, Tumors obtained at necropsy were evaluated for the percentage of infiltrating CD8⁺ T cells among CD45⁺ T cells. C, Tumor-infiltrating CD8⁺ T cells were evaluated for 4-1BB and PD-1 expression by flow cytometry. D, HLA-A2-expressing transgenic mice (HHD-II-DR1) were implanted with SSX-2-expressing tumor cells. After 2 days mice were immunized intradermally weekly with control vector (pTVG4) or DNA encoding SSX2 protein encoding epitopes with high affinity for HLA-A2 (pTVG-SSX2 opt) and delivered alone or codelivered with TLR1/2 or TLR7 agonist. Shown are the growth curves for each group (n = 6 animals per group). *, P < 0.05. Results are representative of two independent experiments with 12 total mice per group.
T-cell effector function characterized by increased expression of IFNγ, T-bet, but lower expression of IL10 and EOMES (42), consistent with our gene-expression studies.

Our findings demonstrate that specific TLR stimulation can affect the transcriptional profile of CD8+ effector T cells, potentially favoring the establishment of effector memory cells with high expression of T-bet and lower expression of EOMES. The effect of treatment on the establishment of T-cell memory will be evaluated in future studies. Nevertheless, our findings suggest that TLR agonists might be used, alone or with other cytokines, to increase the antitumor efficacy of CD8+ effector memory cells with vaccination. Moreover, given the findings that TLR stimulation can lead to reduced expression of molecules associated with T-cell regulation, such as PD-1 and IL10, future studies should explore whether combinations of TLR ligands, or TLR stimulation with checkpoint blockade, can further improve CD8+ T-cell effector function and antitumor efficacy.

Disclosure of Potential Conflicts of Interest

D.G. McNeel has ownership interest (including stock, patents, etc.) in and is a consultant/advisory board member for Madison Vaccines, Inc. No potential conflicts of interest were disclosed by the other authors.

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