B cells imprint adoptively transferred CD8+ T cells with enhanced tumor immunity

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ABSTRACT

Background  Adoptive T cell transfer (ACT) therapy improves outcomes in patients with advanced malignancies, yet many individuals relapse due to the infusion of T cells with poor function or persistence. Toll-like receptor (TLR) agonists can invigorate antitumor T cell responses when administered directly to patients, but these responses often coincide with toxicities. We posited that TLR agonists could be repurposed ex vivo to condition T cells with remarkable potency in vivo, circumventing TLR-related toxicity.

Methods  In this study we investigated how tumor-specific murine CD8+ T cells and human tumor infiltrating lymphocytes (TILs) are impacted when expanded ex vivo with the TLR9 agonist CpG.

Results  Herein we reveal a new way to reverse the tolerant state of adoptively transferred CD8+ T cells against tumors using TLR-activated B cells. We repurposed the TLR9 agonist, CpG, commonly used in the clinic, to bolster T cell–B cell interactions during expansion for ACT. T cells expanded ex vivo from a CpG-treated culture demonstrated potent antitumor efficacy and prolonged persistence in vivo. This antitumor efficacy was accomplished without in vivo administration of TLR agonists or other adjuvants of high-dose interleukin (IL)-2 or vaccination, which are classically required for effective ACT therapy. CpG-conditioned CD8+ T cells acquired a unique proteomic signature hallmarkened by an IL-2Rα(lo)ICOS(hi)CD39(hi) phenotype and an altered metabolic profile, all reliant on B cells transiently present in the culture. Likewise, human TILs benefitted from expansion with CpG ex vivo, as they also possessed the IL-2Rα(lo)ICOS(hi)CD39(hi) phenotype. CpG fostered the expansion of potent CD8+ T cells with the signature phenotype and antitumor ability via empowering a direct B–T cell interaction. Isolated B cells also imparted T cells with the CpG-associated phenotype and improved tumor immunity without the aid of additional antigen-presenting cells or other immune cells in the culture.

Conclusions  Our results demonstrate a novel way to use TLR agonists to improve immunotherapy and reveal a vital role for B cells in the generation of potent CD8+ T cell-based therapies. Our findings have immediate implications in the clinical treatment of advanced solid tumors.

BACKGROUND

Only a decade ago, the treatment options for patients diagnosed with late-stage solid malignancies were mostly ineffective and patient outcomes were bleak. With the advent of immunotherapies, including checkpoint blockade and adoptive T cell transfer (ACT) therapy, a new era of cancer care is underway. Many patients with advanced cancer, like metastatic melanoma, experience objective responses, undergo long-term remissions and can sometimes be cured of their disease following delivery of T cell-based therapies.1 However, as some patients receive ACT as a last resort, after progressing on multiple lines of therapy, and only 20% experience durable complete responses, it is paramount to find ways to improve cell therapy.1 Host preconditioning with chemotherapy or radiotherapy is critical to the success of ACT. The preconditioning regimen promotes engraftment and antitumor activity of transferred T cells.2 Beyond depletion of host immune cells that compete for homeostatic cytokines and/or suppress transferred cells (T regulatory cells or myeloid-derived suppressor cells), host preconditioning also alters gut microbiota homeostasis.3-5 The systemic release of gut microbes and microbial products, via Toll-like receptor (TLR) ligands, in turn, activates the innate immune system and augments the effectiveness of infused T cells via pathogen recognition receptors.6

Additional insights from the microbiome field have led to improvements in immunotherapy, such as the use of TLR ligands to induce immunity.7,8 We and other groups have shown that TLR activation via synthetic microbial ligands improves ACT therapy when administered directly to mice.9,10 While these findings are important, delivering TLR agonists alongside ACT therapy may induce
toxic side effects in patients and delivering TLR ligands in vivo may induce unwanted effects on other immune cells or even tumor cells. Indeed, TLR agonists can profoundly augment immunotherapy, the safest and most effective ways to use them for ACT remain unknown.

CpG DNA agonists of TLR9 have been widely used in both preclinical and clinical settings in combination with various therapies (eg, vaccines, checkpoint blockade therapies, immune-agonistic antibodies, chemo/radiation) and as a monotherapy to induce antitumor responses (NCT03445533, NCT03618641, NCT03007732, NCT03831295, NCT03410901). Initially, several clinical trials reported activation of immune cells in patients with TLR9 agonist administration, but few individuals experienced a complete durable antitumor response. Three recent trials, however, reported patient responses (partial response and complete response) to TLR9 agonists in combination with either low-dose local irradiation of a single tumor site, pembrolizumab, or ipilimumab. Importantly, all three reports noted treatment-emergent adverse events (AEs) of grade 3 and/or 4 in a portion of the patients. In these and other reports, CpG is directly administered to the patient and the route and timing of treatment are likely important. CpG is typically administered locally to tumor lesions, either subcutaneously or intratumorally, in clinical trials to promote targeted action to the tumor and avoid bioavailability issues that arise with systemic intravenous injection. While local administration strategies such as these may be a valuable therapeutic option for some patients, they do somewhat limit the patient population to those with readily accessible tumor sites. Further, as severe AEs arise in many patients treated with combination therapies which include TLR9 agonist administration, determining the best way to exploit these agonists therapeutically while bypassing in vivo toxicity is paramount.

Herein, we developed a novel method in which CpG promotes efficacy of cell therapy without in vivo administration. We hypothesized that the efficacy of T cells could be improved by incorporating a TLR9 agonist into the ex vivo expansion protocol. This approach obviates the need to determine route and timing of CpG delivery and negates any unwanted off-target effects of CpG in vivo. ACT with CD8+ T cells expanded with CpG was effective without in vivo interleukin (IL)-2 and vaccine adjuvants, which are typically necessary. To our surprise B cells played an essential role in imprinting CD8+ T cells with potent immunity. Overall, we describe how addition of CpG in culture propagates CD8+ T cells which display overt immunity against solid tumors in vivo and reveal mechanisms underlying their potency.

METHODS

Mice and tumor cell lines

Pmel-1 TCR transgenic mice were purchased from the Jackson Laboratories and bred in the in-house animal facilities (comparative medicine department) at the Hollings Cancer Center of the Medical University of South Carolina (MUSC) or Emory University. C57BL/6 mice were purchased from Jackson laboratories for use in in vivo tumor experiment studies. Mice used for tumor experiments were between 6 and 10 weeks old. All animals were housed and underwent experimentation in accordance with the Institutional Animal Care and Use Committee (IACUC) at MUSC or Emory, and under the supervision and support of the Division of Laboratory Animal Resources at MUSC or the Division of Animal Resources at Emory. IACUC approval was obtained prior to all animal studies and procedures. B16F10 (H-2b) melanoma and B16F10 (H-2B) cell lines were gifted from Nicholas P. Restifo for use in in vivo tumor studies. Cell lines were confirmed pathogen and mycoplasma free prior to use in experimental studies.

T cell culture

Pmel-1 cells

Pmel-1 transgenic T cells were obtained via the culture of whole Pmel-1 splenocytes. Pmel-1 splenocytes were seeded at 1e6/mL in a 24-well plate and activated with 1μM human gp100 (hgp100) peptide (unless indicated otherwise) in the presence of 100IU/mL IL-2 (NIH repository). At the time of activation (unless otherwise stated) either mouse CpG-ODN 1668 (5′-tcctagacctcttgatgtc-3′) (class B) was added to the culture at 0.5μg/mL or vehicle control (endotoxin-free water) was added at a matched volume (InvivoGen). On day 2 of culture, half of the culture media was replaced with fresh media with 100IU/mL IL-2 in the new media. From day 3 of culture on cells were split to a concentration of 1e6 cells/mL and supplemented with fresh media containing 100IU/mL IL-2. On day 7 of culture, T cell cultures were assayed using flow cytometry and then directly used for in vivo tumor experiments where indicated.

CD8+ T cell negative isolation

For experiments in which T cells were purified from the bulk pmel-1 splenocytes before culturing we used the EasySep Mouse CD8+ T cell Isolation Kit (Stem Cell, Cat#: 19853A) following the manufacturer’s protocol. Prior to cell culture, the T cell isolate was assayed for the purity of the product which was always more than 90% pmel-1 transgenic T cells (identified via expression of the Vβ13 chain of the TCR).

In vitro blockade of soluble factors or CD40L

Pmel-1 splenocyte cultures were established. Prior to activating with hgp100 peptide, IL-2 (100IU/mL) and adding CpG or vehicle control, antibodies directed against IFN-γ (BioXCell BE0312), IL-6 (BioXCell BE0046), Timp-1 (R&D AF980), CXCL10 (R&D AF-466-NA), IL-10 (BioXCell BE0049) or relevant isotype controls (IgG1 (BioXCell BE0088), polyclonal Armenian hamster IgG (BioXCell BE0091), Normal Goat IgG (R&D AB-108-C)) were added to cell culture at 10μg/mL. Blocking antibody for CD40L (BioXCell BE0017-1) or isotype control Armenian
hamster IgG (BioXCell BE0091) antibody was added to cell culture at 25 µg/mL. Antibodies were added every day for the duration of cell culture and IL-2 was added at 100 IU/mL in fresh media whenever cells were split.

**Supernatant transfer**

Cell culture supernatant was collected from bulk pmel-1 splenocytes activated with hgp100 peptide, IL-2 and treated with vehicle or CpG at 7, and 48 hours post seeding, spun down to remove any cells, and stored at −20°C. Fresh pmel-1 splenocytes or isolated CD8⁺ T cells (Stem Cell, Cat# 19853A) were resuspended in supernatants, after supernatants were thawed and at room temperature. Additional IL-2 (100 IU/mL) and hgp100 were added to activate cell cultures and pmel-1 T cells were expanded for 7 days. Control cell cultures were established concomitantly by activating with hgp100 peptide, IL-2, and fresh CpG or vehicle control.

**Bypass antigen-presenting cells**

To bypass antigen-presenting cell (APC) mediated activation of pmel-1 T cells (via major histocompatibility complex (MHC) class I presentation of peptide to the TCR) we used bead-bound or plate-bound antibodies. αCD3/αCD28 beads from Gibco (DynaBeads 11453D) were added at the start of culture at 1:1 ratio of beads to pmel-1 cells. Cell culture plates were coated overnight with αCD3 alone or αCD3 and αCD28 (10 µg/mL αCD3 and 2 µg/mL αCD28) in sterile phosphate-buffered saline (PBS) at 4°C and used the next day for cell culture after gently washing the wells with sterile PBS. Control pmel-1 cultures were activated with hgp100 peptide and all groups received 100 IU/mL IL-2 and CpG or vehicle control as in previous assays.

**Immune cell subset depletion**

From the bulk pmel-1 splenocytes we depleted either CD4⁺ T cells, natural killer (NK) cells, dendritic cells (DCs), macrophages, or B cells using subset marker-specific PE labeling followed by anti-PE microbead targeting and positive selection via column isolation. The negative fraction was used for culture as it does not contain the positively labeled cell subset. Briefly, pmel-1 splenocytes are labeled with PE-conjugated antibodies: either αCD4-PE, αNK1.1-PE, αCD11c-PE, αF4/80-PE, or αCD19-PE (antibodies listed in table 1) to mark CD4⁺ T cells, NK cells, DCs, macrophages, or B cells, respectively. Cell mixtures were then further labeled with Anti-PE MicroBeads from MACS Miltenyi Biotec (130-048-801) and then underwent magnetic separation with the MACS LS Columns (130-042-401) according to the manufacturer’s instructions. Both positive and negative fractions were collected into 15 mL tubes, and assayed via flow cytometry for the presence of the PE-conjugated cell specific marker as well as another secondary marker, when applicable, to identify the presence of each subset in each fraction. The average per cent depletion of each subset or dual depletion was as follows: 97.6, 94.4, 99.8, 98.8, 82.2, and 96.9 of F4/80⁺ macrophages, CD11c⁺ DCs, CD19⁺ B cells, CD4⁺ T cells, NK1.1⁺ NK cells and F4/80⁺ macrophages/CD11c⁺ DCs dual depletion, respectively. The negative fraction was cultured as described above, using hgp100 peptide activation, 100 IU/mL IL-2, vehicle or CpG treatment on day 0. Bulk pmel-1 splenocytes were cultured in the same manner as controls.

**B cell negative isolation**

B cells were negatively isolated using the EasySep Mouse B cell Isolation Kit (Stem Cell, Cat# 19854) following the manufacturer’s instructions. B cell purity was assayed post isolation and prior to cell culture with purified CD8⁺ T cells. In recombination experiments, CD8⁺ T cells were cultured with B cells at a 1:4 T:B cell ratio and activated and expanded in culture as described above.

**Human oral cavity squamous cell carcinoma (OCSCC) tumor infiltrating lymphocyte culture**

Our tumor infiltrating lymphocyte (TIL) culture method was adapted from the method reported by Dudley et al.25 Briefly, tumor specimen was collected fresh from a patient with oral cavity squamous cell carcinoma and cut into 1–3 mm² fragments before seeding in complete media (RPMI) supplemented with 6000 IU/mL recombinant IL-2 (NCI repository). At the time of seeding three tumor fragments were additionally treated with either vehicle control (endotoxin-free water) or CpG ODN 2006 (InvivoGen) at 0.5 µg/mL. Tumor fragments were left untouched for 5 days in culture to allow cell egress from the tumor piece. After 5 days 1 mL/well (24-well plate) was removed without disturbing the cells settled on the bottom of the well and replaced with fresh media with 6000 IU/mL IL-2. Cells were monitored and media was replaced every 3–5 days. Once cells became confluent in the well, the tumor piece was removed and cells were replated and maintained at 1–1.5×10⁶ cells/mL in a 24-well plate.

**Adoptive cell therapy**

Tumor cells (B16F10 or B16F10 KVP) cells from culture were washed twice, resuspended in sterile PBS, and injected subcutaneously on the abdomen at 5×10⁵ cells/mouse (in 200 µL). Tumors were established for 5 days (unless otherwise noted in the figure legend) in vivo prior to ACT and mice were irradiated with 4–5 Gy total body irradiation the day before ACT. Prior to treatment, mice were randomized according to tumor size to distribute tumor size evenly among groups. Average tumor size for parental B16F10 tumors was 25 mm² among mice on the day of cell therapy (D0) unless otherwise indicated in the figure legend. If no tumor was detectable 4 days post tumor cell injection, mice were not included in the study. 5×10⁶ pmel-1 cells expanded 1 week in vitro were resuspended in sterile PBS and transferred via tail vein injection (unless cell number noted otherwise in the figure legend). Starting from day 0 of ACT, tumors were measured 2x per week with handheld calipers by a laboratory member blinded to the treatment.
Mouse blood and tissue collection

Peripheral blood

Mouse peripheral blood was collected at the indicated time points post ACT from the mandibular vein into 1.5 mL Eppendorf tubes containing 0.125 M EDTA. All blood was pelleted, and red blood cells were lysed using

group until tumors reached protocol endpoint (400 mm²). Mice euthanized prior to endpoint for tissue collection and biodistribution analysis were identified and allocated prior to commencing treatment.

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APC, antigen-presenting cell; IFN, interferon; IL, interleukin; TLR, Toll-like receptor.
RBC Lysis buffer (Biolegend). Immune cells were then assayed via flow cytometry.

Tumor collection
Tumors were excised and placed in culture media on ice until tissue dissociation. Tumor single cell suspensions were acquired using the MACS mouse Tumor Dissociation Kit (130-096-730), following the manufacturer’s instructions, and the gentle MACS Octo Dissociator with Heaters. The single cell suspension was washed with FACs buffer (PBS+2% H1-FBS) and filtered over a 70µm screen and collected into 50mL tubes. Cell suspensions were further filtered through a 40µM filter prior to flow cytometry analysis to prevent clumping. Vβ13,CD8+ or Thy1.1+ staining was used to detect pmel-1 donor CD8+ T cells in the peripheral blood and tissues.

Proteomics
Sample preparation
Four biological replicates of day 7 T cells from vehicle or CpG-treated cultures were collected, washed with sterile PBS, and pelleted. Cells were lysed in 9M urea, 50mM Tris pH 8, with 100 units/mL Pierce Universal Nuclease (Thermo Scientific) added. The concentration of protein was measured using the BCA assay (Thermo Scientific). Fifty micrograms of protein from each sample were brought to the same final volume using the lysis buffer and collected into 50mL tubes. Cells suspensions were further filtered through a 40µM filter prior to flow cytometry analysis to prevent clumping. Vβ13,CD8+ or Thy1.1+ staining was used to detect pmel-1 donor CD8+ T cells in the peripheral blood and tissues.

Liquid chromatography and mass spectrometry data acquisition parameters
Dried peptides were dissolved in 15µL of 2% acetonitrile (ACN)/0.2% formic acid (FA) and 5µL of this was injected. Peptides were separated and analyzed on an EASY nLC 1200 System (Thermo Scientific) in line with the Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific) with instrument control software V.4.2.28.14. Peptides were pressure loaded at 1180 bar, and separated on a C18 reversed phase column (Acclaim PepMap RSLC, 75µm×50cm (C18, 2µm, 100Å) (Thermo Fisher) using a gradient of 2%–35%B in 180min (Solvent A: 0.1% FA, 2% ACN; Solvent B: 80% ACN/0.1% FA) at a flow rate of 300nL/min. The column was thermostatted at 45°C. Mass spectra were acquired in data-dependent mode with a high resolution (60,000) Fourier transform mass analyzer (FTMS) survey scan, mass range of m/z 375–1575, followed by tandem mass spectra (MS/MS) of the most intense precursors with a cycle time of 3s. The automatic gain control target value was 4.0e5 for the survey scan. Fragmentation was performed with a precursor isolation window of 1.6m/z, a maximum injection time of 50 ms, and higher-energy collisional dissociation (HCD) collision energy of 35%; the fragments were detected in the Orbitrap at a 15,000 resolution. Monoisotopic-precursor selection was set to ‘peptide’. Apex detection was not enabled. Precursors were dynamically excluded from resequencing for 15s with a mass tolerance of 10 ppm. Advanced peak determination was not enabled. Precursor ions with charge states that were undetermined, 1, or >7 were excluded from fragmentation.

Mass spectrometry data processing
Protein identification and quantification were extracted from raw LC-MS/MS data using the MaxQuant platform V.1.6.3.3 with the Andromeda database searching algorithm and label-free quantification (LFQ) algorithm.26–28 Data were searched against a mice Uniprot reference database UP000005898 with 54425 proteins (April, 2019) and a database of common contaminants. The false discovery rate (FDR), determined using a reversed database strategy, was set at 1% at the protein and peptide level. Fully tryptic peptides with a minimum of seven residues were required including cleavage between lysine and proline. Two missed cleavages were permitted. The LFQ feature was on with ‘Match between runs’ enabled for those features that had spectra in at least one of the runs. The ‘stabilize large ratios’ feature was enabled, and ‘fast LFQ’ was disabled. The first search was performed with a 20ppm mass tolerance; after recalibration, a 4.5 ppm tolerance was used for the main search. A minimum ratio count of 2 was required for LFQ protein quantification with at least one unique peptide. Parameters included static modification of cysteine with carbamidomethyl and variable N-terminal acetylation and oxidation of methionine. The protein groups text file from the MaxQuant search results was processed in Perseus V.1.6.8.0.29 Identified proteins were filtered to remove proteins only identified by a modified peptide, matches to the reversed database, and potential contaminants. The normalized LFQ intensities for each biological replicate were log2 transformed. Quantitative measurements were required in at least two out of three biological replicates in at least one of the treatment groups. Gene ontology and Reactome pathway annotations were added from the murine Uniprot reference proteome. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE30 partner repository with the data set identifier PXD022909. Principal component analysis (PCA) analysis was performed in Perseus. A volcano plot was generated in Perseus using a significance threshold of a two-sided t-test adjusted p value (<0.05 FDR) and a S0 parameter of 0.1. Protein set enrichment analysis was performed using the ToppFun function of the ToppGene Suite (https://toppgene.cchmc.org/enrichment.jsp), where the proteins increased significantly (two-sided
RESULTS

Potent antitumor T cells are generated with ex vivo CpG stimulation

We hypothesized that the TLR9 agonist, CpG, could be used ex vivo to augment T cell-based antitumor therapy. To test this idea, we employed the pmel-1 mouse model of ACT in which CD8+ T cells express a transgenic TCR that recognizes the gp100 epitope expressed by melanoma and healthy melanocytes.\(^2\) On day 0, CpG or vehicle (endotoxin-free water) was added to whole pmel-1 splenocytes at the time of TCR stimulation with 1 µM hgp100 peptide. The culture was expanded in the presence of IL-2 for 1 week to preferentially expand pmel-1 CD8+ T cells (henceforth referred to as pmel-1) to >95% purity. After 1 week of expansion, pmel-1 were infused into lymphodepleted mice bearing established B16F10 melanomas (figure 1A). Pmel-1 expanded with CpG were remarkably therapeutic in vivo, as CpG-treated pmel-1 regressed melanoma in mice while vehicle-treated pmel-1 treatment had little therapeutic impact (figure 1B). Mice infused with CpG pmel-1 survived significantly longer than mice given vehicle pmel-1 or mice left untreated (figure 1C). Moreover, pmel-1 engrafted and persisted at higher frequencies in the blood and periphery of mice if they were expanded in the presence of CpG ex vivo (figure 1D,E). Of note, this robust response was achieved without coadministration of IL-2 or vaccine adjuvants in vivo—previously deemed necessary for durable immunity via pmel-1 ACT.\(^3\) We corroborated these findings in another model: a neoantigen TIL therapy model developed by Hanada et al.\(^4\) In this model B16F10 cells expressed a modified version of gp100 in which three amino acids of the protein epitope have been replaced making the protein resemble the human version and thus confer higher affinity to the pmel-1 CD8+ T cell receptor when presented on MHC class 1 (online supplemental figure S1A). In this model CpG expanded cells mediated robust and sustained antitumor immunity against large tumors (~100 mm\(^2\)) (online supplemental figure S1B). While traditional cell therapy controlled tumors transiently, the tumors eventually relapsed. In contrast, no mice treated with a CpG-expanded CD8+ T cell therapy relapsed and survival was still 100% in this group over 60 days post T cell infusion (online supplemental figure S1C). Thus, adoptively transferred CD8+ T cells can mediate remarkable responses to tumors and robustly persist in vivo when expanded ex vivo with CpG.

In vitro CpG stimulation generates T cells with a unique proteomic signature

As stark differences in antitumor activity were observed in vivo, we hypothesized that CpG treatment leads to the expansion of a CD8+ T cell product phenotypically distinct from conventionally expanded cells. To broadly assess how CD8+ T cells expanded from a CpG-treated culture were different from traditionally expanded cells, we compared these T cell products using proteomic analysis. As portrayed in figure 2A, proteins were extracted,
purified and change in protein abundance from CpG treatment were determined using label-free LC-MS/MS-based proteomics of day 7 CD8⁺ T cells. Not surprisingly, the proteomic profile of vehicle and CpG-expanded CD8⁺ T cells were clearly divergent based on PCA (figure 2B). Of over 2000 proteins identified between vehicle and CpG-expanded T cells, 77 proteins were significantly different between the 2 groups (Student t-test with permutation-based FDR cut-off of 0.01 and S0=0.1) (figure 2C). Several proteins involved in the processing of fatty acids (ACADLI, ACAD9, CLYBL) and GO molecular functions related to fatty acid oxidation were enriched in CpG-derived pmel-1 (figure 2C-D). There was also a marginally greater population of MitoTracker+TMRM+.
**Figure 2**  In vitro CpG stimulation generates T cells with a signature phenotype which is maintained post ACT. (A) Design of proteomic analysis of T cells; vehicle or CpG-expanded T cells were collected on day 7 of culture (n=4 mice/group), subjected to protein extraction and purification, and then analyzed using LC-MS/MS. (B) Principle component analysis of vehicle or CpG-generated pmel-1 T cell proteomes. (C) Volcano plot comparing protein expression between vehicle and CpG-expanded T cells. (D) Top 5 GO: molecular functions enriched from proteins expressed more in CpG-generated pmel-1 T cells compared with vehicle-generated cells (using https://toppgene.cchmc.org/enrichment.jsp). (E) Representative flow cytometry histogram (left) and biological replicates (right) of extracellular expression markers from day 7 of cell culture of vehicle or CpG-treated pmel-1. (F) MFI of ICOS (top) or CD39 (bottom) of donor pmel-1 cells in the blood on day 4 or day 12 post ACT. (G) Percentage of human OCSCC TIL expressing IL-2Rα and ICOS, but not CD39 after expansion with CpG. (H) Diagram of CpG-generated T cell characteristics. Statistics: (C) significance threshold was set using two-sided t-test adjusted p value (<0.05 FDR) and a S0 parameter of 0.1. (E) Two-sample t-test, (F) Mann-Whitney U test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. ACT, adoptive T cell transfer; CoA, coenzyme A; FDR, false discovery rate; GO, gene ontology; ICOS, inducible T cell Ccstimulator; IL, interleukin; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MFI, mean fluorescence intensity; ns, not significant; OCSCC, oral cavity squamous cell carcinoma; TBI, total body irradiation; TIL, tumor infiltrating lymphocytes.
pmel-1 TIL, in mice treated with a CpG-derived product compared with mice that received a vehicle derived pmel-1 (online supplemental figure S2C). However, the most markedly increased protein in pmel-1 expanded with CpG was IL-2Rα (figure 2C). As IL-2Rα is required to form the high-affinity receptor for the T cell growth factor IL-2, this protein may be important for the anti-tumor function of CpG-generated T cells.

We validated the expression of IL-2Rα and further investigated the T cell phenotype of CpG pmel-1. We screened more than 20 phenotypic surface markers using flow cytometry to test which proteins were induced in pmel-1 cultures treated with CpG in vitro (online supplemental figure S2A). In agreement with our proteomics analysis, CpG-expanded pmel-1 expressed heightened IL-2Rα (figure 2E). In addition, we found the inducible T cell costimulator (ICOS) was expressed to a higher degree on the surface of CpG-generated CD8+ T cells. IL-2Rα and ICOS have been reported to augment T cell engraftment and function.33–35 CpG pmel-1 also expressed less CD39, an immunosuppressive ectoenzyme expressed on exhausted CD8+ T cells,36 37 than vehicle pmel-1. Thus, when expanded in the presence of CpG, pmel-1 cells display a signature phenotype (IL-2RαhighICOShighCD39low) (figure 2E). Note, in experiments henceforth, we tracked and used this signature profile as a metric to predict the effect of CpG on pmel-1 CD8+ T cells. Importantly, expression of the signature markers is maintained post ACT as heightened ICOS and diminished CD39 remained characteristic of the CpG-treated donor cells persisting in the peripheral blood as well as the tumor (figure 2F and online supplemental figure S2B). IL-2Rα could not be detected on transferred cells from either group in vivo, possibly due to cytokine binding or rapid receptor cycling from the surface. Further, tumor-infiltrating CpG-generated CD8+ T cells had reduced expression of other markers of suppression or exhaustion including programmed cell death protein 1 (PD-1), lymphocyte activation gene 3 protein (LAG-3), and T cell immunoglobulin and mucin domain-3 (TIM-3), but similar expression of Granzyme B (online supplemental figure S2B). Systemically, inflammatory cytokines including interferon (IFN)-γ and IL-6 were also higher in the plasma of mice that received CpG pmel-1 therapy, indicative of a heightened immune response (online supplemental figure S2D).

To determine if this phenotype would be recapitulated in human cells we cultured TIL from a patient with oral cavity squamous cell carcinoma with human CpG or vehicle at the start of TIL seeding in culture. Similar to their mouse counterparts, both human CD4+ and CD8+ TIL had larger proportions of ICOSIL2RαCD39 T cells post ex vivo culture with CpG (figure 2G and online supplemental figure S2E). Thus, both mouse and human T cells gain IL-2Rα and ICOS, and lose CD39 after CpG treatment ex vivo (figure 2H). While the functional significance of this signature phenotype on CpG-generated T cell products remained incompletely elucidated, individually each marker has been associated with more or less fit T cells. Thus, we turned our attention to uncovering how addition of CpG to cell cultures could bolster T cell potency.

**CpG treatment indirectly alters the CD8+ T cell phenotype and improves tumor immunity**

We first questioned whether CpG was directly acting on pmel-1 T cells or if CpG activates other immune cells present at the onset of the culture, imprinting pmel-1 T cells with the signature phenotype and antitumor capacity. Using the online database, ImmGen, we queried TLR9 expression via available RNA sequencing data sets and found that many B cell, DC, and myeloid subsets express TLR9 transcripts (online supplemental figure S3A). Conversely, T cell subsets, from both healthy mice and those recently exposed to lymphocytic choriomeningitis virus (LCMV), express trace or undetectable TLR9 transcripts in the mouse (online supplemental figure S3A). In our mouse model, we corroborated these findings; TLR9 is not expressed in pmel-1 CD8+ T cells but is expressed by professional APCs, which are present only at the start of the TCR-stimulated culture (online supplemental figure S3B). Thus, pmel-1 likely acquire their unique phenotype (IL-2RαhighICOShighCD39low) after CpG conditioning via TLR9-mediated activation of APCs. The TLR9-expressing APCs in the spleen—B cells, macrophages, and DCs—are present only at the start of culture. These APCs rapidly diminish while CD8+ T cells clonally expand due to peptide-mediated triggering of the pmel-1 TCR. Thus, 3 days after adding peptide to the pmel-1 culture, few APCs remain (not shown).

Using this kinetic information, we designed an experiment to determine whether CpG can act directly on the T cells to confer the signature phenotype and improved tumor immunity. CpG was added at the start of culture on day 0 (early CpG) when APCs and T cells are present, or CpG was added on day 3 (late CpG) when pmel-1 CD8+ T cells dominated the majority of the culture (figure 3A). Pmel-1 CD8+ T cells were assayed for surface IL-2Rα, ICOS, and CD39 1 week after expansion. As anticipated, pmel-1 treated with CpG early expressed high IL-2Rα and ICOS and low CD39 1 week after expansion (figure 3B). Conversely, when CpG was added on day 3 of culture when nominal APCs remained, Late CpG pmel-1 expressed these surface markers to the same extent as vehicle-treated pmel-1 (figure 3B). Early CpG pmel-1 persisted in the blood and tumor and improved therapeutic outcomes and survival (figure 3C-F). However, Late CpG pmel-1 failed to persist in mice and did not improve survival (figure 3D-F).

Our data suggested that either the APCs present at the start of culture or the presence of CpG at priming were critical to the effects it imparted to antitumor CD8+ T cells. Therefore, we performed another experiment to determine if CpG could act directly on pmel-1 in the absence of APCs. Purified pmel-1 CD8+ T cells (negative isolation, >90%) were activated and expanded in the
absence or presence of CpG; cells expanded logarithmically as expected (online supplemental figure S4A). As anticipated, purified pmel-1 CD8+ T cells did not gain the signature phenotype or enhanced antitumor efficacy when expanded in the presence of CpG (online supplemental figure S4B,C). Moreover, the engraftment and persistence of enriched pmel-1 T cells treated with CpG were poor (online supplemental figure S4D). Taken

Figure 3  CpG treatment indirectly alters the CD8+ T cell phenotype and improves tumor immunity. (A) Schema of experimental design; pmel-1 splenocytes were treated with CpG at the time of activation (early) or 3 days post activation (late) with peptide or treated with vehicle at the time of activation. (B) Phenotypic marker expression on day 7 of culture (n=3 biological replicates). (C) Tumor area over time (single curves represent one mouse) treated with NT, vehicle pmel-1, early CpG pmel-1, or late CpG pmel-1. Single curves are combined from two individual experiments; n=9–12 mice/group. (D) Survival of mice in (C). (E) Percentage of donor cells in the blood of mice from (C) on day 7 and day 14 post ACT. (F) Percentage of donor cells in the tumor of mice treated in (C) on day 21 post ACT. Statistics: (B) Two-sample t-test. (D) Log-rank test. (E, F) Mann-Whitney U test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. ACT, adoptive T cell transfer; ICOS, Inducible T Cell Costimulator; IL, interleukin; MFI, Mean Fluorescence Intensity; NT, No Treatment.
together, these findings indicate that CpG does not modulate T cell phenotype directly. Instead, we reasoned that the CpG-mediated effects occur via other APCs present in the culture. Therefore, we questioned whether CpG improved direct or indirect immune cell interactions in the culture to drive the expansion of potent antitumor CD8+ T cells.

**Soluble factors are not required for the CpG-improved APC—CD8+ T cell interaction**

Specific cytokines secreted by innate immune cells in culture can skew T cell phenotypes. The phenotype we observed in our CpG-treated cells recapitulated, in part, a report of pmel-1 conditioned with IL-12.38 Thus, we hypothesized that CpG treatment induced the secretion of pro-inflammatory cytokines and chemokines in the culture by TLR9 expressing immune cells and that these soluble factors were responsible for promoting the pmel-1 CD8+ T cell phenotype, IL-2Ra\textsuperscript{high}ICOS\textsuperscript{high}CD39\textsuperscript{low}.

To first get a broad idea of the soluble factors induced post-CpG treatment, we collected and froze supernatant at 48 hours after treatment with CpG or vehicle. Supernatant was analyzed for the presence of 44 chemokines and cytokines via a multiplex array (Eve Technologies). Many cytokines and chemokines were increased in the supernatant of CpG-treated cultures on day 2 post treatment compared with vehicle treated cells (online supplemental figure S5A,B). To determine if the most abundant factors were responsible for skewing the CpG-associated phenotype, we blocked the top hits associated with CpG. We individually targeted the top five proteins from our array, which included IFN-\(\gamma\), IL-6, TIMP-1, CXCL10, and IL-10, during cell culture. Each blocking antibody (or isotype control) was added immediately prior to the addition of hgp100 peptide and vehicle or CpG on day 0 and daily thereafter for the duration of culture. To our surprise, CpG-generated CD8+ T cells retained their IL-2Ra\textsuperscript{high}ICOS\textsuperscript{high}CD39\textsuperscript{low} phenotype independent of blockade of any of these targets, suggesting that these soluble factors were not required for the effects of CpG on T cells (online supplemental figure S5C). As mentioned previously, some of our phenotype was similar to that of IL-12 conditioned T cells, thus we tested IL-12 blockade as well, but this cytokine was also dispensable for the signature phenotype (not shown).

However, in criticizing our approach to this question, we noted that perhaps individual cytokines and chemokines might be insufficient to drive CpG-mediated effects on donor antitumor CD8+ T cells. Instead, we hypothesized that a collection of these factors, in the supernatant was analyzed for the presence of 44 chemokines and cytokines in the culture by TLR9 expressing immune cells and that these soluble factors were responsible for promoting the pmel-1 CD8+ T cell phenotype, IL-2Ra\textsuperscript{high}ICOS\textsuperscript{high}CD39\textsuperscript{low}.

To first get a broad idea of the soluble factors induced post-CpG treatment, we collected and froze supernatant at 48 hours after treatment with CpG or vehicle. Supernatant was analyzed for the presence of 44 chemokines and cytokines via a multiplex array (Eve Technologies). Many cytokines and chemokines were increased in the supernatant of CpG-treated cultures on day 2 post treatment compared with vehicle treated cells (online supplemental figure S5A,B). To determine if the most abundant factors were responsible for skewing the CpG-associated phenotype, we blocked the top hits associated with CpG. We individually targeted the top five proteins from our array, which included IFN-\(\gamma\), IL-6, TIMP-1, CXCL10, and IL-10, during cell culture. Each blocking antibody (or isotype control) was added immediately prior to the addition of hgp100 peptide and vehicle or CpG on day 0 and daily thereafter for the duration of culture. To our surprise, CpG-generated CD8+ T cells retained their IL-2Ra\textsuperscript{high}ICOS\textsuperscript{high}CD39\textsuperscript{low} phenotype independent of blockade of any of these targets, suggesting that these soluble factors were not required for the effects of CpG on T cells (online supplemental figure S5C). As mentioned previously, some of our phenotype was similar to that of IL-12 conditioned T cells, thus we tested IL-12 blockade as well, but this cytokine was also dispensable for the signature phenotype (not shown).

However, in criticizing our approach to this question, we noted that perhaps individual cytokines and chemokines might be insufficient to drive CpG-mediated effects on donor antitumor CD8+ T cells. Instead, we hypothesized that a collection of these factors, in the supernatant of CpG-treated cultures, were causing the acquisition of the signature phenotype. Thus, we next performed a supernatant transfer experiment. For this experiment, we froze supernatant from CpG or vehicle treated bulk pmel-1 splenocytes at 7 and 48 hours post activation with hgp100 peptide. Stored supernatant was then used to stimulate fresh bulk pmel-1 splenocytes or CD8+ isolated pmel-1 T cells. As controls, we also stimulated bulk pmel-1 splenocytes or CD8+ isolated pmel-1 T cells directly with CpG or vehicle to ensure that CpG had the typical effect on the pmel-1 CD8+ T cells generated from a bulk culture (online supplemental figure S5D). Each group was then grown for 7 days in culture and assayed for surface phenotypic markers. We suspected that bulk pmel-1 splenocytes treated directly with CpG would generate CD8+ T cells with the IL-2Ra\textsuperscript{high}ICOS\textsuperscript{high}CD39\textsuperscript{low} phenotype while CD8 purified T cells would not acquire this phenotype when treated with CpG. Similarly, we thought that supernatant collected at the early time point from the culture (7 hours), when CpG is likely still available, would confer the phenotypic alterations when transferred to bulk pmel-1. However, we posited that supernatant collected at a later time (48 hours) from CpG-treated cultures, would likely be depleted of CpG and thus not induce the typical changes to CD8+ T cells derived from bulk pmel-1 cultures. In contrast to when CpG is added directly to purified T cells, we thought that transferring supernatant from CpG-treated cultures, which is rich in cytokines and chemokines would expand CD8+ T cells with the IL-2Ra\textsuperscript{high}ICOS\textsuperscript{high}CD39\textsuperscript{low} phenotype. CD8+ pmel-1 T cells from bulk splenocytes acquired the CpG associated phenotype after transfer of CpG-supernatant that was collected at the early time point (7 hours), but not the late time point (48 hours). However, no supernatant transfer conditions reproduced the signature phenotype on purified CD8+ T cells (online supplemental figure S5E). Additionally, supernatant from vehicle treated cultures did not induce IL-2Ra\textsuperscript{high}ICOS\textsuperscript{high}CD39\textsuperscript{low} CD8+ T cells under any conditions (online supplemental figure S5E). This finding indicates that cytokines and chemokines secreted as a result of CpG treatment do not support the generation of CD8+ T cells with high expression of IL-2Ra and ICOS and low expression of CD39, suggesting the CpG facilitated interaction between T cells and APCs in culture is not mediated by a soluble factor.

**A direct interaction between T cells and APCs is critical for the CpG induced effects**

As secreted factors did not appear to foster the CpG-associated phenotype in antitumor T cells, we hypothesized the direct interaction between APCs and pmel-1 T cells in culture was required. Initially, we tested this idea by blocking a costimulatory pair critical for bolstering B and CD4+ T cell interactions: the CD40/CD40L pathway. However, blocking this pathway did not impact the outgrowth of CD8+ T cells with the CpG signature phenotype nor did it hinder their in vivo potency (online supplemental figure S6A-C). Thus, we next tested this ‘interface’ idea using a series of experiments designed to bypass the direct interaction between T cells and APCs during antigen presentation. Specifically, we activated pmel-1 cultures with one of the following strategies: (1) hgp100 peptide (positive control—mediates B–T cell communication via MHC-I-TCR signaling), (2) plate-bound anti-CD3 (bypasses MHC-I-TCR signaling), (3) plate-bound...
anti-CD3/anti-CD28 (bypasses MHC-I-TCR signaling), or (4) bead-bound anti-CD3/anti-CD28 (bypasses MHC-I-TCR interactions) in the presence or absence of CpG (figure 4A). After 7 days of expansion, pmel-1 cells activated with any strategy that bypassed the use of an APC, did not acquire the same phenotype as those activated via APCs and hgp100 peptide when in the presence of CpG (IL-2RαhighICOShighCD39low). Instead, when plate-bound or bead-bound antibodies were used in place of peptide activation, the phenotype was similar between vehicle and
CpG-expanded T cells (figure 4B-C and online supplemental figure S7A).

Based on this finding, we questioned if bypassing the APC in vitro would also abrogate the in vivo antitumor efficacy of CpG-generated T cells. Indeed, we found that the antitumor efficacy gained with CpG treatment of peptide activated cells was not recapitulated when CpG was used alongside CD3/CD28 beads, plate-bound CD3/CD28 or plate-bound anti-CD3 activation (figure 4D and online supplemental figure S7B). Mice that received cell therapies derived from any strategy in which the MHC-I-TCR interaction was bypassed had significantly reduced survival compared with those who received T cells that were activated with peptide and CpG (figure 4E and online supplemental figure S7C). Though the engraftment and persistence of the donor T cells were bolstered in mice which received the peptide/CpG stimulated culture product, neither metric was enhanced in mice that received a product generated in the presence of CpG when the APCs were bypassed (figure 4F). Together, these findings indicate an intimate, direct, interaction between APCs and T cells in CpG-treated cultures drive the expansion of potent CD8+ T cells.

The phenotype of CpG-generated CD8+ T cells is largely dependent on B cells

While many different immune cells are present in the early stages of CD8+ TIL cultures, intracellular TLR9 expression is limited to B cells, DCs, and macrophages in the mouse (online supplemental figure S3B). Thus, we next sought to identify which APC was important for generating pmel-1 with the signature phenotype associated with CpG-promoted tumor immunity. We hypothesized that DCs were critical, as they are potent TLR9+ APCs that secrete cytokines and express heightened MHC I and II molecules when activated with CpG.39–42

To address this question, B cells, DCs, or macrophages were individually depleted or DCs and macrophages were dually depleted from the starting cell culture prior to expansion for 7 days and phenotypic analysis. As additional controls, we also removed CD4+ T cells or NK cells before the start of culture as these populations are also present in pmel-1 splenocytes. In contrast to our hypothesis, depleting DCs from the CpG-treated cell culture did not hinder the outgrowth of pmel-1 with the signature phenotype. Instead, only B cell depletion prevented the expansion of IL2RαhighICOShighCD39low pmel-1 CD8+ T cells (figure 5A). Indeed, whether macrophages, CD4+ T cells, or NK cells were depleted, CpG treatment could still propagate IL2RαhighICOShighCD39low pmel-1 cells (figure 5A and online supplemental figure S8B). Dual depletion of DCs and macrophages did not disrupt the acquisition of the IL2RαhighICOShighCD39low phenotype with CpG expansion (online supplemental figure S8F).

To examine global changes in CpG-expanded pmel-1 on depletion of B cells, we used proteomics to determine specific characteristics of CpG-expanded T cells in the presence or absence of B cells. We included traditionally-expanded T cells (bulk vehicle) as a control to see whether removing B cells essentially reverts the phenotypic effects of CpG to that of a vehicle-derived population. By PCA, the ACT products (vehicle, CpG-expanded, or CpG-expanded CD19 depletion) did not overlap, indicating that each of these groups was distinctly different from one another at the proteomic level (figure 5B). We next tested if depleting B cells results in the expansion of a cell product that more closely resembles the ineffective vehicle-derived CD8+ T cells. We found that a majority of the proteins which were significantly enriched in vehicle-expanded pmel-1 were also more abundant in CD19-depleted CpG-treated cells compared with T cells expanded from a bulk culture treated with CpG (figure 5C). Under certain circumstances, several of these proteins that were abundant in both the bulk vehicle and CD19-depleted CpG expanded pmel-1 have been associated with highly activated or terminally exhausted T cells including: GZMB, TNFRSF9, and PIK3CD.43 44 However, these same proteins can also promote activation, costimulation, and cytotoxic function of T cells. These results indicate that CD19+ B cells, in the context of CpG, drive broad phenotypic alterations in T cell products, but do not reveal whether B cells are critical for the potent tumor immunity seen after expansion with CpG.

Removal of B cells from the culture ablates the improved antitumor efficacy with CpG

Since B cells were critical to the CpG-mediated phenotypic impact on CD8+ T cells, we hypothesized that B cells would be similarly critical for the antitumor activity of CpG-expanded pmel-1. To test this idea, we depleted B cells, CD11c+ DCs, F4/80+ macrophages, CD4+ T cells or NK1.1+ NK cells from the starting culture. These cultures were then peptide activated with or without the addition of CpG, expanded and adoptively transferred into tumor-bearing mice (figure 6 and online supplemental figure S8). We found that when B cells were removed from the culture, CpG pmel-1 cells were as ineffective as vehicle pmel-1 cells at regressing tumors in vivo (figure 6A). Conversely, when DCs, macrophages, CD4+ T cells, or NK cells were individually depleted, the therapeutic efficacy of CD8+ T cells with CpG treatment was maintained (figure 6A and online supplemental figure S8C). We also depleted DCs and macrophages concomitantly and found that CpG expanded cells still improved the survival of mice (online supplemental figure S8G). No survival advantage was achieved in mice treated with CpG pmel-1 that were expanded in the absence of B cells ex vivo (figure 6B). Engraftment and persistence of pmel-1 in the blood were improved if cultures were treated with CpG, even in the absence of DCs, macrophages, NK or CD4+ T cells, at the start of culture (figure 6C and online supplemental figure S8E). In contrast, the engraftment and persistence of CpG-treated pmel-1 cultures depleted of B cells were poor (figure 6C). These findings uncover
Figure 5  The phenotype of CpG-generated CD8⁺ T cells is largely dependent on B cells. (A) Histograms (left) and biological replicates (n=6) (right) of the expression of signature phenotype markers on the cell surface on day 7. T cells were expanded from bulk, F4/80-depleted, CD11c-depleted, or CD19-depleted cell cultures treated with vehicle control or CpG. (B) Principle component analysis of proteomic signatures of day 7 expanded T cells derived from a bulk vehicle or CpG-treated culture, or a CD19-depleted CpG-treated culture (n=4 biological replicates). (C) Heatmap comparing Z scores (of the Log2 protein intensity value) from the T cells expanded from the same groups as in (B). Displayed are proteins that were expressed significantly higher (determined using two-sided t-test adjusted p value (false discovery rate <0.05) and an S0 parameter of 0.1) in bulk vehicle generated T cells compared with bulk CpG-generated T cells. Statistics: (A) Mann-Whitney U test.*p<0.05, **p<0.01. ICOS, Inducible T Cell Costimulator; IL, interleukin; MFI, Mean Fluorescence Intensity; ns, not significant; SCR, scramble ODN.
a key role for TLR9-activated B cells in generating CD8+ T cells with potent antitumor immunity.

**B cells alone are sufficient to improve antitumor T cells after activation via TLR9**

We assumed purified B cells could enhance pmel-1 CD8+ T cells for ACT via CpG activation of TLR9, but it remained unknown if the same extent of tumor immunity would be mediated if B cells were the sole APC. Thus, we examined the signature phenotype and antitumor properties of CpG-generated cells in bulk and purified B cell/T cell co-cultures. As seen in bulk cultures treated with CpG, when CD8+ T cells were expanded from a co-culture of CD8+ T cells...
and B cells treated with CpG, T cells also expressed more IL2Rα and ICOS. CD39 expression was similar between both CpG and vehicle treated groups in the purified B plus T cell co-culture, but the overall expression of both groups was lower than that of T cells generated from a bulk culture (figure 7A and online supplemental figure S9A). Mice given either of the CpG-derived pmel-1 products arrested tumor outgrowth and enhanced survival time compared with mice treated with vehicle treated cells or those that were not treated (figure 7B–D). Survival did not differ between the two CpG-treatment groups, suggesting that B cells are sufficient to induce the potency of pmel-1 CD8+ T cells via CpG. Of note, pmel-1 generated without CpG, but from a B and T cell co-culture, were more effective antitumor T cells than their bulk vehicle counterparts, as demonstrated by both the slower outgrowth of tumors and longer survival (figure 7B–D). There was no significant difference in tumor control or survival in mice given the bulk CpG-treated product over those given the pure B/T cell CpG-treated product (figure 7B–D). Also, mice treated with either CpG-generated cell product had more donor pmel-1 cells in their blood 1 week after infusion, indicating better engraftment of CpG-generated T cells (figure 7E).

Collectively, we found that a highly effective CD8+ T cell product can be achieved by simply adding the TLR9 agonist, CpG, to cell expansion protocols. Moreover, the mechanism of action of CpG is mediated by B cells, and B cells alone are sufficient to promote this powerful antitumor T cell product via CpG.
DISCUSSION

Herein we describe a novel use for a microbial ligand, the synthetic TLR9 agonist CpG DNA, for adoptive cellular therapy. We reveal CpG can be added to the ex vivo cell expansion process to reverse the tolerant state of CD8+ T cells to tumors in vivo and augment cancer treatment. Perhaps most surprising, B cells were essential to the manifestation of this potent T cell product as observed through our cell depletion and purified co-culture studies.

The properties that drive CpG-generated T cells to robust antitumor responses in vivo are still unclear. Given our finding that CpG-induced T cells increase IL-2Rα and ICOS expression but express less CD39, a natural question follows: Do these molecules influence the potency of antitumor T cells? Our team previously reported that IL-2Rα on activated T cells allows them to respond in an improved and temporal fashion to IL-2, in vivo, and mediate antitumor immunity.\(^{33}\) Further overexpression of IL-2Rα improves T cell tumor control.\(^{24}\) ICOS, which is also highly elevated on CpG-treated pmel-1 T cells, supports the function and antitumor activity of various types of cell products, including TIL and antigen receptor (CAR or TCR) therapies.\(^{34,35}\) ICOS signaling on T cells is also crucial for an antitumor response post-CTLA4 blockade, Th17 and Tc17 therapies.\(^{34,35,45}\) Finally, CpG-mediated downregulation of the ectoenzyme CD39 may improve T cell tumor immunity. As CD39 is a key enzyme in the catabolism of pro-inflammatory adenosine triphosphate (ATP) to immunosuppressive adenosine, a diminishment of CD39 may lead to less inhibitory adenosine within the tumor.\(^{46}\) Moreover, CD39 marks effector T cells that have an exhausted phenotype in patients with infectious disease and cancer and have reduced survival after activation in older individuals.\(^{36,37,47}\)

In patients with metastatic melanoma, Krishna et al demonstrated that CD39 and CD69 expression can be used to bifurcate patient response to cell therapy and that some CD39+ TIL from these patients are neoantigen recognizing stem-like T cells, which are known to potentiate powerful antitumor responses.\(^{38}\) Thus, it is likely that the reduced CD39 and overt expression of IL-2Rα and ICOS on T cells post CpG treatment potentiate T cell function, metabolism, and persistence in vivo. Critically, this phenotype was shared by human TIL, both CD4+ and CD8+ T cells, that were expanded in the presence of CpG. The contribution of these surface molecules (separately or in combination) on the efficacy of this therapy will be explored in future studies.

Our proteomic screen of T cell products points also to increased fatty acid oxidation (FAO) in CpG-expanded cultures compared with traditionally-expanded T cells. Several reports demonstrate FAO is important for the development of memory T cells and their enhanced survival.\(^{19-21}\) As CpG-derived T cells survive far longer in vivo compared with vehicle-derived T cells, this advantage may in part be due to enhanced mitochondrial FAO-based energetics. In the tumor, there was a marginally greater population of MitoTracker™TMRM™ pmel-1 TIL in mice that received CD8+ T cells from a CpG-treated culture compared with a vehicle-treated culture. However, further investigation is required to determine how CpG indirectly modulates T cell metabolism and if it plays a role in the effectiveness of this cellular therapy.

Using two experimental approaches, one in which CpG was added to purified T cells and one in which CpG was added when few APCs remained in culture, we discovered that CpG does not directly alter the CD8+ T cell phenotype or antitumor ability via TLR9 signaling. This finding was not surprising as CD8+ T cells express nominal TLR9.

Instead, we found that B cells in the CpG-treated culture were most vital for enhancing the antitumor potency of T cells for ACT. This finding is illuminated by our discovery that CpG-generated cells were completely ineffective in vivo when B cells were removed from the culture. In contrast, the CpG product remained effective against melanoma when F4/80+ macrophages, CD4+ T cells or NK1.1+ NK cells were removed from the culture. Likewise, removing CD11c+ DCs (with or without F4/80+ macrophages), did not impair the antitumor potency of CpG-treated pmel-1 T cells. It should be appreciated however that various DC subsets, such as myeloid and plasmacytoid DCs (pDCs), can differentially impact tumor immunity. In our experiments, we removed CD11c+ DCs, yet, pDCs express intermediate levels of CD11c, so this subset may not have been completely removed from our T cell product. As pDCs express high TLR9, follow-up experiments may shed light on the specific role of pDCs in CpG-conditioned ACT products. Most notably, however, when bulk B cells were depleted by targeting CD19, the antitumor potency of CpG-T cells was completely lost. However, B cell subsets, such as marginal zone and follicular B cells, respond differently to TLR9 stimulation and impact T cell immunity in distinct ways.\(^{39}\) Thus, further investigations are warranted to determine if a specific B cell subset is uniquely positioned to empower CD8+ T cells with robust antitumor activity via CpG.

What remained striking to us was the ability of TLR-activated B cells to foster the development of potent antitumor CD8+ T cells for ACT. However, depleting B cells from the cell culture did not satisfy the question of whether B cells alone were able to generate this T cell product via CpG. This distinct question is of critical importance, as the translation of this finding would be more feasible if isolated B cells could impart this biology on T cells via CpG without the need for other immune cells. When purified B and CD8+ T cells were co-cultured, the addition of CpG improved their activity when infused into mice with large tumors. Of note, B cells slightly enhanced CD8+ T cell immunity even when CpG was not present. This work further underscores that B cells could be considered in the manufacturing of T cell products for adoptive immunotherapy.

The finding that B cells were critical for the efficacy of CpG-improved T cell therapy leads to several questions of the mechanism of these cells’ interaction. Studies...
herein reveal that the potency of this therapy is reliant on a contact dependent CpG-promoted B cell/T cell interaction mediated by MHC-I-peptide-TCR signaling, as bypassing this crosstalk using αCD3/αCD28 beads (or plate-bound antibodies) abrogated the CpG-induced benefit to treatment. In contrast, CpG facilitated the B–T cell crosstalk via MHCI-TCR signaling via tumor antigen (hgp100) peptide. This CpG induced interaction was needed to potentiate the phenotype of antitumor CD8\(^+\) T cells, and in turn enhance their capacity to regress and even ablate tumors in vivo. Interestingly, we found that although CpG induced many inflammatory factors in our cultures, they were not the main contributors to the CpG augmentation of T cell products. Follow-up studies will be required to more intimately understand how CpG empowers B–T cell interactions to direct the fate, function, and antitumor activity of adoptively transferred CD8\(^+\) T cells in immunotherapy.

B cells are emerging as a potentially critical presence in successful immunotherapies. In fact, several exciting studies have revealed that the presence of B cells in tertiary lymphoid structures in multiple malignancies served as a powerful prognostic indicator of successful immune checkpoint blockade (ICB) therapies.\(^52-54\) Several features unique to ICB responders arose: the presence of specific B cell subsets, altered B cell receptor (BCR) clonality and diversity, and B cell/T cell interactions within the tumor microenvironment (TME). However, whether or how each of these observations drive response to checkpoint blockade and if their presence is predictive of response to ICB in other malignancies remains to be determined. As B cells can play multiple roles in the immune response—antibody production to tumor antigens, presentation of antigens to CD4\(^+\) and CD8\(^+\) T cells, and cytokine/chemokine production—it is likely that they contribute to tumor immunity in vivo in a number of ways. Indeed, tumor-infiltrating B cells (TIL-B) have been shown to act as APCs ex vivo to CD4\(^+\) TIL from human non-small cell lung cancer tumors.\(^55\) Thus, exploiting and enhancing this interaction by incorporating TLR agonists, is a logical future direction. The findings from these clinical reports alongside our findings prompt another question of whether B cells, in vivo, are also critical for the response of adoptive T cell therapies for cancer.

Our findings can be directly translated to improve cell therapies. In TIL-based ACT therapies, two approaches could be used: (1) at the onset of culture when tumor pieces harbor a TIL-B population or (2) during rapid expansion in which T cells are expanded in the presence of peripheral blood mononuclear cell (PBMC) feeder cells to large numbers before returned to the patient. We posit that incorporating CpG into either step of ex vivo expansion could lead to improved cellular therapies. Importantly, B cell populations vary widely among tumors, so strategies to exploit them in an ex vivo TIL culture may depend on the starting number and phenotype. One-way to account for this variability could be to supplement TIL cultures with B cells from the peripheral blood or tumor draining lymph nodes. Ongoing studies in our laboratory seek to understand how to best use CpG in clinical trials at various institutions. Collectively, our work demonstrates, for the first time, the importance of B cells in generating potent CD8\(^+\) T cell products for cancer immunotherapy.

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REFERENCES
adoptive T cell transfer antitumor efficacy in a lymphodepleted host.  


Supplemental Material

Supplemental Figure 1. CpG-expanded CD8\(^+\) T cells regress large, neoantigen-expressing melanomas. A) Schematic of experimental design; pmel-1 splenocytes were activated with hgp100 peptide in the presence of CpG or Vehicle control and IL-2, expanded over 7 days in culture, and infused into mice bearing subcutaneous B16\(_{KVP}\) melanoma (~100mm\(^2\)) that were preconditioned with 5Gy TBI 1 day prior to cell therapy. B) Tumor area over time of mice treated with Vehicle pmel-1 or CpG pmel-1 or NT. C) Survival of mice in B. Statistics: C) Log-rank test. ns, not significant, \(*p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001.\)

Supplemental Figure 2. The CpG-signature phenotype is preserved in mouse and human TIL. A) Phenotypic analysis by flow cytometry of 21 cell surface markers comparing Vehicle or CpG expanded pmel-1 CD8\(^+\) T cells on day 7 of culture. B) Biological replicates of donor tumor-infiltrating lymphocyte (TIL) cell surface phenotypic analysis on day 4 post transfer. C) Representative flow plots (top) and biological replicates (bottom) of the metabolic phenotype (mitochondrial mass and TMRM) of donor TIL on day 4 post transfer. D) Cytokines in the plasma of mice treated in B on day 4 post ACT. E) Representative flow plots of human OCSCC CD4\(^+\) and CD8\(^+\) TIL expanded with Vehicle or human CpG-ODN 2006 on week 4 of ex vivo expansion. Statistics: B,C,D) Unpaired T-test. ns, not significant, \(*p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001.\)

Supplemental Figure 3. Mouse T cells express nominal TLR9. A) Murine TLR9 transcript expression by RNAseq was queried using an online database of the Immunological Genome Project (immgen.org), ImmGen ULI RNASeq, under the Gene Skyline databrowser. Various immune and non-immune cell types are shown with their expression of Tlr9 where expression value ranges are categorized as ‘trace’ (blue), ‘very low’ (green), ‘low’ (yellow), and ‘medium’
Intracellular expression of TLR9 at baseline in various pmel-1 immune cell types by intracellular flow cytometry (representative histograms on left and biological replicates on right).

**Supplemental Figure 4. CpG does not confer purified T cells with an altered phenotype or enhanced antitumor ability**

A) Cell counts over time during culture of purified T cells activated and expanded with Vehicle or CpG. B) Expression of surface markers on day 7 of cell culture of purified CD8+ pmel-1 treated on day 0 with Vehicle (n=9) or CpG (n=14). C) Tumor area over time of mice treated with NT, Vehicle treated purified pmel-1, or CpG treated purified pmel-1. D) Percentage of donor cells (V13_CD8+) in the blood of mice from D on D4, 12 and 25 post ACT.

Statistics: B) Unpaired T-test, D) Mann-Whitney test. ns, not significant, *p< 0.05.

**Supplemental Figure 5. CpG-induced soluble factors do not confer the IL-2RαhighICOShighCD39low phenotype on CD8+ T cells.**

A) Soluble factors in the pmel-1 cell supernatant 48hrs post activation and CpG or Vehicle treatment. B) Concentration of the top 5 cytokines and chemokines that were more abundant in CpG compared to Vehicle treated cultures by fold change and total amount. C) Flow cytometry of the signature phenotype markers (IL-2Rα, ICOS, CD39) on day 7 of pmel-1 cells expanded from cultures treated with Vehicle or CpG when the top 5 soluble factors were individually blocked for the duration of culture (representative of 3 biological replicates). D) Schematic of cell supernatant transfer experiment: Supernatant was collected from Vehicle or CpG treated pmel-1 cultures, stored at -20 degrees Celsius, thawed and then used to expand bulk pmel-1 splenocytes, or CD8+ isolated pmel-1 T cells. E) Flow cytometry analysis of signature phenotypic markers on day 7 of cell culture of cultures that received 7hr or 48hr supernatant (Vehicle control or CpG), or fresh cell media controls (supplemented with Vehicle or CpG day-of) (representative of 3 biological replicates). Statistics: B) Unpaired T-test. ns, not significant, *p< 0.05, **p< 0.01, ***p< 0.001.
Supplemental Figure 6. Blocking CD40L does not abrogate the CpG-mediated phenotype or antitumor immunity. A) Phenotype of day 7 T cells expanded from pmel-1 B cell/T cell co-cultures treated with Vehicle or CpG and isotype control antibody or aCD40L blocking antibody. B) Tumor area over time of mice bearing subcutaneous B16KVP melanoma treated with CpG pmel-1 CD8+ T cells expanded from cultures treated with isotype control antibody or aCD40L blocking antibody over the duration of culture. C) Survival of mice in B. Statistics: C) Log-rank test. **p<0.01.

Supplemental Figure 7. Direct interaction between APCs and CD8+ T cells is critical for improved antitumor immunity with CpG. A) Biological replicates of signature phenotype marker expression of pmel-1 CD8+ T cells expanded from cultures activated via APC mediated MHC-I – peptide presentation to TCR (hgp100), or that bypass the APC (plate-bound αCD3/αCD28 or plate-bound αCD3) on day 7 of culture. B) Tumor area over time of mice bearing subcutaneous B16F10 melanoma treated with Vehicle pmel-1 or CpG pmel-1 CD8+ T cells expanded from cultures activated via a direct APC interaction (hgp100 peptide), or which bypass the APC (plate-bound αCD3/αCD28 or plate-bound αCD3). C) Survival of mice in B. Statistics: A) Two-sample T-test, C) Log-rank test. ns, not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Supplemental Figure 8. The signature CpG phenotype and enhanced antitumor efficacy do not rely on CD4+ T cells, NK cells, or the combination of Macrophages and DCs A) Representative column depletion efficacy on day 0, prior to plating. B) Representative histograms showing expression of a given marker on day 7 of cell culture. C) Tumor area over time of mice treated with NT, or Vehicle or CpG-generated T cell products from CD4 or NK cell-depleted cultures (n=7-10 mice/group). D) Survival of mice in C. E) Percentage of donor cells in the blood of mice treated in C over time post-ACT. F) Representative histograms showing expression of
signature markers on day 7 of cell culture. G) Survival of mice treated with NT, Vehicle, or CpG-generated T cell products from cultures depleted of macrophages and DCs on day 0 of culture. Statistics: D,H) Log-rank test. E) Mann-Whitney U at each time point, G) Log-rank test. ns, not significant, *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001.

**Supplemental Figure 9. Co-cultured B and T cells induce the signature phenotype with CpG.** A) Expression of phenotypic markers on day 7 of cell culture from Bulk or purified B + T cell cultures treated with CpG or Vehicle control on Day 0 (3 biological replicates). Statistics Unpaired T-tests *p< 0.05, **p< 0.01, ***p< 0.001, ****p< 0.0001.
Supplemental Figure 1. CpG-expanded CD8⁺ T cells regress large, neoantigen-expressing melanomas

A

Mice with B16KVP melanoma

Vehicle expand w/ IL-2

CpG expand w/ IL-2

D0

D7 ACT

B

B16KVP

Tumor area (mm²)

0 100 200 300 400

0 11 22 33 44 55 66

Day Post-ACT

C

B16KVP

% Survival

0 25 50 75

Day Post-ACT

NT

Veh Pmel

CpG Pmel
Supplemental Figure 2. The CpG-signature phenotype is preserved in mouse and human TIL

A

**Day 7 phenotype in vitro (first gate on live CD8+VB13+)**

- CD28
- CD27
- PD-1
- LAG-3
- TIM-3
- TIGIT
- OX-40
- SLAMF6
- KLRG1
- CD69
- CD26
- CD73
- CD38
- CXCR3
- CCR7
- CCR6
- IL-23R
- IL-7R
- CXCR5
- CD44
- CD62L

**Day 4 post ACT TIL phenotype in vivo (first gate on live pmel-1 donor cells)**

B

- **ICOS**
  - MFI
  - Veh: 400
  - CpG: 600

- **CD39**
  - MFI
  - Veh: 700
  - CpG: 1000

- **GRZMB**
  - ns
  - Veh: 800
  - CpG: 1200

- **PD-1**
  - MFI
  - Veh: 2500
  - CpG: 5000

- **LAG-3**
  - MFI
  - Veh: 7000
  - CpG: 12000

- **TIM-3**
  - ns
  - Veh: 600
  - CpG: 400

C

**MitoTracker**

- Veh: 27.5
- CpG: 42.6

**TMRM**

- Veh: 20
- CpG: 20

**% of Pmel-1**

- Veh: 10
- CpG: 20

D

- **IFN-γ**
  - Veh: 1000
  - CpG: 1000

- **IL-6**
  - Veh: 200
  - CpG: 400

- **TNFα**
  - Veh: 12
  - CpG: 12

- **GM-CSF**
  - Veh: 10
  - CpG: 10

E

**Human CD4+ICOS+ TIL**

- Veh: 29
- CpG: 55

**Human CD8+ICOS+ TIL**

- Veh: 42
- CpG: 64
Supplemental Figure 3. Mouse T cells express nominal TLR9

A

TLR9 Expression Value Normalized by DESeq2

B

T cell

B cell

DC

Mac

Expression Value Range

Intracellular TLR9

1.08%

10.5%

31.2%

52.1%

0%

20%

40%

60%

% TLR9+
Supplemental Figure 4. CpG does not confer purified T cells with an altered phenotype or enhanced antitumor ability

A. Cell Count x10^6

B. MFI

C. Tumor area (mm^2)

D. % Pmel-1 live
Supplemental Figure 5. CpG-induced soluble factors do not confer the IL-2Rα\textsuperscript{high}ICOS\textsuperscript{high}CD39\textsuperscript{low} phenotype on CD8\textsuperscript{T} cells

A. A scatter plot showing the Log\textsubscript{2} of CpG normalized to control.

B. Bar charts showing the concentration of IFN-\gamma, IL-6, TIMP-1, CXCL10, and IL-10 for Veh and CpG conditions.

C. Flow cytometry histograms comparing the expression of IL-2Rα, ICOS, and CD39 in control, αIFN-\gamma, αIL-6, αTIMP-1, αCXCL10, αIL-10, Iso-IgG1, Iso-AH IgG1, and Iso-Goat IgG conditions.

D. Diagram showing the effects of fresh (control) and supplemented (7hr and 48hr) conditions on bulk and CD8 pure cells.

E. Flow cytometry histograms comparing the expression of IL-2Rα, ICOS, and CD39 in bulk and CD8 pure cells under different conditions.

Supplemental material
Supplemental Figure 6. Blocking CD40L does not abrogate the CpG-mediated phenotype or antitumor immunity

A

Control

IL-2Rα ICOS CD39

αCD40L

IL-2Rα ICOS CD39

B

Tumor area (mm²)

0 7 14 21 28 35

NT CpG αCD40L

C

% Survival

0 50 100

0 7 14 21 28 35

Days Post-ACT

NT CpG αCD40L

Days Post-ACT

NT CpG αCD40L

Supplemental Figure 7. Direct interaction between APCs and CD8+ T cells is critical for improved antitumor immunity with CpG

A. Flow cytometry analysis of IL-2Rα, ICOS, and CD39 expression in CD8+ T cells.

B. Tumor growth over time in mice treated with different conditions.

C. Survival rates of mice treated with different conditions.
Supplemental Figure 8. The signature CpG phenotype and enhanced antitumor efficacy do not rely on CD4+ T cells, NK cells, or the combination of Macrophages and DCs.
Supplemental Figure 9. Co-cultured B and T cells induce the signature phenotype with CpG

A

IL-2Rα

**** ns

***

***

ICOS

**

ns

****

***

ns

CD39

*

ns

****

**

ns
B cells imprint adoptively transferred CD8⁺ T cells with enhanced tumor immunity

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In Brief
During the expansion of tumor-reactive T cells (left) CpG can be added to cell culture to improve the potency of cell therapy in vivo (right). Ex vivo B cells are responsible for directly improving the T cell product via CpG and T cells gain a unique phenotype: IL-2RαhighICOShighCD39low. Once transferred, CpG-generated T cells display enhanced engraftment, persistence, and tumor killing compared to a traditionally-expanded cell product.