Amplifying TLR-MyD88 Signals within Tumor-Specific T Cells Enhances Antitumor Activity to Suboptimal Levels of Weakly Immunogenic Tumor Antigens

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Abstract

The efficacy of T cell–based immunotherapy to treat cancer patients remains a challenge partly because of the weak activity toward subdominant tumor antigens (TAg) and to tumors expressing suboptimal TAg levels. Recent reports indicate that Toll-like receptor (TLR) stimulation on T cells can lower the activation threshold. In this study, we examined the antitumor activity and survival of TLR2-MyD88–stimulated CD8 T cells derived from melanoma patients and T-cell receptor transgenic pmel mice. TLR2-stimulated pmel CD8 T cells, but not TLR2−/− pmel or MyD88−/− pmel T cells, responded to significantly lower TAg levels and resulted in increased production of effector molecules and cytotoxicity. Wild-type or MyD88−/− mice treated with TLR2 ligand and pmel T cells, but not TLR2−/− pmel or MyD88−/− pmel T cells, showed tumor regression of an established melanoma tumor. Overexpressing TLR2 in TAg-specific T cells eradicated tumors; four times fewer cells were needed to generate antitumor responses. The enhanced antitumor activity of TLR2-MyD88–stimulated T cells was associated with increased effector function but perhaps more importantly with improved survival of T cells. Activating TLR-MyD88 signals in patient-derived T cells also reduced the activation threshold to several weakly immunogenic TAgS, resulting in increased cytokine production, expansion, and cytotoxicity. These data highlight a previously unappreciated role for activating TLR-MyD88 signals in tumor-reactive T lymphocytes. Cancer Res; 70(19); 7442–54. ©2010 AACR.

Introduction

Recent advances highlight the potential for using T cell–based immunotherapies to treat patients with metastatic cancers. However, several challenges in achieving effective antitumor responses stem from the fact that tumor-reactive T cells can display reduced avidity for tumor antigens (TAg), are generally present at low frequencies, and can exhibit diminished cytolytic function (1–6). In addition, some tumors can downregulate MHC class I TAg expression and consequently evade detection by T cells. Therefore, strategies aimed at amplifying T-cell responses to weakly expressed or subdominant TAgS are a major goal in designing effective cancer immunotherapies.

Emerging studies from several groups, including ours, indicate that activating TLR-MyD88 signals within CD4 or CD8 T cells can lower the activation threshold. Toll-like receptors (TLR) recognize pathogen-associated molecular patterns derived from all known microorganisms. Each TLR can recognize and form homodimers or heterodimers that presumably aid in the detection of a broader array of microbial products (7). In CD4 T-helper cells, TLR1/2, TLR5, TLR7/8, and TLR9 engagement has been shown to enhance interleukin-2 (IL-2) production (8–10). TLR2 or TLR9 ligation on CD4 and CD8 T cells also enhances survival by modulating the expression levels of the antiapoptotic protein including A1, bcl2, and bcl-xL (11–14). TLR1/2 stimulation on CD8 T cells has also been shown to enhance IFN-γ production and increase cytotoxicity in vitro (11, 12). We recently showed that TLR1/2 engagement on OT-1 T-cell receptor (TCR) transgenic CD8 T lymphocytes enhanced the antitumor activity against an established tumor expressing the ovalbumin protein (11). Admittedly, one of the major limitations to those studies was that the ovalbumin model does not represent an authentic TAg, as it is an immunodominant xenoantigen and therefore does not deal with the parameters of tolerance or low avidity.

We report that activating TLR2-MyD88 signals within a bona fide population of human and murine tumor-specific...
CD8 T cells enhances responses against suboptimal concentrations of weakly immunogenic TAggs. We used the synthetic ligand [triplepalmitoyl-S-(bis[palmitoxy]propyl)-Cys-Ser-(Lys) 3-Lys] as a TLR1/2 agonist because this TLR agonist has been shown to enhance CD4 and CD8 T-cell response in vitro and in vivo (10−12, 15, 16). TLR2 stimulation on patient CD8 T cells or T cells from TCR transgenic “pmel” mice, which recognize the weakly immunogenic melanoma TAg gp100(25-33), lowered the activation threshold consequently, increasing T-cell expansion, cytokine production, and cytolytic activity in vitro and in vivo (17). Adoptive cell transfer (ACT) of pmel T cells in combination with TLR1/2 ligand, but not TLR1/2 ligand and TLR2−/− pmel or MyD88−/− pmel cells, into wild-type (WT) or MyD88−/− reduced tumor growth kinetics. Furthermore, overexpressing TLR2 on tumor-reactive T cells cured mice bearing an established melanoma tumor, and four times fewer pmel T cells were required to generate anti-tumor responses. The enhanced antitumor effects seemed to be due in part to enhanced T-cell survival. These results reveal that the activating TLR2-MyD88 signals within tumor-specific T cells lowers the activation threshold to weakly immunogenic TAggs and increases their efficiency by enhancing the duration and magnitude of T-cell responses.

Materials and Methods

Mice

Studies were approved by Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee. C57BL6 mice were obtained from Charles River Laboratories, MyD88−/− mice were a kind gift from Dr. Douglas Golenbock (Boston University, Boston, MA), and B6.129-TLR2tm1kir/J (TLR2−/−) mice and pmel (B6.Cg-Thy1/Cy Tg (TcraTcrb)8Rest/J) mice were purchased from the Jackson Laboratory. MyD88−/− and TLR2−/− pmel mice were generated by crossing with pmel for over nine generations.

T-cell sorting and functional studies

CD8 T cells were purified by negative selection (Stemcell Technology) followed by positive selection (Miltenyi Biotec) and activated with MyD88−/− splenocytes pulsed with mouse gp100(25-33) peptide (EGRSNQDWL; GenScript Corp.) with or without TLR1/2 agonist Pam3Cysk4 (10 μg/mL; Invitrogen) or plate-bound anti-CD3 antibodies. T-cell proliferation was determined by measuring [3H]thymidine (0.5 μCi/well) uptake. Cytokine production after 96 hours was determined by staining with phycoerythrin-labeled Annexin V (BD Pharmingen) and analyzed by flow cytometry. For in vitro T-cell survival/proliferation studies, T cells were labeled with 10 μmol/L CFSE dye and pulsed with the gp10025-33 peptide (EGSRNQDWL; GenScript Corp.) with or without anti-CD40 antibody (50 μg). The percentage of targets recovered 24 hours after cell transfer (ACT) was evaluated by fluorescence-activated cell sorting (FACS) analysis.

B16 melanoma tumor challenge

MyD88−/− or WT mice were injected (s.c.) with 5 × 104 B16 tumor cells in the rear leg flank and were allowed to grow to 50 mm², by irradiation (400 cGy) and i.v. injection with 10⁷ T cells; 4 days earlier, T cells were activated in vitro with gp100-pulsed splenocytes, which served as antigen-presenting cells (APC). B16 tumor cell lines were purchased and used within 6 months of use from the ATCC. One day later, mice were injected i.p. with mgp100 (5 μg) anti-CD40 antibody (50 μg) and TLR1/2 ligand (5 μg) or peritumorally injected with TLR1/2 ligand (5 μg) or control PBS weekly. The anti-CD40 antibody was included in vaccine formulations, as the combination of anti-CD40 antibody and TLR agonists synergistically enhances T-cell responses (18). Tumor sizes (mm²) were analyzed using a mixed model approach for repeated measurements; mean comparisons at each time interval were performed using the SAS TUKEY option to adjust the P values for multiple comparisons; mouse survival data were analyzed with the exact log-rank test. MyD88−/− or WT mice were injected (s.c.) with 5 × 10⁴ B16 tumor cells in the rear leg flank and were allowed to grow to 50 mm² followed by irradiation (400 cGy) and i.v. injection with 10⁷ T cells, activated with gp100-pulsed splenocytes, which served as APCs, in vitro 4 days earlier. One day later, mice were injected i.p. with mgp100 (5 μg) anti-CD40 antibody (50 μg) and TLR1/2 ligand (5 μg) and injected peritumorally with TLR1/2 ligand.
(5 μg) or control PBS weekly. Tumor sizes (mm\(^3\)) were analyzed using a mixed model approach for repeated measurements; mean comparisons at each time were performed using the TUKY option of SAS to adjust the P values for multiple comparisons, and mouse survival data were analyzed with the exact log-rank test.

**Retroviral vectors and transduction**

The gene encoding murine TLR2 was excised from the pDUO-mTLR1/TLR2 vector (InvivoGen) and ligated into the multiple cloning site, upstream of the internal ribosomal entry site of the Moloney murine leukemia virus–based pBMN-green fluorescent protein (GFP) vector (Orbigen), whereas GFP was constructed downstream.

Retroviruses were produced by calcium phosphate–mediated transient transfection of Phoenix Eco packaging cells (Orbigen). Phoenix Eco packaging cells, purchased within the last 2 years, were expanded, and frozen stock cell lines were generated on receipt. Cells were characterized by our group based on their ability to produce retroviral particles. For transduction, pmel T cells were activated for 48 hours before infection using human gp100 (KVPRNQDWL)–pulsed WT splenocytes and transduced by adding virus, concentrated via ultracentrifugation, in the presence of 8 μg/mL polybrene, centrifuged for 4 hours at 2,000 rpm at room temperature followed by incubation at 37°C, 7% CO\(_2\) for 48 hours in the presence of IL-2 (100 units/mL) and IL-7 (50 ng/mL).

**Isolation, expansion, and activation of patient T cells**

Peripheral blood mononuclear cells (PBMCs) were collected from consented melanoma patients (HLA-A2\(^+\)). T cells were expanded using CD3/CD28-coated beads (Invitrogen) in the presence of IL-2 (250 units/mL), IL-15 (50 ng/mL), and irradiated PBMCs in RPMI 1640 supplemented with 2 mmol/L glutamine, 25 mmol/L HEPES buffer, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% heat-inactivated human antibody sera (Sigma–Aldrich). Mart126-35 (ELAGIGILTV)–specific, MPS160 glycoprotein 100–specific, and tyrosinase188-196 (AFLPWHRLF)–entry site of the Moloney murine leukemia virus–based pBMN-green fluorescent protein (GFP) vector (Orbigen), whereas GFP was constructed downstream.

To validate that effects of TLR2 ligand occurred via a TLR2- and MyD88-dependent manner in CD8 T cells, we generated TLR2\(^{-/}\) pmel and MyD88\(^{-/}\) pmel mice and examined the production levels of effector molecules and proliferation. TLR2-stimulated pmel T cells, but neither TLR2\(^{-/}\) nor MyD88\(^{-/}\) pmel cells, showed substantial expansion and increased production of IFN-γ and granzyme B (Fig. 1D; Supplementary Data S1). The increased levels of effector molecules were due in part to the increased percentage of cells producing cytokines.

**TLR2-ligated CD8 T cells show enhanced cytotoxicity against suboptimal tumor-antigen concentrations**

We examined the cytolytic activity of TLR2-stimulated pmel T cells in vitro and in vivo. TLR2-stimulated T cells showed significantly higher cytotoxicity against B16 melanoma tumor cells at various effector-to-target ratios (Fig. 2A). In contrast, TLR2 ligand did not increase TLR2\(^{-/}\) pmel or MyD88\(^{-/}\) pmel T cell cytotoxicity. Moreover, TLR2 stimulation increased pmel cytotoxicity against EL4 tumor cells expressing suboptimal concentrations of TAg (Fig. 2B). However, at levels below 1 ng/mL, TLR2-stimulated T cells did not lyse target cells, indicating that TLR2 stimulation alone did not enhance nonspecific killing (Fig. 2B).

For in vivo studies, previously activated (but resting) pmel T cells were injected (i.v.) into MyD88\(^{-/}\) mice. One day later, mice received an equal number of CFSE\(^{high}\) target cells (MyD88\(^{-/}\) splenocytes pulsed with gp100 peptide and CFSE\(^{low}\) cells pulsed with an irrelevant peptide and injected with PBS or TLR2 ligand (Fig. 2C). The frequency of each target population was determined by FACS analysis 24 hours after transfer. The histogram shown in Fig. 2C shows that fewer mgp100-pulsed targets were recovered from mice injected with pmel T cells and TLR2 ligand than mice injected with pmel T cells alone.

**Results**

**Stimulating TLR2-MyD88 signals in pmel CD8 T cells lower the activation threshold to a weakly immunogenic tumor antigen**

We sought to determine if TLR2 engagement on a bona fide population of TAg-specific CD8 T cells enhanced responses to the weakly immunogenic mouse TAg gp100\(_{25-33}\). Purified pmel T cells were activated with MyD88\(^{-/}\)– splenocytes pulsed with varying concentrations of gp100\(_{25-33}\). The use of MyD88\(^{-/}\)–APCs ensures that the costimulatory effects of TLR1/2 ligand occur through TLR stimulation on CD8 T cells, but not APCs (15, 20).

Significantly higher levels of IFN-γ, granzyme B, IL-2, and granulocyte macrophage colony-stimulating factor (GM-CSF) were detected in supernatants from TLR2-ligated CD8 T cells (Fig. 1A; P < 0.005). Furthermore, TLR2-ligated pmel T cells responded to 30 to 70 times lower levels of antigen (Fig. 1A; P < 0.005). TLR2 engagement also increased T-cell proliferation in response to suboptimal concentrations of antigen (Fig. 1B, left). To confirm that effects of TLR2 ligand occurred in the absence of APCs, purified pmel T cells were stimulated with varying concentrations of plate-bound anti-CD3 antibodies in the absence or presence of TLR2 ligand. TLR2-activated T cells showed enhanced expansion (Fig. 1B, right) and cytokine production (data not shown).

To validate that effects of TLR2 ligand occurred via a TLR2- and MyD88-dependent manner in CD8 T cells, we generated TLR2\(^{-/}\) pmel and MyD88\(^{-/}\) pmel mice and examined the production levels of effector molecules and proliferation. TLR2-stimulated pmel T cells, but neither TLR2\(^{-/}\) nor MyD88\(^{-/}\) pmel cells, showed substantial expansion and increased production of IFN-γ and granzyme B (Fig. 1D; Supplementary Data S1). The increased levels of effector molecules were due in part to the increased percentage of cells producing cytokines.

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However, similar number of target cells was recovered from mice injected with PBS or TLR2 ligand alone (Fig. 2C, right). The average percentage lysis from five mice per group is shown in Fig. 2D. In contrast, TLR2 ligand did not increase TLR2−/− pmel or MyD88−/− pmel cytotoxicity. It is worth noting that TLR2 stimulation did not increase the killing of target cells pulsed with 10-fold lower levels of antigen (data not shown).

Figure 1. Activating TLR2-MyD88 signals in tumor-reactive CD8 T cells lowers the activation threshold to a weakly immunogenic tumor antigen. Purified pmel, TLR2−/− pmel, and MyD88−/− pmel CD8 T cells were activated with MyD88−/− splenocytes pulsed with varying concentrations of the mgp100 peptide or plate-bound anti-CD3 antibody with or without TLR2 agonist. Four days later, cytokine levels determined were by ELISA, whereas proliferation was determined by [3H]thymidine uptake. D, the intracellular level of IFN-γ and granzyme B were determined by flow cytometry 4 d following activation of mgp100-pulsed APCs. Top right, the percentage of cytokine-positive cells. All data are representative of three or more independent experiments each yielding identical trends.

Overexpressing TLR2 in T Cells Enhances Antitumor Activity

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Treatment with pmel T cells and TLR2 ligand enhances antitumor responses compared with treatment with TLR2−/− pmel or MyD88−/− pmel T cells

We examined the antitumor activity of TLR2-stimulated pmel T cells in tumor-bearing mice. As depicted in Fig. 3A, WT B6 harboring an established B16 tumor (∼50 mm²) were irradiated (21–24) and injected (i.v.) with pmel, TLR2−/− pmel, or MyD88−/− pmel T cells followed by injection (i.p.) with mgp10025-33 and anti-CD40 antibody with or without TLR2 ligand. Mice received weekly injections (peritumoral) of PBS or TLR2 ligand.

Tumor growth and mouse survival were comparable in mice receiving pmel, TLR2−/− pmel, or MyD88−/− pmel T cells in the absence of TLR2 ligand (Fig. 3B). These observations are in agreement with reports indicating that transfer of TAg-specific T cells alone is insufficient to mediate significant antitumor responses (25). The lack of antitumor responses is likely due to the initial size and aggressive nature of this established tumor, especially in the absence of TLR and CD40 signals, which are required to generate potent T-cell responses (18, 26).

Treatment with pmel CD8 T cells with TLR2 ligand delayed tumor growth (Fig. 3C, circles) compared with vaccinated mice not receiving T cells (Fig. 3C, asterisk). Although we observed tumor regression lasting up to 20 days after...
ACT, tumor growth resumed after this time point. The median survival time in mice receiving pmel T cells and vaccine/TLR2 ligand increased to 34 days compared with 13 days in mice receiving T cells without TLR2 ligand (Fig. 3E). It is worth noting that the absence of antiCD40 antibody significantly reduced the antitumor activity of pmel (data not shown) despite injection with TLR2 ligand. These observations emphasize a critical need for APC activation with TLR and CD40 signals, as suggested by others (18). We also examined whether a second injection of pmel
T cells delayed tumor growth and found that, although tumor growth was delayed and mouse survival increased compared with mice receiving a single injection of T cells, mice ultimately succumbed to tumor within 60 days (Supplementary Data S2).

Mice receiving TLR2+/− pmel CD8 T cells and TLR2 ligand also showed delayed tumor growth (Fig. 3C, triangles) compared with mice receiving TLR2 ligand alone (Fig. 3C, asterisks). Still, this treatment regimen was not as effective as pmel T cells and TLR2 ligand (Fig. 3C, circles; P < 0.001). The median survival time in mice receiving TLR2+/− pmel T cells plus TLR2 ligand was 27 days.

Mice receiving MyD88+/− pmel CD8 T cells and TLR2 ligand (squares) showed moderate but statistically insignificant antitumor responses than mice treated with TLR2 ligand alone (Fig. 3C). Mice treated with TLR2 ligand or PBS alone showed similar tumor growth kinetics, indicating that stimulating TLR2 on WT APCs was insufficient to suppress tumor development (Fig. 3B and C).

We next examined the contribution of activating TLR2-MyD88 signals in CD8 T cells in MyD88−/− mice. Administration of pmel T cells plus TLR2 ligand delayed tumor growth compared with mice treated with TLR2 ligand or pmel T cells (Fig. 3D; P ≤ 0.04). Treatment with TLR2 ligand plus pmel T cells moderately enhanced mouse survival (26 days) over mice receiving pmel T cells (21 days; P < 0.001) or TLR2 ligand (20 days; P < 0.0001).

Collectively, these data show that activating TLR2-MyD88 signals in T cells enhances antitumor activity and that eliminating MyD88 signals in T cells severely reduces antitumor responses. These results also emphasize the importance for activating TLR2-MyD88 signals in APCs for generating antitumor T-cell responses.

Antitumor activity of TLR-MyD88–activated T cells is associated with enhanced T-cell survival

We examined if the improved antitumor activity in TLR ligand/T-cell–treated mice was associated with increased pmel T-cell numbers in tumor-bearing mice. As shown in Fig. 4A, more pmel T cells (CD90.1+) were detected in the draining lymph nodes and tumors following treatment with TLR2 ligand. The increase in T-cell numbers correlated with reduced tumor size at this time point (day 12) compared with mice not receiving TLR2 ligand (Fig. 3C). However, the tumor size began to increase 19 days after T-cell transfer (Fig. 3). In accordance with increased tumor size, we found that the number of pmel T cells in the draining lymph nodes and tumor were reduced to an average of 937 ± 26 pmel T cells per 105 tumor cells and 12 ± 3 per 105 LN cells 30 days after T-cell transfer. The number of TLR2+/− pmel T cells was also higher in TLR2 ligand–treated mice than TLR2−/− pmel T cells in mice not receiving TLR2 ligand (Fig. 4A, left; P < 0.01). However, TLR2+/− pmel T-cell numbers were less than pmel T cells, suggesting that TLR2 engagement on T cells contributed to increased numbers (Fig. 4A, left; P < 0.05). In sharp contrast, MyD88−/−/pmel T-cell numbers remained similar in TLR2 ligand–treated and untreated mice (Fig. 4A, left; P < 0.05). Furthermore, MyD88−/− pmel T-cell numbers were significantly lower than pmel and TLR2−/− pmel T-cell numbers.

We tested whether the elevated T-cell numbers, in response to the TLR2 stimulation, was due to enhanced cell division and/or survival. CFSE-labeled pmel, TLR2+/− pmel, and MyD88−/− pmel T cells were injected (i.v.) into WT mice followed by injection with mgp10010−15 peptide and TLR2 ligand. To quantify the number of transferred T cells, we coinjected CD45.1+ spleen cells and set the instrument gates to count a fixed number of CD45.1 cells (Fig. 4B). We detected 2.5-fold more pmel cells than TLR2+/− pmel and 7-fold more than MyD88−/− pmel T cells (Fig. 4B, bar graph). Although we detected more pmel T cells, the number of pmel T cells that underwent multiple divisions were only moderately increased compared with TLR2+/− pmel or MyD88−/− pmel T cells, suggesting that increased pmel T-cell numbers was primarily due to the enhanced survival.

We next examined cell division and apoptosis in vitro. CFSE-labeled pmel T cells were activated with antigen-pulsed MyD88−/− splenocytes with or without TLR2 ligand. TLR2 ligated pmel T cells showed significantly reduced apoptosis (12%) compared with non–TLR2-stimulated T cells (39%; Fig. 4C). Identical trends were observed when staining cells with propidium iodide and Annexin V (data not shown). In contrast, the addition of TLR2 ligand to TLR2+/− pmel or MyD88−/− pmel T cells did not reduce cell death. Surprisingly, the absence of MyD88 in pmel T cells rendered cells more susceptible to death than WT or TLR2−/− T cells. Additionally, whereas pmel and TLR2−/− pmel T cells underwent apoptosis after two cell divisions, MyD88−/−/pmel T cells began to die before dividing (Fig. 4C, left). TLR2 engagement on pmel T cells moderately increased cell division (Fig. 4C). Noteworthy, the enhanced survival of TLR2-ligated T cells correlated with increased levels of bcl-2 and bcl-xl at the protein and RNA level and a reduction in bim transcript levels (Supplementary Data S3).

To determine the requirement for intrinsic MyD88 signals within T cells, we cotransferred MyD88−/− pmel (CD45.2−/CD90.2+) and pmel T cells (CD45.2+CD90.1+) into CD45.1+ mice and compared T-cell numbers at different time points, as depicted in Fig. 4D. pmel T cells outnumbered MyD88−/− pmel T cells as early as 14 days after ACT and became more pronounced over time (Fig. 4D). These results indicate that MyD88−/− T cells could not be rescued despite conditions sufficient to promote the expansion/survival of pmel T cells within the same environment.

Altogether, these data indicate that activating MyD88 via TLR2 on T cells in vivo augments antitumor responses in part by rescuing T cells from death and that MyD88 in T cells is critical for their long-term survival.

Increased CTL effector function is achieved through overexpressing TLR2 on CD8 T cells

We sought to determine if overexpressing TLR2 on T cells increased antitumor responses. pmel T cells infected with retroviruses, engineered to express TLR2-GFP (pmel-RV-TLR2)
or GFP (pmel-RV-GFP), showed higher TLR2 levels (Fig. 5A). TLR2 stimulation on pmel-RV-TLR2 T cells increased TCR sensitivity as shown by significantly higher IFN-γ, granzyme B, and IL-2 levels (Fig. 5A, right; *P < 0.0001). In the absence of TLR2 agonist, pmel-RV-TLR2 and pmel-RV-GFP T cells produced similar cytokine levels.

We examined the antitumor responses of pmel-RV-TLR2 and pmel-RV-GFP T cells. Treatment with 10⁶ RV-TLR2-pmel cells and TLR2 ligand successfully reduced tumor size in 7 of 10 mice bearing an established melanoma tumor (Fig. 5B, solid lines). Mice treated with 10⁵ pmel-RV-TLR2-GFP cells and TLR2 ligand delayed tumor growth; however, all mice succumbed to tumor by 45 days (Fig. 5B, hashed lines). Treatment with 0.5 × 10⁶ pmel-RV-TLR2 cells and TLR2 ligand reduced tumor growth and prolonged mouse survival (survival, 59 days) compared with mice treated with pmel-RV-GFP plus TLR2 ligand (median survival, 32 days; *P < 0.0001). Mice receiving 0.25 × 10⁶ pmel-RV-TLR2 and TLR2 ligand also showed tumor growth delay and prolonged survival (median survival, 38 days) than the control group (survival, 25 days).

Interestingly, mice treated with pmel-RV-TLR2 in the absence of TLR2 ligand also showed a statistically significant reduction in tumor growth kinetics, although overall survival was not improved compared with mice treated with pmel-RV-GFP (Fig. 5C). Collectively, these data indicate that overexpressing TLR2 on tumor-specific T cells could improve current approaches to treat cancer patients by augmenting sensitivity to subdominant TAggs and by requiring fewer T cells.

Human melanoma–specific T cells respond to suboptimal concentrations of weakly immunogenic tumor antigens following TLR2 engagement

We examined the costimulatory effects of TLR2 engagement on TAg-specific CD8 T cells derived from melanoma.
Figure 5. Overexpressing TLR2 on tumor-reactive T cells enhances the therapeutic efficacy of adoptively T-cell transfer. A, pmel CD8 T cells transduced with RV-expressing TLR2 and GFP (pmel-RV-TLR2) or GFP only (pmel-RV-con) were sorted by FACS. Cytokine production was determined by ELISA. T cells were activated with MyD88$^{-/-}$ APCs pulsed with varying concentrations of antigen in the absence or presence of TLR2 ligand. B and C, BL6 mice bearing an established B16 tumor were treated with varying numbers of pmel-RV-TLR2 or pmel-RV-con with (B) or without (C) the TLR2 ligand. All mice were vaccinated with mgp100, anti-CD40 antibody, and TLR2 ligand, as described in Fig. 3. Data are compiled from three independent experiments, with each experiment yielding similar results. *, $P < 0.001$, MIXED procedure of SAS.
patients. Sorted T cells were cocultured with the A375 melanoma cell line pulsed with various concentrations of the following weakly immunogenic TAGs: Mart126-35, tyrosine188-196, and melanoma gp100 antigen (MSP160; Fig. 6A). In the presence of TLR2 ligand CD8 T cells produced higher levels of IFN-γ, GM-CSF, and IL-2 and responded to significantly lower levels of TAG (Fig. 6B). It is worth noting that, although the increases in cytokine production were more compelling at

Figure 6. TLR2 stimulation on melanoma-specific patient T cells lowers the activation threshold to subdominant tumor antigens. Tumor antigen-specific CD8 T cells from melanoma patients were expanded and sorted as described in Materials and Methods. Cytokine production, proliferation, and cytolytic activity of Mart1-, gp100-, and tyrosinase-specific CD8 T cells were tested against peptide-pulsed HLA-A2+ A-375 melanoma target cells in the presence or absence of TLR2 ligand. The A375 melanoma cell line was pulsed with varying concentrations of peptides starting at 1 μg/mL and diluted 10-fold or pulsed with 0.001 μg/mL peptide for in vitro cytotoxicity assays. B, cytokine production was evaluated using a Milliplex cytokine array 48 h after stimulation, whereas T-cell proliferation was measured at the end of 3 days via [3H]thymidine incorporation. C, cytotoxicity was determined in a 6-h 51Cr release assay at an effector-to-target ratio of 1:1. D, alternatively, gp100-specific CD8 T cells were mixed with melanoma cells (SK-MEL-23) expressing endogenous gp100 at varying effector-to-target ratios. Blood samples were collected from 10 melanoma patients at the time of diagnosis. The data presented are from three (B and C) and two (D) different donors from which the highest number of tumor-specific T cells could be generated. All experimental determinations were performed in triplicate; averages ± SDs were consistently within 15% of the mean. Error bars represent mean ± SD of triplicate samples. *, P < 0.01, ANOVA.
the higher antigen concentrations, TLR2 stimulation significantly enhanced T-cell expansion (Fig. 6B, bottom) and augmented cytolytic activity (Fig. 6C). To obtain a better understanding of the cytotoxic capacity of TLR2-stimulated T cells, we examined the lytic activity of gp100-specific T cells at varying effector-to-target ratios against an HLA-A2* melanoma line that expresses the weakly immunogenic tumor antigen gp100. As shown in Fig. 6D, TLR2 stimulation significantly augmented cytotoxicity at various effector-to-target ratios. These data highlight the potential for augmenting human T-cell responses against suboptimal expression levels of low-avidity TAg by activating TLR-MyD88 signals within T cells.

Discussion

For several reasons, TAg-specific CD8 T cells do not always persist in vivo or function effectively against established tumors. The present studies highlight that stimulating TLRs on TAg-specific T cells occurs in vivo, enhances longevity, lowers the activation threshold to suboptimal concentrations of TAg, and augments antitumor responses. Furthermore, overexpressing TLR2 enhances antitumor responses above WT T cells.

These results highlight several key points. First, although several studies have shown the costimulatory effects of stimulating TLRs on T cells in vitro, the present studies emphasize the importance of activating TLR-MyD88 signals in T cells in vivo. For example, WT mice receiving pmel CD8 T cells plus TLR2 ligand, but not TLR2+/− pmel or MyD88−/− pmel T cells, improved tumor regression and augmented the numbers of transferred T cells (Fig. 3). The contribution of ligating TLR2 on T cells is further highlighted in experiments showing that injection of TLR2 ligand and pmel T cells into MyD88−/− mice augmented T-cell cytotoxicity (Fig. 2) and delayed tumor growth (Fig. 4). Studies by Tak and colleagues suggested that TLR9 and TLR2 agonists in the synovium of rheumatoid arthritis costimulated T cells resulting in enhanced cytokolytic function and IFN-γ production (27). Conversely, these findings stress a physiologic role for activating TLR/MyD88 signals in T cells and could make possible new approaches for the development of more effective immunotherapies by manipulating TLR signaling within T cells.

Secondly, the studies presented here also show that overexpressing TLR2 on pmel T cells (without provision of TLR2 ligand) significantly delayed tumor growth compared with mice receiving control pmel T cells transduced with GFP alone. Because TLR2 can also recognize endogenous TLR ligands released from dying cells such as heat shock proteins and high-mobility group box-1 proteins (29–31), we speculate that overexpressing TLR2 on T cells could have enhanced the detection of these endogenous danger-associated signals, which may have served as a costimulatory signal to T cells.

The studies presented here also show that the enhanced antitumor responses of TLR2-stimulated pmel T cells correlated with improved persistence and accumulation at the tumor site (Fig. 4). This seemed to be primarily a result of enhanced cell survival rather than increased proliferation. Recent studies by Bartholdy (32) and Rahman (33) show that MyD88 expression in lymphocytic choriomeningitis virus–specific CD8 T cells plays a critical role in maintaining T-cell expansion. Furthermore, Zhoa and colleagues (34) show that vaccinia virus–specific MyD88−/− CD8 T cells failed to accumulate in vivo and underwent slow expansion in response to infection, highlighting a critical role for MyD88 signals in T cells. Our present studies add to these findings by showing that ligating TLR2 directly on CD8 T cells occurs in vivo and enhances MyD88-mediated survival, thus promoting antitumor immune responses. Zanin-Zhorov and colleagues (31) reported that TLR2-ligated T cells change the chemokine receptor expression profile, which alters T-cell migration/retention patterns, suggesting that the accumulation of T cells within the tumor may have also been due to enhanced recruitment and/or retention.

The mechanisms by which TLR2 stimulation on T cells influences cell survival are unknown but most likely involve the regulation of apoptosis-related molecules (12–14). Studies by several groups, including ours, showed TLR3 or TLR9 stimulation on CD4 T cells increased cell survival and was associated with the enhanced expression of Bcl-xL (13, 14, 35). In CD8 T cells, TLR2 engagement increased the expression of bcl-XL and A1 (12). The importance of modulating apoptosis-related protein in TAg-specific T cells is highlighted by Rosenberg and colleagues, who reported that overexpression of Bcl-2 in tumor-specific T cells augmented survival, consequently enhancing tumor regression (36). The data in Supplementary Data S3, showing an increase in bcl-2 and bcl-xl protein and mRNA expression levels and a reduction in bim, are in agreement with these studies.

Understanding the integration of signaling cascades that can enhance CD8 T-cell cytotoxicity against weakly immunogenic TAg is critical for developing effective cancer immunotherapies. TLR2 ligation on T cells enhanced the production of various cytokines and increased cytotoxicity (Figs. 1, 2, and 6; refs. 11, 12). Recent studies by our group suggest that TLR activation amplifies TCR signals in part by increasing the expression levels of several transcription factors including T-bet (Tbx21), known to transcriptionally regulate the expression of IFN-γ, perforin, and granzyme B (37). However, it is worth noting that MyD88 activation in T cells may have occurred via TLR-independent signals such as IL-1 or IL-18 or perhaps other endogenous danger-associated molecular patterns.

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These findings reveal a novel role for TLR-MyD88 signals within tumor-specific T cells, which may inspire new approaches for enhancing immunotherapies by targeting or manipulating TLR-MyD88 signaling within T cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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