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Adoptive immunotherapy combined with intratumoral TLR agonist delivery eradicates established melanoma in mice

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Abstract

Toll-like receptor (TLR) agonists can trigger broad inflammatory responses that elicit rapid innate immunity and promote the activities of lymphocytes, which can potentially enhance adoptive immunotherapy in the tumor-bearing setting. In the present study, we found that Polyinosinic:Polycytidylic Acid [Poly(I:C)] and CpG oligodeoxynucleotide 1826 [CpG], agonists for TLR 3 and 9, respectively, potently activated adoptively transferred T cells against a murine model of established melanoma. Intratumoral injection of Poly(I:C) and CpG, combined with systemic transfer of activated pmel-1 T cells, specific for gp100_{25–33}, led to enhanced survival and eradication of 9-day established subcutaneous B16F10 melanomas in a proportion of mice. A series of survival studies in knockout mice supported a key mechanistic pathway, whereby TLR agonists acted via host cells to enhance IFN- γ production by adoptively transferred T cells. IFN- γ , in turn, enhanced the immunogenicity of the B16F10 melanoma line, leading to increased killing by adoptively transferred T cells. Thus, this combination approach counteracted tumor escape from immunotherapy via downregulation of immunogenicity. In conclusion, TLR agonists may represent advanced adjuvants within the setting of adoptive T-cell immunotherapy of cancer and hold promise as a safe means of enhancing this approach within the clinic.

Keywords

Tumor immunology; Immunotherapy; Pmel; Toll-like receptor; Adoptive immunotherapy; Adjuvant

Introduction

Adoptive T-cell immunotherapy (ATI) has held great promise as a cancer therapy since evidence was obtained that T cells can specifically recognize and destroy tumor cells [1]. In its simplest form, ATI involves isolating tumor-specific T cells from patient tumors or peripheral blood, activating and expanding them *ex vivo*, and then transferring them back into the patient [2]. The key challenge of this therapy is the requirement for adjuvants to maximize T-cell survival and function *in vivo*.

A range of innovative adjuvant approaches for ATI including the use of cytokines, vaccines, and lymphodepletion have been used to enhance the antitumor activity of T cells [3–5]. As a result of these combination approaches, ATI has shown stunning success in the treatment of advanced human metastatic melanoma [6, 7]. However, considerable scope remains to improve adjuvant therapies. IL-2 and lymphodepleting preconditioning are used to enhance adoptive transfer, but a pressing concern is the morbidity associated with high-dose IL-2 delivery and immunodepletion [8]. Co-administration of specific vaccines can also enhance T-cell transfer, but these vaccines need to be tailored to the MHC haplotype and TAA expression profile of individual patient tumors.

We hypothesized that Toll-like Receptor (TLR) agonists represent an opportune set of reagents from which to expand the arsenal of adjuvant approaches for ATI. TLR agonists are conserved components of pathogens that the immune system has evolved to recognize through receptors known as Toll-Like Receptors (TLRs) [9]. The TLR family is expressed on a wide variety of leukocytes and stromal cells, and its members are notable for their ability to signal via pathways that result in strong inflammatory reactions [10, 11]. Activation of leukocytes through TLRs can facilitate progression from innate to adaptive immune responses [12].

The TLR agonists selected for testing in this work were Poly(I:C), a synthetic dsRNA sequence recognized by TLR 3, and CpG 1826, and a DNA oligonucleotide rich in CpG motifs that is recognized by TLR 9 [3, 4]. These compounds function as markers of viral and bacterial infection for the immune system, respectively. The receptors for Poly(I:C) and CpG display a disparate distribution pattern among leukocytes, with TLR 3 expressed on myeloid dendritic cells (mDCs) and possibly natural killer (NK) cells and mast cells, and TLR9 on plasmacytoid cells (pDCs), B cells, and murine macrophages.

Of the many TLR agonists that exist, Poly(I:C) and CpG were specifically chosen, firstly because it is hypothesized that the broad inflammation they trigger will promote T-cell cytotoxicity and cell-mediated immunity, in vivo [13, 14]. Secondly, in vitro evidence is beginning to emerge to suggest that they may be able to act synergistically in their capacity to drive gene expression and cytokine release [15, 16]. Thirdly, in vivo evidence has been presented as to their efficacy in the tumor setting both in the mouse models [17–19] and in the clinic [4, 20].

TLR agonists stimulate a plethora of responses from host systems. They can directly act on tumor cells [21], host stroma [22, 23], and a broad range of leukocyte populations [24, 25]. They can thereby trigger waves of inflammation with a profound range of impact, including on host vasculature and leukocytes. Previous studies have correlated many effects of TLR agonist delivery with improved therapeutic outcome. However, there is a need to move beyond the identification of changes that occur in tumor microenvironments upon TLR agonist administration and to progress to functional evidence that these changes are fundamental to their role in enhancing ATI.

The key purpose of the current study was to directly pair ATI with tumor-localized delivery of Poly(I:C) and CpG, and to study this therapeutic regimen against a model of established, aggressive murine B16F10 melanoma. In this report, we examine the impact of Poly(I:C) and CpG on the tumor microenvironment and use gene-deficient mice to obtain functional insight into their role as adjuvants of T-cell antitumor activity.

Materials and methods

Cell lines and mice

B16F10 is a mouse melanoma cell line and MC38 a mouse colon carcinoma cell line, both syngeneic to C57BL/6 mice. Tumor lines were maintained at 37°C, 10% CO₂ in DMEM, supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. In experiments measuring the impact of IFN- γ on tumor immunogenicity, 1.4×10^7 B16F10 or MC38 tumor cells were preincubated overnight in the presence of 1–100 IU/ml recombinant mouse IFN- γ (Roche, Basel, Switzerland).

Wild-type C57BL/6 mice were purchased from the Walter and Eliza Hall Institute of Medical Research (Bundoora, VIC, Australia), and the Animal Resources Centre (Perth, Western Australia, Australia). Gene-deficient and TCR-transgenic mice were bred on the C57BL/6 background at the Peter MacCallum Cancer Centre (East Melbourne, Australia). MyD88^{-/-} mice were kindly provided by Shizuo Akira (Osaka, Japan), and IFN- α 1^{-/-} mice by Paul Hertzog (Clayton, Australia). All gene-deficient mice were backcrossed onto a C57BL/6 background for at least 10 generations. All experiments were initiated using mice 6–12 weeks of age unless otherwise indicated and were performed under specific pathogen-free conditions, according to the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee guidelines.

Peptide and antibodies

The gp100_{25–33} peptide (KVPRNQDWL) [26] was purchased from Auspep (Parkville, VIC, Australia) and stored frozen at -20°C at 1 mg/ml in dimethyl sulfoxide (Sigma–Aldrich, St. Louis, MO, USA) until use. Monoclonal antibodies used to deplete IFN- γ (clone H22) and TNF α (clone TN3-19.2) in vivo were a kind gift from R. Schreiber (Washington University School of Medicine, MO, USA). Mice received 250 μg i.p. twice weekly for the duration of the experiment.

In vitro expansion and activation of gp100_{25–33}-specific T cells

Spleens dissected from pMel/Thy1.1 mice [27] were crushed in 5 ml of hypotonic ACK lysis buffer (0.15 M NaCl, 1 mM KHCO_3 , 0.1 mM EDTA, pH = 7.2–7.4) to remove red blood cells. Cell suspensions were filtered through a 70- μm filter and washed twice in RPMI (Gibco, Grand Island, NY, USA) at 338 g, 5 min at room temperature. Cells were pulsed with 1 $\mu\text{g}/\text{ml}$ gp100_{25–33} in 10 ml of RPMI for 2 h at room temperature, and then washed twice in 10 ml RPMI to remove unbound peptide. Splenocytes were cultured at a density of $1.5\text{--}2 \times 10^6$ cells in 2 ml RPMI, supplemented with 10% (v/v) FCS, 2 mM Glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 50 IU/ml recombinant human interleukin-2 (National Cancer Institute, Frederick, MD, USA) for 2 weeks at 37°C , 5% CO_2 . Fresh IL-2 was added every second day and T cells restimulated at a 2:1 ratio with gp100_{25–33}-pulsed irradiated (5 Gy) C57BL/6 splenocytes after the first week of culture.

In vivo tumor studies

Wild-type mice or gene-deficient mice on the C57BL/6 background were inoculated with 100 μl of a single-cell suspension of 4×10^5 B16F10 melanoma cells in Ca^{2+} and Mg^{2+} -free phosphate-buffered saline (PBS), s.c. (for localized disease) or i.v. (to establish lung colonies). Mice bearing tumors $\sim 25 \text{ mm}^2$, 9 days postinoculation, received a single dose of 5×10^6 activated pMel T cells, i.v. in 200 μL PBS. A total of four injections of 25 μg each of Poly(I:C) (Sigma–Aldrich) and CpG 1826 (Coley Pharmaceutical, Canada) were delivered in a 30 μl total volume of PBS intratumoral (i.t.) or 200 μl total volume of PBS (i.v.), on days 9, 11, 13, and 15 after tumor inoculation. Mice were culled when their tumors reached 150 mm^2 . Mice displaying complete responses to B16F10 treatment with ATI and TLR agonists (>50 days) were rechallenged via s.c. injection of 4×10^5 B16F10 cells in 100 μl PBS on the contralateral flank to the primary inoculum.

Digestion of ex vivo tissues and flow cytometry

Ex vivo tumor samples were isolated as s.c. masses from C57BL/6 mice, weighed, physically minced in PBS on ice and then digested, shaking for 30 min at 37°C in 10 ml DMEM containing 1.5 mg/ml Collagenase A (Roche) and 1.5 mg/ml Collagenase IV (Worthington Biochemical Corporation, Lakewood, NJ, USA). Digested samples were filtered through a 70- μm bucket filter and washed twice in 10 ml DMEM.

Tumor-draining (inguinal) and tumor-remote (auricular) lymph nodes were harvested 1 day posttherapy for DCs enrichment via gradient centrifugation, and 3 days post-therapy for intracellular cytokine staining (ICS) of adoptively transferred T cells. Pools of eight lymph nodes/ treatment group were digested, shaking at 37°C for 25 min in RPMI containing 50 $\mu\text{g}/\text{ml}$ Collagenase IV (Worthington Biochemical Corporation). Digested samples were filtered through a 40- μm filter and washed in 10 ml of FACS buffer (PBS with 0.5% bovine serum albumin) for 7 min at 300 g and DCs enriched via gradient density centrifugation. Samples analyzed for intracellular IFN- γ were cultured in RPMI for 6 h in the presence of

10^{-8} M gp100_{25–33}, or with plate-bound anti-CD3 antibody (clone 145-2C11; Becton–Dickinson Biosciences (BD); San Jose, CA, USA) as a positive control.

Flow cytometry

Cells were stained at the cell surface, or intracellular staining carried out using a Cytofix/Cytoperm Plus kit (BD) according to manufacturer's instructions. Antibodies used in this study were sourced from AbCAM (Cambridge, MA, USA), BD Biosciences (San Jose, CA, USA), BioLegend (San Diego, CA, USA), and eBioscience (San Diego, CA, USA) (see Supplementary Table 1). During quantification, cells were spiked with a known number of Calibrite unlabeled beads (BD). A FACS LSRII flow cytometry system (BD) was used to acquire 10,000 live, gated events, and data analyzed using FCS Express, version 3 (Denovo Software, Los Angeles, CA, USA). The incidence of CD45⁺ leukocytes or Thy1.1⁺ pMel T cells was expressed as mean number of cells/mg tumor and mean number of cells/lymph node. During phenotypic analysis of DCs, plasmacytoid DCs (pDCs) were defined as live, single, CD11c⁺CD45RA⁺CD19[−] cells. Comparisons between independent experiments were enabled by normalizing the mean fluorescence intensity (MFI) values for pools of treated lymph nodes to those observed for vehicle-control mice.

Cytotoxicity assays

The cytotoxicity of activated T cells toward tumor targets was assessed in 6 h ⁵¹Cr-release assays [28]. Tumor targets were labeled with 100 μ Ci sodium [⁵¹Cr] chromate (Perkin Elmer, Boston, Massachusetts, USA), washed, then plated in triplicate into 96-well round-bottom plates at incremental ratios of T cells:tumor, and incubated for 6 h at 37°C, 5% CO₂. Spontaneous ⁵¹Cr-release was measured in wells containing tumor targets in media alone and total release assessed by adding 100 μ L of 5 mM HCl to relevant wells. Radioactivity within the supernatants was measured using a Wallac 1470 automatic gamma counter (Wallac, Finland), and cytotoxicity was expressed as percentage specific ⁵¹Cr release after subtraction of spontaneous ⁵¹Cr release.

Software and statistical analysis

Graphs were generated using GraphPad Prism, version 5.02 for Windows (GraphPad Software, San Diego, CA, USA). SPSS, version 13 (SPSS Inc., Chicago, IL, USA) was used to obtain statistical data. Statistics for survival studies were calculated using the Log-Rank (Mantel–Cox) test. All other statistics were calculated using the nonparametric Mann–Whitney *U* test.

Results

The TLR agonists, Poly(I:C) and CpG, are effective adjuvants for ATI of established melanoma

In the present study, the TLR agonists, Poly(I:C) and CpG, were assessed for their ability to act as adjuvants for adoptively transferred T cells against established s.c. melanoma. In Fig. 1a, it can be seen that untreated mice injected s.c. with 4×10^5 cells of the B16F10 melanoma line survived until day 7 through 9 after the start of therapy in other groups, which is equivalent to day 16 through 18 posttumor inoculation.

Adoptive transfer of large numbers of highly activated tumor-specific T cells did not in itself significantly extend the survival of mice with established s.c. disease (Fig. 1a; $P = 0.28$, relative to untreated mice; Log-Rank (Mantel–Cox) test). A significant enhancement in survival of mice was achieved when adoptively transferred T cells were combined with intratumoral (i.t.) injection of the TLR 3 agonist Poly(I:C) or with the TLR 9 agonist CpG (Fig. 1a, $P = 0.015$ and $P < 0.001$, respectively, relative to therapy with T cells alone; Log-

Rank (Mantel–Cox) test). The greatest survival benefit was conferred when the two agonists were delivered in conjunction with adoptive T-cell transfer. In the four independent *in vivo* experiments compiled in Fig. 1a, ATI together with Poly(I:C) and CpG induced complete responses in 7/31 mice (23%). These complete responses were found to be durable over an observation period of >200 days ($P < 0.001$ and $P = 0.003$, respectively, relative to therapy with T cells and Poly(I:C) and T cells and CpG; Log-Rank (Mantel–Cox) test). Some variability was observed in the percentage of long-term survivors between individual experiments, which was likely due to variable purity and/or activity of pMel T-cell cultures.

The functional importance of host responses to the TLR9 agonist, CpG, was assessed in survival experiments in mice lacking the MyD88 adaptor protein—a critical intermediate in TLR 9 signaling (Fig. 1b). Survival of wild-type mice was assessed alongside as a measure of therapeutic impact on each independent occasion. In the Kaplan–Meier survival plot shown in Fig. 1b, it can be seen that untreated wild-type and MyD88^{−/−} mice displayed equivalent survival kinetics ($P = 0.872$; Log-Rank (Mantel–Cox) test), but treated MyD88^{−/−} mice died significantly earlier than treated wild-type mice ($P = 0.025$; Log-Rank (Mantel–Cox) test). This indicated that host inflammatory responses to CpG, via TLR 9, made a functional contribution to enhancing ATI.

In Fig. 1c, wild-type mice that had undergone complete responses to therapy beyond 50 days were assessed for the presence of immunological memory via s.c. rechallenge with 5×10^6 B16F10 cells in the contralateral flank. It was observed that 20% of rechallenged mice did not develop tumors over the course of a 150-day follow-up period—an outcome that is never observed when B16F10 is inoculated in this manner into naïve mice. Those rechallenged mice that did develop tumors reached the ethical cull point significantly later than control mice undergoing primary tumor challenge (Fig. 1c; $P < 0.001$; Log-Rank (Mantel–Cox) test). Thus, combination immunotherapy with adoptive T-cell transfer and i.t. TLR agonist injection induced complete, durable regression of established melanomas in a proportion of mice, generating protective immunological memory against the highly aggressive B16F10 melanoma line.

Both TLR agonists are necessary and they must be delivered intratumorally, not systemically during ATI

The increase in antitumor activity of the combination of TLR agonists was not solely due to a higher overall dose of total agonist, since using 50 µg of either agonist was not as effective as using 25 µg of each together (Supplementary Figure 1a). ($P < 0.05$ Paired Student's *t* test for ATI + CpG + Poly(I:C) vs. all other groups on days 16 and 19). The method of TLR agonist delivery is an important consideration as it may have a bearing on therapeutic efficacy as well as the ease of therapeutic administration in the clinic. An ability to deliver TLR agonists systemically would simplify the process of administering this therapy. As such, i.v. delivery of TLR agonists was assessed as an alternative to the i.t. delivery route. It can be seen from Supplementary Figure 1b that TLR agonists delivered i.v. did not act as effective adjuvants for adoptive T-cell therapy, as all mice treated via this combination approach needed to be culled by day 15 posttherapy. Attempts to deliver TLR agonists i.p. were hindered by toxicity issues in which mice became hunched and ruffled with darkened abdomens (data not shown). Hence, it was concluded that TLR agonists must be delivered i.t., and not systemically, in order to be effective as adjuvants during ATI.

It would also be of benefit in the clinic if i.t. treatment of s.c. tumors was able to establish an immune response that could impact on the progression of remote melanomas. To test this, experimental groups were set up that had both s.c. and lung colonies of B16F10 (see “Materials and methods”). ATI alone enabled mice bearing lung tumors to survive until day 23 after treatment (Supplementary Figure 1c). No significant enhancement in survival was

achieved beyond this effect for mice bearing lung and s.c. tumors that received i.t. injection of the TLR agonists into the s.c. tumor in combination with ATI (Supplementary Figure 1c; $P = 0.928$, relative to mice bearing lung tumors treated with T cells alone; Log-Rank (Mante–Cox) test). In these survival time courses, ATI and TLR agonists delivered to mice with s.c. tumors alone significantly improved their survival kinetics, with 36% of mice undergoing complete responses. As such, it was concluded that successful combination immunotherapy of s.c. tumors did not initiate an immune response that could induce regression of remote tumor burdens.

TLR agonists increase the incidence of leukocytes in tumor-draining lymph nodes during combination immunotherapy

Since inflammatory signals induced by TLR agonists may induce infiltration and/or expansion of leukocytes, we determined the incidence of leukocytes in tumors and lymph nodes in the presence or in the absence of therapy. Quantitative flow cytometry was used to track changes in leukocyte incidences in both tumor-draining lymph nodes and tumors in the 7 days after initiation of immunotherapy. This timeframe was chosen as it represented the period in which tumor regression occurred in response to therapy, but where sufficient tissue was still able to be collected for analysis. The number of leukocytes increased progressively in response to TLR agonist delivery over the 7 days only in lymph nodes draining the delivery site. It can be seen that the incidence of innate neutrophils, macrophages and NK cells, and adaptive $CD4^+$ and $CD8^+$ T cells and B cells in tumor-draining lymph nodes increased dramatically in response to i.t. injection of TLR agonists, and that these increases occurred independently of whether tumor-specific T cells were co-administered (Supplementary Figure 2).

Adoptively transferred pMel T cells were able to be identified in lymph nodes and tumors through their expression of the Thy1.1 congenic marker. In Fig. 2a, it can be seen that i.t. delivery of Poly(I:C) with CpG did not lead to an increase in the mean number of pMel T cells being detected in the tumor-draining lymph node, relative to when therapy consisted of T cells alone. It was surprising to observe that co-administration of TLR agonists corresponded with a significant decrease in the incidence of adoptively transferred T cells in tumor-remote lymph nodes on days 1 and 7 (Fig. 2b, $P = 0.01$; Mann–Whitney U test). Furthermore, on day 3, i.t. injection of Poly(I:C) and CpG coincided with a significant decrease in the incidence of adoptively transferred pMel T cells detected in the tumor site ($P = 0.005$, relative to ‘T-cells alone’; Mann–Whitney test; Fig. 2c). The i.t. incidences of host $CD8^+$ T cells and host leukocytes were unaltered by TLR agonist therapy (Fig. 2c).

In summary, the quantitative flow cytometry data collected in the 7 days following initiation of ATI with Poly(I:C) and CpG confirms that these agonists have a broad impact on leukocytes proximal to the tumor site. Analysis of tumor infiltrate suggests that the adjuvant effects of TLR agonists function independently of a need for enhanced infiltration of adoptively transferred T cells into the tumor microenvironment.

Host DCs, but not host lymphocytes, contribute to the enhancement of ATI by TLR agonists

In light of the quantitative flow cytometry data, the functional contribution made by host lymphocytes during ATI with TLR agonists was investigated via survival experiments in strains lacking mature B cells (μ MT mice), or lacking mature B cells and T cells (RAG-1^{-/-} mice). Both knockout strains displayed a significant enhancement in survival relative to untreated mice of their respective strains ($P < 0.001$ for μ MT mice and $P = 0.002$ for RAG-1^{-/-} mice; Log-Rank (Mantel–Cox) test, data not shown). This indicated that neither host B cells nor T cells played a critical role in the response to ATI with Poly(I:C) and CpG.

A role for host dendritic cells in enhancing the impact of ATI was investigated via phenotypic and survival studies. Flow cytometry performed on DC-enriched pools of tumor-draining lymph nodes, taken 24 h postinitiation of therapy, revealed that host pDC populations (CD11c⁺CD45RA⁺CD19⁻) upregulated their expression of the CD40 and CD86 co-stimulatory receptors in response to i.t. TLR administration (Fig. 3a–c). Non-plasmacytoid DCs, including resident DCs and migratory DCs (CD11c⁺CD45RA⁻CD19⁻), also upregulated CD40 and CD86 (data not shown).

A co-stimulatory role for DC populations was studied using mice unable to cross-present TAA because of a gene modification that knocked out the TAP-1 transporter protein (Fig. 3d). The increase in survival displayed by treated TAP-1^{-/-} mice, in Fig. 3d, failed to reach statistical significance relative to the survival kinetics of untreated TAP-1^{-/-} mice ($P=0.118$; Log-Rank (Mantel–Cox) test). Thus, the survival benefit of combining i.t. TLR agonist administration with ATI was impaired in mice unable to carry out normal processes of antigen cross-presentation.

IFN- γ acts in a critical manner on nonhost tissues during ATI with TLR agonists

To gain crucial functional insight into the therapeutic mechanism underpinning TLR agonist therapy during ATI, the importance of several inflammatory cytokines was measured via survival experiments in gene-targeted mice. Kaplan–Meier survival plots from these experiments indicated that host IL-12 and TNF- α and host responses to type-I interferons did not make a critical contribution during therapy (see Supplementary Figure 3).

When IFN- γ gene-targeted mice were treated via combination immunotherapy and additionally depleted of IFN- γ produced by adoptively transferred T cells, a complete abrogation of survival benefit was observed (Fig. 4a; $P=0.572$, relative to untreated IFN- γ knockout mice; Log-Rank (Mantel–Cox) test). This outcome established that IFN- γ was critical to the mechanism by which Poly(I:C) and CpG enhanced the antitumor impact of adoptively transferred T cells.

The level to which adoptively transferred T cells could act as a source of IFN- γ during combination immunotherapy was measured by treating IFN- γ ^{-/-} mice in the absence of any further IFN- γ depletion (Fig. 4b). The survival kinetics of treated IFN- γ ^{-/-} mice extended significantly beyond those of untreated IFN- γ ^{-/-} mice ($P<0.001$; Log-Rank (Mantel–Cox) test) and were statistically equivalent to those of treated, wild-type mice ($P=0.546$; Log-Rank (Mantel–Cox) test). As such, IFN- γ produced by host lymphocytes was not necessary for therapy, and IFN- γ produced by adoptively transferred T cells was sufficient in order for the tumor regression promoted by Poly(I:C) and CpG to proceed.

In order to determine whether host or nonhost cells were crucial targets of IFN- γ during immunotherapy, IFN- γ R^{-/-} mice were used as hosts in survival experiments. In Fig. 4c, it can be seen that the survival of wild-type and IFN- γ R^{-/-} mice was equivalent following combination immunotherapy ($P=0.533$; Log-Rank (Mantel–Cox) test). As such, nonhost cells (the B16F10 tumor or the adoptively transferred T cells) are the critical targets of IFN- γ during ATI with Poly(I:C) and CpG.

IFN- γ promotes cytotoxic interactions between pMel T cells and the B16F10 melanoma line

In the current work, flow cytometry was used to confirm the expression of the IFN- γ R1 and 2 chains at the surface of the B16F10 line (Fig. 5a). Under basal conditions, the MHC-I H-2D^b allele responsible for presenting the tumor-associated antigen gp100_{25–33} to adoptively transferred pMel T cells could not be detected at the surface of B16F10 (Fig. 5b). This correlated with an inability of the pMel T cells to exhibit cytotoxicity following co-culture with untreated B16F10 tumor cells (Fig. 5c). B16F10 cells cultured overnight in the

presence of IFN- γ displayed upregulation of the MHC-I H-2D^b allele (Fig. 5b), which correlated with a significant enhancement in the ability of pMel T cells to exhibit cytotoxicity toward the B16F10 line (Fig. 5c). Thus, IFN- γ promotes cytotoxic interactions between gp100-specific pMel T cells and the B16F10 melanoma line.

IFN- γ -producing adoptively transferred T cells increase in incidence in tumor-draining LNs in response to TLR agonist therapy

Intracellular cytokine staining was used to measure whether i.t. injected Poly(I:C) and CpG enhanced the level to which IFN- γ was produced by adoptively transferred T cells in vivo. In Fig. 6a and b, it can be seen that 12.77 and 11.46% of adoptively transferred T cells stained positive for intracellular IFN- γ when transferred alone into tumor-free or tumor-bearing mice, respectively. The proportion of IFN- γ -positive pMel T cells increased to 31.72% when adoptive T-cell transfer was combined with i.t. delivery of Poly(I:C) and CpG (Fig. 6c). Thus, i.t. delivered TLR agonists enhanced in vivo production of the critical cytokine, IFN- γ , by adoptively transferred T cells during tumor immunotherapy.

Discussion

In this paper, we report a series of studies into the means by which TLR agonists act as effective adjuvants for ATI of cancer. Intratumoral injection of Poly(I:C) and CpG alongside adoptive T-cell transfer had the capacity to drive complete, durable regression of established s.c murine melanomas and generate memory responses. The feasibility of using TLR agonists in the human setting has previously been established within phase I and II clinical trials [29, 30]. As such, we propose that TLR agonists are opportune reagents for development as adjuvants for ATI in the clinic. The use of two or more TLR agonists in combination with ATI is indicated in future trials, as combined administration of Poly(I:C) and CpG enhanced ATI more effectively than either agonist alone.

The literature pertaining to the use of TLR agonists in tumor therapies is split among therapies in which the agonists are delivered systemically [31–33] or local to the tumor environment [19, 30, 34]. On the basis of evidence presented in this study, we propose that TLR agonists function best on a local level, rather than a systemic one. Delivery of TLR agonists via systemic routes did not considerably enhance the therapeutic effect of ATI and was associated with adverse effects. Meanwhile, i.t. delivery resulted in dramatic expansion of leukocytes in tumor-draining, but not remote lymph nodes. This spatial constraint on the range of TLR agonist influence may be the key to why tumor-localized delivery of TLR agonists is best. It is notable that clinical trials that have reported therapeutic outcomes when applying CpG in the treatment of cancer also delivered CpG local to the tumor site [30, 35]. Indeed, a recent study involving low-dose radiotherapy in combination with locally administered CpG in 15 patients demonstrated one complete response, 3 partial responses, and 2 with stable disease [36].

Curiously, leukocytes within the tumor site did not increase significantly in response to i.t. TLR agonist injection, in spite of the dramatic increases seen in tumor-draining lymph nodes. Most notable and surprising was that the incidence of adoptively transferred T cells was reduced in tumors and tumor-remote lymph nodes in the week following i.t. delivery of TLR agonists. Detailed investigations into T cell and leukocyte behavior in tumors during therapy were beyond the scope of the current study, but reduced numbers of pMel T cells in tumors may be due to the existence of physical and/or biochemical immunosuppressive factors within the B16F10 site that subvert antitumor immunity.

A previous study targeting spontaneous insulomas expressing SV40 large T antigen (Tag) demonstrated that, in contrast to the current study, administration of CpG

oligodeoxynucleotides enhanced tumor localization of adoptively transferred Tag-specific T cells [37]. The observed paucity of adoptively transferred T cells in B16F10 in our study was not taken to contradict this and other reports that have correlated successful immunotherapy with increases in T-cell numbers at the tumor site [38, 39]. Rather, it is proposed that in the present study, TLR agonists achieve a degree of therapeutic enhancement even without an apparent increase in T cell entry and expansion in the tumor site.

Differences in the level of infiltration by adoptively transferred T cells may have been due to the use of different tumor types or different routes of CpG administration—systemic in the previous study compared with intratumoral in the current study. Or it may be a product of the use of additional therapeutic components, including delivery of recombinant viral vaccines and cytokines or chemotherapy in combination with TLR agonists and T cells. Alternatively, in the present study, highly activated pMel T cells may undergo activation-induced cell death shortly after entering tumors and engaging tumor cells, leading to an apparent reduction in their numbers. The literature reports that chemokines that can attract T cells, such as CXCL9-11, are produced by leukocytes in response to Poly(I:C) and CpG [40–42]. It remains of interest to determine whether in our hands, the adoptively transferred T cells retain expression of receptors for these cytokines and a capacity to migrate in response to them after the two-week in vitro activation period. Future experiments comparing localization of pMel T cells with nonspecific T cells may enable further interpretation of any effect that activation-induced cell death may be having.

An important aspect of this study was its potential to pinpoint factors of the inflammatory response to TLR agonists which made functional contributions to enhancing ATI. Gene-targeted survival studies revealed that host B and T lymphocytes, the cytokines IL-12, TNF- α , host IFN- α R, and host IFN- γ Rs, were not critical to the therapeutic effect. In contrast, host or nonhost IFN- γ and host TAP-1 were critical in order for any survival benefit to be seen. We propose that TLR agonists enhance ATI in two steps. First, by promoting better interaction between activated host DCs and adoptively transferred T cells and thereafter, by enhancing tumor clearance via an IFN- γ -dependent mechanism.

Evidence that the TLR agonists function via host cells were obtained using MyD88 knockout mice, and the activation of host DC populations in the tumor-draining LN was confirmed via flow cytometry. It is difficult to measure the importance of host responses to Poly(I:C), as this agonist is recognized by several intracellular RIG-like Receptor (RLR) proteins in addition to TLR 3 [43]. Double knockout mice, which lack the TRIF signaling intermediate of the TLR 3 pathway and the IPS protein of RLR pathways, may be an option for the future [44].

DCs have previously been thought to be a population important for augmenting the activity of adoptively transferred T cells during combination immunotherapy with immunodepletion and systemic vaccination with TAA and Poly(I:C) [18]. DCs fulfill a multitude of roles in the immune response, bearing the capacity to provide co-stimulation, cytokine support, and homing instructions to T cells in addition to TCR-specific stimulation. The preliminary evidence that TAP-1 was critical to therapy enabled us to hypothesize that TAA cross-presentation on host DCs is a fundamental step in the mechanism by which ATI is enhanced. However, given that TAP-1 deficient mice may have other dysfunctions in T cells and NK cells, and the current study supports the requirement for cross-presentation but does not definitively prove it.

Four observations have been made in this study concerning the importance of IFN- γ during ATI with TLR agonists. First, abrogation of both host and nonhost IFN- γ during survival

studies revealed that IFN- γ was absolutely critical in order for any therapeutic effect to be seen. Second, adoptive transfer of IFN- γ wild-type pMel T cells into IFN- γ knockout hosts in combination with TLR agonist delivery demonstrated that the IFN- γ produced by adoptively transferred T cells was sufficient for the needs of the therapy. Third, the observation that therapy was effective in IFN- γ knockout mice demonstrated that nonhost B16F10 tumors or pMel T cells must be the critical targets of IFN- γ activity. Finally, ex vivo intracellular cytokine staining conducted on pMel T cells isolated from tumor-draining LNs 3 days postimmunotherapy confirmed an enhancement in IFN- γ production when TLR agonists were used as adjuvants.

The ability of IFN- γ to enhance the immunogenicity of B16F10 cells is well documented [45]. The implication of this response for the current therapy was demonstrated, with pMel T cells effecting significantly greater levels of cytotoxicity against IFN- γ pretreated B16F10 cells than the untreated line. Dighe et al. [46] have previously used a dominant-negative IFN- γ R1 chain to demonstrate that the ability of the TLR 4 agonist, lipopolysaccharide, to treat murine Meth-A fibrosarcomas is dependent on the effects of IFN- γ . However, the level to which ATIs being trialed in the clinic are reliant on such mechanisms is unclear.

If TLR agonists are to be used in therapies for a broad range of cancer types, it is promising to note that the literature contains a number of papers reporting that non-melanoma tumors retain the capacity to respond to IFN- γ [47–49]. The broader implications of our finding are that it provides one means of identifying which patient tumors are and are not likely to respond to this approach and that it opens up the possibility of exploit the beneficial effects of IFN- γ by finding more targeted means of enhancing its production [50] or delivering it directly into the tumor microenvironment [51–53].

Thus, the TLR agonists Poly(I:C) and CpG are successful adjuvants during ATI for established melanoma. We propose that TLR agonists enhance ATI in two functional phases—first, by promoting better interaction between activated host DCs and adoptively transferred T cells and second, by enhancing the antitumor activity of the T cells via an IFN- γ -dependent mechanism. It will be worthwhile to test whether TLR agonists can replace peptide vaccines and IL-2 in current combination ATI approaches [27]. In conclusion, TLR agonists represent a promising adjuvant approach for the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Intratumoral delivery of Poly(I:C) and CpG in combination with adoptive T-cell transfer enhances the survival of mice with established s.c. melanoma. In vivo survival experiments were conducted as described in “Materials and methods”. Kaplan–Meier survival analysis indicated that **a** the survival of mice bearing established s.c. melanoma was significantly enhanced when Poly(I:C) and CpG were delivered i.t. in combination with i.v. ATI (* $P < 0.001$ relative to T cells alone group; Log-Rank (Mantel–Cox) test; data compiled from four independent experiments). **b** Loss of host responsiveness to CpG had a detrimental effect on therapy, with MyD88^{-/-} mice dying significantly earlier than treated, wild-type controls ($P = 0.025$; data compiled from two independent experiments). **c** Complete tumor

responses, durable >50 days, were accompanied by the formation of immunological memory, with mice exhibiting enhanced survival kinetics when rechallenged with B16F10 s.c. in the contralateral flank (data collected in one experiment)

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Fig. 2.

TLR agonists do not enhance the number of adoptively transferred T cells in lymph nodes or the tumor microenvironment. Flow cytometry was used to quantify pMel T cells in the tumor microenvironment, as described in “Materials and methods”. **a** Delivery of TLR agonists in combination with adoptive T-cell transfer did not increase the number of pMel T cells in tumor-draining lymph nodes and coincided with reductions in their number in **b** non-draining LNs (* P 0.01, days 1 and 7, relative to ‘T-cells alone’) and **c** s.c. tumors (** P = 0.005, relative to ‘T-cells alone’). **c** The incidences of host CD8⁺ T cells and CD45⁺ leukocytes in the tumor site were not altered as a result of therapy. Data were compiled from 3 independent experiments, n = 6–9 at each timepoint; P values calculated using the Mann–Whitney test

Fig. 3.

Antigen cross-presentation by TLR agonist-activated host DCs contributes to antitumor immunity during combination immunotherapy. Flow cytometry was used to phenotypically assess the activation status of pDCs enriched from pools of tumor-draining lymph nodes, taken 24 h posttherapy (see “Materials and methods”). Mean fluorescence intensity (*MFI*) readouts were used as a measure of the level of expression of the **a** CD40 and **b** CD86 cell-surface activation markers. **c** When normalized to the vehicle-control group to allow comparison between independent experiments, *MFI* values for both CD40 (3 experiments shown) and CD86 (6 experiments shown) increased in response to TLR agonist therapy and independently of adoptive T-cell transfer. **d** Disruption of antigen cross-presentation in TAP-1^{-/-} mice negated the survival benefit of ATI with TLR agonists (**P* = 0.118, relative to untreated TAP-1^{-/-} and *P* = 0.02, relative to treated wild-type C57BL/6; Log-Rank (Mantel–Cox) test)

Fig. 4.

IFN- γ is a critical contributor to the mechanism underpinning ATI with TLR agonists. The importance of IFN- γ during ATI was assessed using IFN- γ and IFN- γ^R knockout mouse strains and antibody-mediated cytokine depletion, according to the schedule described in “Materials and methods”. Survival times for B16F10-bearing wild-type mice were also assessed, with graphs representing three independent experiments ($n = 8-10$, P values were calculated using the Log-Rank (Mantel-Cox) test). **a** Complete abrogation of IFN- γ was achieved by delivering 250 μg of the H22 antibody clone i.p. twice weekly for the duration of the experiment. The resulting Kaplan-Meier survival curve demonstrated that IFN- γ was critical during ATI with TLR agonists. **b** Treatment of IFN- γ knockout mice with IFN- γ -competent pMel T cells and TLR agonists further demonstrated that IFN- γ produced by pMel T cells was sufficient for the needs of the therapy. **c** Successful treatment of IFN- γ^R knockout mice via ATI with TLR agonists demonstrated that host responses to IFN- γ were not necessary for therapy.

Fig. 5.

IFN- γ upregulates the immunogenicity of the B16F10 melanoma line. Flow cytometry was used to measure the density of **ai** the IFN- γ R1 and **aii** IFN- γ R2 chains and **b** upregulation of the MHC-I H-2D^b allele at the surface of in vitro cultured B16F10 cells, in response to IFN- γ . **c** The capacity of gp100₂₅₋₃₃-specific pMel T cells to exhibit cytotoxicity toward B16F10 was augmented by preincubating the B16F10 line with 100 IU/ml IFN- γ overnight, prior to co-culture with T cells in 51Cr-release assays (data compiled from 3 independent experiments)

Fig. 6.

TLR agonists enhance in vivo production of IFN- γ by tumor-specific pMel T cells. ICS was used to measure IFN- γ production by Thy1.1+ pMel T cells isolated from tumor-draining lymph nodes of **a** tumor-free C57BL/6 recipients of pMel T cells, or B16F10+ mice treated with **b** pMel T cells alone or **c** pMel T cells, Poly(I:C) and CpG. Data are representative of two independent experiments