Inhaled TLR9 Agonist Renders Lung Tumors Permissive to PD-1 Blockade by Promoting Optimal CD4⁺ and CD8⁺ T-cell Interplay

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Abstract

Currently approved inhibitors of the PD-1/PD-L1 pathway represent a major advance for the treatment of lung cancers, yet they are ineffective in a majority of patients due to lack of preexisting T-cell reactivity. Here, we show that a TLR9 agonist delivered by inhalation is able to prime T-cell responses against poorly immunogenic lung tumors and to complement the effects of PD-1 blockade. Inhaled TLR9 agonist causes profound remodeling in tumor-bearing lungs, leading to the formation of tertiary lymphoid structures adjacent to the tumors, CD8⁺ T-cell infiltration into the tumors, dendritic cell expansion, and antibody production. Inhalation of TLR9 agonist also increased the pool of functional PD-1lowT-bethigh effector CD8⁺ T cells in tumor-bearing lungs. Effector CD8⁺ T cells generated by inhaled TLR9 agonist treatment were licensed by PD-1 blockade to become highly functional CTLs, leading to a durable rejection of both lung tumors and tumor lesions outside the lungs. CD4⁺ T cells activated in response to inhaled TLR9 play a critical role in this process by controlling the proliferation, preventing exhaustion, and guiding the differentiation of optimally functional CTLs. This study characterizes a strategy to apply localized TLR9 stimulation to a tumor type not accessible for direct injection, a strategy that may expand the therapeutic potential of PD-1 blockade in non–small cell lung cancer.

Significance: These findings demonstrate that local delivery of a toll-like receptor 9 agonist can change the immune content of an entire organ and enhance the efficacy of immune checkpoint inhibition.

Graphical Abstract: http://cancerres.aacrjournals.org/content/canres/78/17/4943/F1.large.jpg

Introduction

Studies in mice have demonstrated that local intratumoral injection of an oligonucleotide containing immunostimulatory CpG motifs (CpG-ODN) signaling through Toll-like receptor-9 (TLR9) can control tumor growth by coordinated activation of both innate and adaptive responses (1). In phase I/II clinical trials in patients with lymphoma, intratumoral injection of CpG-ODN in combination with low-dose radiotherapy resulted in shrinkage of untreated tumor lesions (abscopal responses), demonstrating that localized stimulation through TLR9 is an effective strategy to...
increase systemically active tumor immunity (2). In contrast, systemic administration of TLR9 agonists has generally proven unsuccessful, both in humans (3) and in mouse tumor models (4). This limits the application of CpG-ODN to tumor types with at least one lesion accessible for repeated injections. For lung tumors, this is rarely possible in practice, but delivery by inhalation represents a promising alternative. The TLR9 agonist AZD1419 developed for the treatment of allergic asthma has been delivered safely and effectively by inhalation in both humans and mice (5) and is currently being evaluated in a phase II study of asthmatic patients (ClinicalTrials.gov, NCT02898662). A similar approach for delivering a TLR9 agonist to patients with lung cancer thus appears feasible.

Although monotherapy with intratumoral CpG-ODN can have significant activity on the injected lesion, systemic anti-tumor responses require combination with complementary immunoregulatory treatments, including immune checkpoint inhibitors such as PD-1/PD-L1 inhibitors (6, 7), T-cell activators, such as anti–OX-40 (8), or IDO blockade (9). In animal models, such combinations can lead to a high frequency of durable complete responses in animals with both treated and untreated lesions.

The combination of an inhaled CpG-ODN with antibodies blocking PD-1/PD-L1 pathway represents a particularly promising avenue for clinical evaluation as five such antibodies have been approved for use in advanced non–small cell lung cancer (NSCLC). Although PD-1/PD-L1 blockade does represent a significant advance in the treatment of NSCLC, only about 20% of unscreened patients respond (10). Demonstrating the need for combinations that are able to increase the frequency and durability of clinical responses. Antibodies to PD-1/PD-L1 act by blocking a key immune checkpoint restraining the effectiveness of preexisting T-cell responses to tumor antigens. Clinical response to these agents is correlated with the expression of PD-L1 on tumors and tumor-infiltrating immune cells, the presence of CD8+ T-cell infiltration, and an elevated IFNγ-dependent T-cell gene signature at baseline (11, 12). In addition, a positive correlation between the response to PD-1 blockade and a high mutational tumor burden, suggesting a higher frequency of tumor neoantigens, has been reported in NSCLC (13).

In this study, we demonstrated that delivery of a TLR9 agonist through the airways can stimulate effective immunity against lung tumors and complement the actions of PD-1/PD-L1 blockade to generate durable, systemic anti-tumor immunity and delineate the distinctive mechanisms of action of these agents in the lung environment. A short course of inhaled CpG-ODN induced significant structural changes in tumor-bearing lungs, characterized by the formation of prominent tertiary lymphoid structures (TLS) adjacent to the tumor and the infiltration of CD8+ T-cells into the tumor mass. Combining inhaled SD-101 with systematic anti–PD-1 led to long-term cure in two different mouse lung tumor models, mediated by systemic immunity that eradicated tumors both in the lung and in distal organs. The therapeutic effect of inhaled CpG-ODN plus anti–PD-1 on established tumors unexpectedly required CD4+ as well as CD8+ T-cells. When CD4+ T-cells were depleted, effector CD8+ T-cells proliferated rapidly, but did not develop into functional cytotoxic T-cells and acquired a severely exhausted phenotype. These findings substantially expand the potential clinical applications of localized TLR9 to include primary lung cancer and other cancer types with lung metastases and may provide a strategy for enhancing the frequency of patients able to benefit from currently approved PD-1 pathway inhibitors.

Materials and Methods

Mice
Six to 8-week-old female BALB/c mice were supplied by Harlan and housed at MuriGenics. All experimental procedures involving live animals were approved by the Institutional Animal Care and Use Committees of MuriGenics.

Cell lines and reagents
4T1 mammary carcinoma cells (CRL-2539) and CT26 colon carcinoma cells (CRL-2639) were purchased from the ATCC and authenticated by ATCC using COI analysis. Cell lines were routinely tested for Mycoplasma. After thawing, cell lines were passage twice before using for in vivo experiments. Antibodies for in vivo use [anti-mouse PD-1 (clone RMPI-14), anti-mouse CD8 (YTS 169.4, BE0117), and anti-mouse CD4 (clone GK1.5, BE0003)] were purchased from Bio X Cell. The CpG-C oligodeoxynucleotide SD-101 was synthesized and purified by standard techniques as described previously (14).

Tumor challenge and treatments
4T1 cells (1.0 × 106) were injected subcutaneously in the flank of BALB/c mice. Primary tumors were surgically removed, as described previously (15), with the following modifications: primary tumors were removed 15 days after 4T1 inoculation when they reach 8 mm in diameter. Two days after the surgery, mice started receiving intranasal SD-101 treatment (10 μg/50 μL in saline) or saline control twice a week for a total of 6 doses. The intranasal dose volume of 50 μL was selected to maximize exposure to the lung and is routinely used to mimic inhalation in mice (16). In experiments assessing T-cell infiltration and TLS formation by immunofluorescence, SD-101 treatment was started at day 26 (11 days after surgical removal of primary tumor) and given twice a week for 2 weeks. Lung harvest was performed 3 days after last treatment. To establish CT26 pulmonary metastasis, mice were given an injection of 1.0 × 106 tumor cells intravenously. CT26 tumor-bearing mice were treated with intranasal administrations of SD-101 (10 μg/50 μL in saline), starting at day 7, twice a week for a total of 8 doses. Mouse anti–PD-1 blocking antibody was administered at 250 μg per injection. Anti-CD4 and anti-CD8 in vivo depleting antibodies were used at 250 μg per injection and given daily for the first 2 days and then twice weekly until the end of the experiment.

Metastasis assessment and isolation of leukocytes from tumor-bearing lungs
Lungs or liver were harvested and processed using the gentleMACS dissociator (Miltenyi Biotec). To facilitate tissue disruption, cells were enzymatically digested with 1 mg/mL type D collagenase (Sigma) and 250 μg/mL DNase I (Sigma) in HBSS medium for 20 minutes at 37°C in 5% CO2. Cell suspension from lung and liver was used for metastasis quantification. Number of metastatic cells in lung and liver was determined using the clonogenic metastasis assay in which dissociated organ cells were cultured in medium supplemented with 6-thioguanine. Isolated cells from tumor-bearing lungs were also used in FACS analysis, T-cell function assay, RNA extraction, and CD8+ T-cell purification.
Flow cytometry

Cells isolated from tumor-bearing lungs were processed for surface labeling with several antibody panels staining for CD45, CD3, CD4, CD8, CD11b, CD11c, CD19, B220, CD49b-DX5, 78-TCR, PD-1, CD73, MHCI-I, MHCI-II, CD44, CD107a, Ly6-G, Ly6-C, CD80, CD86, CD69, CD64, MAR-1, CD39, LAC-3, and TIM-3 markers. FC receptors were blocked using Fc-Block (BD Biosciences). Cells were further permeabilized using Transcription Factor Buffer Set (BD Biosciences) and stained for Ki-67, FoxP3, T-bet, granzyme B, TNF, and IFNγ. Data were acquired using the LSRII Flow Cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar).

Analysis of cytokine production

For functional analysis of tumor-bearing lungs infiltrating lymphocytes, 5 × 10^5 cells from lung suspension were stimulated with Leukocyte Activation Cocktail (BD Biosciences) or BD GolgiPlug alone (BD Biosciences) for 4 hours. After restimulation, lymphocytes were processed for flow cytometry as above.

NanoString gene expression analyses

RNA from cells isolated from tumor-bearing lungs was purified using RNeasy Mini Kit (Qiagen). Isolated RNA was hybridized with the NanoString nCounter PanCancer Immune Profiling mouse panel code set and quantified using the nCounter Digital Analyzer at the Core Diagnostics, Inc. Data were processed with nSolver Analysis Software, using the Advanced Analysis module. NanoString results (raw and normalized counts) were produced from RCODE using nSolver software (version 3.0). Exploratory analysis and summary statistics were calculated to identify variation in the data and relationships among replicates and conditions in each study.

Immunofluorescence staining

To prepare frozen sections, lungs were perfused with 4% paraformaldehyde and excised. Fixed lungs were then incubated in 30% sucrose in PBS (w/v) overnight and frozen in optimum cutting temperature gel (Tissue-Tek). Sections of 8-μm thickness were cut on Leica CM1860 cryostat. Slides were blocked in PBS with 10% normal goat serum (Abcam), 1% BSA, and 0.1% Tween-20 (Thermo Fisher Scientific) and stained for Ki-67, FoxP3, T-bet, granzyme B, TNF, and IFNγ. Sections were counterstained with DAPI (4,6-diamidino-2-phenylindole; Life Technologies), for 5 minutes at room temperature. The slides were then washed and coverslips were mounted, using ProLong Diamond anti-fade reagent (Life Technologies). Stained sections were visualized by a Leica DM4-B microscope using 10×, 20×, or 40× objectives and analyzed with Leica Las X software.

Bronchoalveolar lavage collection and determination of immunoglobulin and IL21 concentration

Bronchoalveolar lavage (BAL) samples were collected by injecting 3 mL of PBS intratracheally. BAL samples were subsequently centrifuged to separate cells, and the supernatants were lyophilized using Speed-Vac to dryness. The dry samples were rehydrated by adding 300 μL of milli-Q water. Immunoglobulin analyses were performed using the MGAMMAG-300K Isotyping Multiplex Assay (Millipore Sigma), according to the manufacturer’s protocol. IL21 was detected using the IL-21 Mouse ProcartaPlex Simplex Kit (Thermo Fisher Scientific), according to the manufacturer’s protocol.

In vitro binding assays

4T1 cells were fixed in 4% PFA for 30 minutes and incubated with BAL containing immunoglobulins from SD-101–treated mice for 1 hour at 4°C. After washing, cells were stained with secondary anti-mouse IgG-Alexa Fluor 700–conjugated Ab (1:200). The A mean fluorescence intensity (ΔMFI) was obtained subtracting the MFI of the cells incubated with the secondary antibody only from the MFI of the cells incubated with BAL containing immunoglobulins from SD-101–treated mice. Data were acquired using the LSRII Flow Cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar).

Microarray analysis

Lung CD8+ T cells were purified by negative selection using Miltenyi Biotec magnetic beads. Isolated CD8+ T cells were further enriched for CD44+ subset by positive selection using Miltenyi Biotec CD44-PB antibody and anti-PE magnetic beads. The purity of the isolated CD8+CD44+ cells was 90% to 97%, as determined by flow cytometry analysis. RNA extraction was performed using RNeasy Mini Kit (Qiagen). RNA purity and integrity of pooled samples was assessed by Bioanalyzer. The cDNA synthesis and hybridization onto Affymetrix GeneChip Mouse Genome 430 2.0 Array were performed at the Microarray Core Facility of the Scripps Research Institute (San Diego, CA). R package affy was used to process the raw microarray data, and rma method was used for background correction. Differential expression analysis was performed by using limma package (17) and moderated t test. Benjamini–Hochberg method was used to adjust the raw P values for multiple testing. Only genes with log2 fold change (up- and downregulated) >0.6 and FDR <0.05 were considered as differentially expressed. Venn diagram of overlapping differentially expressed genes (DEG) was built using iPathwayGuide software (AdvaitaBio). Principal component analysis (PCA) was conducted using Partek genomic studio software. List of DEGs was used to carry out gene set enrichment analysis using Partek Genomic Studio software (Partek). Data are deposited in Gene Expression Omnibus database (GEO accession number GSE13987).

Statistical analysis

All statistical analyses were performed using Prism software v5 (GraphPad Software). A two-tailed unpaired Mann–Whitney U test was used to compare two groups. Multiple comparisons
were performed using the one-way ANOVA test with Tukey post hoc test or Kruskal–Wallis test with Dunn post hoc test when a nonparametric test was required. Survival curves were plotted using Kaplan–Meier method and compared using the log-rank test. P values lower than 0.05 were considered statistically significant ( *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001).

**Results**

Inhaled SD-101 reduces lung tumors and increases T-cell responses in tumor-bearing lungs

To evaluate the antitumor efficacy of CpG-ODN delivered to the lung, we have used the highly metastatic 4T1 mouse tumor as the primary model. The 4T1 model is a triple-negative breast cancer line that spontaneously metastasizes from an orthotopic subcutaneously growing tumor to multiple tissue sites. Surgical removal of the primary 4T1 tumor at an early time results in multiple metastases primarily, but not exclusively, in the lung and liver (18). 4T1 tumors are poorly immunogenic due to the low number of coding region mutations and the highly immunosuppressive tumor microenvironment of these tumors (19). 4T1 lung tumors also exhibit an immune-excluded phenotype with CD45 + leukocytes, CD8 + T cells, and PD-L1–expressing cells located around tumor perimeter (Supplementary Fig. S1). Although there are autochthonous lung models that are more reflective of primary lung cancer, this 4T1 model is particular suited to understand whether local administration of a TLR9 agonist through the airways generates, by local stimulation, antitumor responses that can migrate to and control metastases throughout the body.

The TLR9 agonist chosen for these studies, SD-101, is a C class CpG-ODN that is capable of inducing high levels of type I IFNs as well as efficient dendritic cell (DC) and B-cell maturation (6). Twice-weekly treatment with inhaled SD-101 was initiated after surgical removal of the primary tumor (Fig. 1A), a time point when tumors are already disseminated to multiple organs (18). Treatment with SD-101 resulted in approximately 90% decrease

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**Figure 1.**

Inhaled SD-101 suppresses lung tumors and promotes long-term survival of treated mice. **A,** Illustration of the models. Primary 4T1 tumors were resected at day 16 postimplantation. SD-101 treatment was started 2 days after surgery and continued biweekly for a total of six treatments. Mice were followed up for long-term survival or sacrificed at day 36, and lungs were collected for metastasis evaluation, histologic analysis, phenotypic analysis, or gene expression. CT26 cells were injected intravenously to induce dissemination into the lungs, and mice received intranasal administration of SD-101 or saline biweekly from day 7 after cell inoculation. Mice were either followed up for long-term survival or sacrificed 4 days after the last treatment for phenotypic analysis. **B,** Number of 4T1 tumor cells per lungs and livers in treated mice. Cumulative data from two independent experiments, n = 12 to 17 lungs or livers per group. Error bars, SEM. **C,** Long-term survival of treated mice bearing 4T1 lung tumors (left; n = 8/mice/group) and CT26 lung tumors (right; data from three independent experiments; n = 21).
in tumor burden in both the lung and liver (Fig. 1B) and a significant increase in survival time (Fig. 1C, left). As oligonucleotides administered to normal lungs do not readily enter systemic circulation (20), the antitumor activity on liver metastases suggests that local administration of SD-101 to lung generates an antitumor T-cell response capable of controlling tumor growth beyond the lung itself. The antitumor activity of SD-101 was confirmed in a second model based on intravenous injection of CT26 colon carcinoma cells. Treatment of mice bearing CT26 lung tumors with inhaled SD-101 showed a significant prolongation of survival compared with controls (Fig. 1C, right).

SD-101–treated tumor-bearing lungs showed a 1.8-fold increase in the number of CD45^+ leukocytes compared with saline (Fig. 2A). However, the relative distribution of leukocyte subsets was largely unchanged, with the exception of a slight decrease in CD4^+ T and natural killer (NK) cells, and an increase in CD11b^+ cells (Fig. 2B–G; Supplementary Fig. S2A), suggesting that SD-101 treatment leads to a general influx of leukocytes into the lungs. Both CD8^+ T cells and CD4^+ T cells showed substantial increases in effector markers including CD44, Ki67, and CD69, and the cytotoxicity markers granzyme B (GzmB) and CD107a in response to SD-101 treatment (Fig. 2B and C). SD-101 treatment increased the percentage of CD8^+ T cells expressing PD-1; however, PD-1 levels among PD-1^+ CD8^+ T cells were significantly lower than in saline-treated lungs (Fig. 2B). Although PD-1 can be a marker of T-cell exhaustion, it also identifies tumor-reactive CD8^+ T cells infiltrating human (21) and mouse tumors (22). The increase in cells expressing PD-1, especially at relatively low levels, suggests that SD-101 treatment increased the pool of functional tumor-specific CD8^+ T cells in tumor-bearing lungs (23).

The percentage of Foxp3^+ regulatory T cells (Treg) among leukocytes remained unchanged, but the levels of the activation markers CD44 and CD73 were strongly decreased, indicating reduced activation of Tregs upon SD-101 treatment (Fig. 2D; refs. 24, 25). In addition, a significant increase in activation markers, including MHCI and CD69, was observed on B cells (Fig. 2E). Although SD-101 treatments decreased the percentage of NK cells among total CD45^+ cells, this population showed significant increases in cytotoxicity markers (GzmB and CD107a) and the transcription factors T-bet, whose expression is critical for terminal maturation of NK cells (Fig. 2E; ref. 26). SD-101 treatment led to an increased percentage of GzmB^+PD-1^– T-bet^high CD8^+ T cells in tumor-bearing lungs (23).

The presence of this gene profile signature at baseline was recently reported to be positively correlated with response to anti–PD-1 across tumor types (28).

SD-101 induces CD8^+ T-cell homing to lung tumors and TLS development adjacent to the tumors

As inhaled SD-101 was not directly administered into lung tumors, in contrast to direct injection of subcutaneous tumors, it was important to characterize the extent of immunostimulation within the tumor itself. Analysis by IHC revealed a statistically significant increase in the number of CD8^+ T cells (Fig. 3A) and a trend toward increased CD4^+ T cells (Fig. 3B) infiltrating lung tumors in SD-101–treated mice, compared with saline controls. IHC analysis of SD-101–treated lungs revealed the presence of a substantial number of TLSs adjacent to tumors, structures that were virtually absent in saline-treated controls (Fig. 3C). These structures contained a high density of B and T cells in intimate contact with each other (Fig. 3D). They also contained CD11c^+ DCs and stained positive for the chemokines CXCL13 and CCL21 (Fig. 3E), which have been implicated in TLS formation in autoimmune disease and cancer (29). Consistent with these findings, a chemokine gene signature that predicts the presence of TLSs in human tumors (30) is significantly increased upon SD-101 treatment (Fig. 3F), suggesting that the chemokine milieu induced by SD-101 treatment might promote the neogenesis of TLSs. Germinal center B cells were significantly increased in SD-101–treated lungs and antibody titers in BAL of SD-101–treated mice were 2- to 6-fold higher for all Ig classes compared with saline-treated animals (Fig. 3G and H). Antibodies found in the BAL of SD-101–treated mice bind to 4T1 tumor cells, demonstrating tumor specificity (Fig. 3I). These findings are consistent with the presence of B–T-cell contact areas close to tumors and the well-described ability of TLR9 ligation on B cells to increase germinal center response and to induce antibody production (31). Thus, a limited course of inhaled SD-101 in tumor-bearing lungs induced a substantial remodeling of the lung microenvironment focused on the tumor masses, including increased CD4^+ and CD8^+ T effector functions, expansion of mature moDCs, B-cell maturation, and tumor-specific antibody production.

SD-101 combined with PD-1 blockade achieves long-term control of lung tumors

Despite the potent reduction in lung tumor burden initially, this regimen of inhaled SD-101 treatment did not induce complete rejection in most animals. However, the addition of PD-1 blockade led to a profound reduction in lung tumor burden (Fig. 4A) and a substantial increase in the frequency of long-term survivors in both the 4T1 (Fig. 4B) and CT26 models (Fig. 4C). This represents a synergy between anti–PD-1 and SD-101, as neither single-agent therapy achieves durable rejection of lung tumors.

Comprehensive flow cytometric analysis of tumor-bearing lung cell suspensions showed that the leukocyte distribution and phenotype profile in SD-101–treated mice was largely unchanged by the addition of anti–PD-1, with a few notable exceptions. The percentage of proliferating, antigen-experienced CD8^+ T cells (CD8^+PD-1^– and CD8^+PD-1^+Ki-67^+) was further increased by the addition of PD-1 blockade (Fig. 4D). The combination treatment led to an increased percentage of GzmB^+ cells within both the total CD8^+ T cells and the antigen-experienced subset (CD8^+PD-1^+). A further increase of PD-1^hiPD-1^hi T cells (CD8^+PD-1^+)} was observed in the lungs of PD-1^hiPD-1^hi mice, which was accompanied by a decrease in CD8^+PD-1^– T cells and a decrease in the number of TLSs. These findings suggest that the combination of SD-101 and PD-1 blockade promotes an antitumor CD8^+ T-cell response capable of controlling tumor growth.
Figure 2.
Inhaled SD-101 promotes infiltration of activated T cells in tumor-bearing lungs. Mice bearing 4T1 lung tumors were treated as in Fig. 1A and lungs were analyzed by flow cytometry. A, The total number of CD45+ cells in saline or SD-101-treated lungs is represented. Floating bars show the minimum and maximum values and the means. B-G, Phenotypic analysis of leukocyte subsets from saline- or SD-101-treated lungs. Cumulative data from two independent experiments (n = 14 lungs/group). Error bars, SEM. H-L, RNA from individual saline- or SD-101-treated lungs was analyzed for expression of 750 genes in the NanoString Mouse PanCancer Immune Profiling panel (n = 5/group). H-K, Heatmaps showing relative expression levels of T-cell function genes, B-cell function genes, DC-function genes, and MHC genes. L, Heatmap representing relative expression levels of IFNα-regulated genes and a T-cell-inflamed gene signature. Data represent the log2-fold change of expression between SD-101-treated tumor-bearing lungs and saline control.
was also observed in the combination group, a characteristic linked to reinvigorated, low exhausted cells following anti-PD-1 blockade (Fig. 4D; ref. 23). Altogether, these results suggest that SD-101 and anti-PD-1 cooperate to enhance the effector functions of CD8⁺ T cells and to expand antigen-experienced T cells with a low-exhaustion phenotype.
CD4+ T-cell help is essential for SD-101 plus anti–PD-1 antitumor activity and dictates the cellular fitness of effector CD8+ T cells

Depletion of either CD4+ or CD8+ T cells effectively reversed the antitumor activity of the combination of SD-101 and anti–PD-1, as demonstrated by a large lung tumor burden in mice treated with either anti-CD4 or anti-CD8 antibodies (Fig. 5A). This contrasts with studies performed in subcutaneous growing tumors, in which complete rejection after SD-101 plus anti–PD-1 was dependent on CD8+ T cells but not CD4+ T cells (6). Paradoxically, tumor-bearing lungs treated with the combination, but depleted of CD4+ T cells, had higher number and proportion of CD8+ T cells among CD45+ cells compared with nondepleted SD-101 plus anti–PD-1-treated mice (Fig. 5B). In addition, a higher frequency of these CD8+ T cells expressed the effector markers CD44 and KLRG1 (Fig. 5B). To determine why CD8+ T cells were not able to control lung tumor burden, we examined their expression of inhibitory receptors when generated in the absence of CD4+ T cells and found a large increase in the percentage of cells expressing exhaustion markers such as PD-1, CD39, TIM-3, and LAG-3 (Fig. 5C). Within the population of CD8+ T cells defined as antigen-experienced (CD44+), there was a pronounced increase in cells expressing multiple exhaustion markers when generated in the absence of help (‘nonhelped’ T cells; Fig. 5D), implicating severe exhaustion of the CD8+ population (32). Consistent with these data, the fraction of PD-1+ CD8+ T cells expressing high levels of PD-1 (PD-1hi) and decreased expression of GzmB was significantly increased among nonhelped CD8+ T cells (Fig. 5E). GzmB expression was decreased regardless of the number of expressed inhibitory receptors, suggesting a major defect in cytotoxicity in nonhelped CD8+ T cells (Fig. 5E, right). Nonhelped CD8+ T cells expressing multiple exhaustion markers were also less multifunctional, as defined by coexpression of IFNγ and TNFα (Fig. 5F). Taken together, these results point to an essential role of CD4+ T-cell help in shaping the cellular fitness of cytotoxic CD8+ T cells for rejecting lung tumors.

Lack of CD4+ T cells also resulted in the complete absence of TLS formation in response to SD-101 plus anti–PD-1 (Fig. 5G), showing that the ability of SD-101 to induce TLS formation is dependent upon CD4+ T cells. Consistent with the lack of TLSs, the absence of CD4+ T-cell help results in the abrogation of the SD-101–mediated increase of germinal center B cells (Fig. 5H).

Nonhelped CD8+ effector T cells are highly proliferative but functionally impaired

To better understand the differences among CD8+ T cells in mice given different treatments, we profiled gene expression in
CD44+ effector T cells purified from tumor-bearing lungs. PCA of normalized expression data showed that the individual samples clustered according to the treatment and that CD8+ effector T cells in the combination treatment group were quite different if generated in the presence or absence of CD4+ T-cell help (Fig. 6A). The number of DEGs relative to saline-treated controls was much greater in mice treated with SD-101 plus anti–PD-1 than in mice treated with SD-101 alone, 848 versus 50.
Figure 6.
Transcriptional profiles of effector CD8+ T cells generated in the absence of CD4+ T-cell help. Gene expression was performed on purified CD44+CD8+ effector T cells from 4T1 tumor-bearing lungs treated with the indicated treatment regimen with the same schedule as in Fig. 4. Lungs were collected 2 to 4 days after last treatment.

A, Principal component analysis was performed using Partek Genomic Studio software. Saline, gray (n = 5); SD-101, red (n = 6); SD-101+anti–PD-1, blue (n = 5); SD-101+anti–PD-1+a–CD4, purple (n = 4). B and E, Venn diagrams illustrating the overlap of DEG relative to saline (P < 0.05 and log2-fold change of >0.6). C, Gene set enrichment analysis was performed on the DEGs between SD-101+anti–PD-1 versus SD-101 comparison. The genes are categorized into GO terms in two ways: biological process (black) and cellular process (orange). GO terms are shown in vertical axis and enrichment score on the horizontal axis. D, Heatmap of genes contained in two representative GO terms from C. The heatmap represents log2-fold change in gene expression relative to saline. F, Heatmap of selected genes representing log2-fold change in gene expression relative to saline. G, Gene set enrichment analysis was performed on the 702 genes differentially expressed in the “nonhelped” but not in “helped” CD8+ T cells compared with saline (purple arrow, genes in the Venn diagram subjected to the analysis). H, Heatmap of genes contained in one representative GO term from G. The heatmap represents log2-fold change in gene expression relative to saline.
DEGs, respectively (Fig. 6B), demonstrating the marked effect of PD-1 blockade on the transcriptional profile of effector CD8+ T cells. Direct comparison of SD-101–treated versus SD-101 plus anti–PD-1–treated CD8+ effector T cells resulted in 664 DEGs. Gene set enrichment analysis of these differentially regulated genes identified multiple gene sets involved in motility, cytoskeleton organization, cilium, and dynein axonemal assembly (Fig. 6C). These gene sets contain many genes also involved in the organization of the immunologic synapse, a process similar to primary cilia formation (33, 34). When we compared the fold change versus saline of the genes contained in the GO terms shown in Fig. 6C, many genes were found upregulated in CD8+ T cells from SD-101 plus anti-PD-1 combination group, but not in CD8+ T cells from SD-101 monotherapy group or from combination therapy group in absence of CD4+ T cells (two representative GO terms from Fig. 6C are shown in Fig. 6D as heat maps of DEGs vs. saline).

There were considerable differences between CD8+ effector T cells from SD-101 plus anti–PD-1–treated mice generated in the presence or absence of CD4+ T cells. Of 1,036 DEGs (relative to saline-treated controls) in mice treated with the combination but depleted of CD4+ T cells, only 334 (32%) were common to the 848 DEGs in the nondepleted group, indicating a substantially different transcriptional profile between these two groups (Fig. 6E). CD8+ T cells from CD4-depleted mice expressed significantly higher levels of inhibitory receptors, including Pdcd1 (PD-1), Havs2 (TIM-3), Cila4, Lg3, and Cd39 (Fig. 6F), confirming flow cytometric measurements of the corresponding surface proteins (Fig. 5C and D). These dysfunctional CD8+ T cells also showed increased expression of IL10, suggesting they actively contribute to immunosuppression within the tumors.

Markers associated with cell proliferation (Prc1, Kif67, Calu2) and apoptosis-mediating genes (Bcl2l11, Casp1-3) were also increased in these cells, whereas the transcription factor Tcf1, involved in the prevention of T-cell exhaustion and the persistence of T-cell responses (35), was markedly decreased (Fig. 6F). We then interrogated the functional significance of the genes differentially expressed in the “nonhelped” but not in “helped” CD8+ T cells when compared with saline and found the most striking overrepresentation within cell-cycle proliferation GO terms (Fig. 6G). Cell cycle–related genes were not overrepresented in any other treatment group (Fig. 6H), a finding that is consistent with the selective CD8+ T-cell expansion found in the absence of CD4+ T-cell help (Fig. 5B).

Discussion

A large body of preclinical data has demonstrated that intratumoral injection of agents that activate the innate immune response can stimulate both local and systemic antitumor T-cell responses. These findings have been translated to the clinic, where abscopal effects in patients with lymphoma and melanoma have been described in response to intratumoral TLR9 agonists and oncolytic virus therapies, given as single agents or in combination with PD-1 blockade (1, 36, 37). This promising approach is most feasible for tumors with readily accessible lesions for repeated injection, but is far more challenging to apply to lung tumors. Here, we demonstrate that delivery of an inhaled TLR9 agonist, SD-101, to mice bearing tumors in the lung and liver results in a reversal of tumor progression, recapitulating the effect obtained with direct injection into subcutaneous tumors. A different CpG-ODN, delivered intratracheally, has been reported to have activity on lung tumors when formulated as a microparticle, but not as a solution (38). In a model of B16 melanoma lung metastasis, an inhaled formulation of poly (C:C) TLR3 agonist and CpG-ODN TLR9 agonist was shown to decrease lung metastasis restoring NK-cell effector function (39).

A limited regimen of inhaled SD-101 exerts a pronounced remodeling effect on tumor-bearing lungs, affecting multiple aspects of the immune response, including the activation of T cells and their influx into lung tumors. In addition, we describe the ability of a TLR9 agonist to induce neogenesis of TLSs, composed of highly compacted B cells, DCs, and T cells in close contact, adjacent to lung tumors. TLSs are correlated with long-term survival in multiple human cancers, including NSCLC, and likely function as the site of initiation and maintenance of humoral and T-cell response against tumor antigens (30, 40). Similarly, de novo TLSs have been demonstrated to function as priming sites of T cells in the lung after mucosal immunization (41).

SD-101–induced TLSs might provide a particularly suitable site for antigen presentation and T-cell priming, as they form in close proximity to the source of tumor antigens, yet may be protected from the highly immunosuppressive environment that occurs within the tumor microenvironment itself. Inhaled SD-101 appears particularly effective at initiating TLS formation, as it induces an ideal milieu of chemokines known to promote their neogenesis (Fig. 3F). In addition, by inducing B-cell activation, SD-101 might promote B-cell organization into structures resembling lymph nodes.

Inhaled SD-101 treatment substantially increases the infiltration of moDCs with high expression of costimulatory markers, suggesting that they may function as fully mature antigen-presenting cells within the lung tumor microenvironment. moDCs can elicit CD8+ T-cell response against tumor antigens as efficiently as conventional DCs (42, 43). The increased infiltration of moDCs is accompanied by a profound reduction in the number of CD11b+Ly6G+Ly6Cint granulocytic cells (gMDSC), which show upregulation of maturation and differentiation markers on their surface (Supplementary Fig. S2C). This population is particularly expanded in 4T1 tumor-bearing lungs, where it was shown to promote tumor growth. Previous studies have shown the effect of TLR9 agonist given by the subcutaneous route in reducing the number and the suppressive function of gMDSCs in tumor-bearing mice, an effect that is mediated by IFNβ (44).

Inhaled SD-101 is quite effective during the period of active treatment; however, long-term survival after cessation of treatment is not observed in most mice. However, treatment with inhaled SD-101 and PD-1 blockade leads to a more complete reduction in lung tumor burden and to durable survival in the majority of treated mice. This represents a synergistic interaction between the two agents, as anti–PD-1 itself has little activity in the models studied here. This synergy may be due, in part, to the ability of SD-101 to generate CD8+ T cells that are PD-1lowT-bethigh, a population known to be selectively invigorated by PD-1 blockade (23). The combination also induces a significant expansion of cytotoxic CD8+ T cells (Fig. 4E and F). Changes in the transcriptional profile of effector CD8+ T cells, from SD-101 plus anti–PD-1 compared with SD-101 group, are consistent with cells undergoing morphologic and functional changes of synapse formation, cytosis, and acquisition of cell motility. Thus, anti–PD-1–induced the effector CD8+ T cells generated by SD-101 treatment to become highly functional CTLs.
leading to the much more robust and durable antitumor response in the combination group versus SD-101 alone. Whether the gene expression changes upon addition of anti–PD-1 resulted from activity of the combination or were mediated only by anti–PD-1 was not addressed in this study.

In striking contrast to studies with subcutaneous tumors treated with PD-1 blockade and intratumoral injections of SD-101 (6), we found an important role for CD4+ T cells in controlling the functionality of effector CD8+ T cells rejecting lung tumors. The population of CD8+ T cells in tumor-bearing lungs depleted of CD4+ T cells was significantly increased and showed upregulation of multiple effector markers, including KLRG1 and CD44, indicative of activated antigen-experienced T cells. However, these CD8+ T cells also exhibited a phenotype characteristic of severe exhaustion, coexpressing high levels of multiple inhibitory receptors and low levels of cytotoxicity markers. This exhaustion of CD8+ T cells may explain the paradox of tumor progression despite expansion of the effector CD8+ T-cell compartment.

The transcriptional profile of CD8+ T cells from CD4-depleted mice confirmed these data, showing upregulation of multiple inhibitory molecules, including surface checkpoint receptors, typical of T-cell exhaustion in the context of infectious disease and cancer (45). Studies in chronic viral infection models show that depletion of CD4+ T cells results in severe exhaustion of CD8+ T cells and loss of control of viral replication (46). Adoptive transfer of lymphocytic choriomeningitis virus (LCMV)-specific CD4+ T cells into chronically LCMV-infected mice restores proliferation and functionality of exhausted virus-specific CD8+ T cells, decreasing viral burdens (47). In human chronic infections, such as HIV and hepatitis C, loss of CD4+ T-cell help has been implicated in CD8+ T-cell dysfunction and increased disease (48, 49). These studies suggest that maintaining the optimal functional state of chronically antigen-stimulated CD8+ T cells requires CD4+ T-cell help. Analogous data for chronic exposure to tumor antigens have not been reported; however, a recent article reported that CD8+ T cells, generated in response to tumor antigen vaccination, are ineffective at killing tumor cells in vitro or in vivo if the vaccine does not include CD4+ T-cell epitopes (50). Some of the characteristics of these nonhelped CD8+ T cells appear similar to those reported here, including elevated expression of inhibitory receptors, and reduced expression of genes encoding cytotoxic functions and cell motility. It is important to note that in the models studied by Ahrends and colleagues, CD4+ T-cell help was absent from the initial priming of the CD8+ T-cell response, whereas in our lung tumor model, CD4+ T cells were depleted just before the initiation of immunotherapy and the initial exposure to tumor antigens took place in the presence of potential CD4+ T-cell help. This demonstrates that the CD8+ T-cell dysfunction in a poorly immunogenic tumor can be reversed by the combination of SD-101 and anti–PD-1 and that CD4+ T-cell help plays a key role in this reversal.

One possible role for CD4+ T cells may be their production of IL21, an essential helper cytokine controlling CD8+ T-cell exhaustion during viral and bacterial infections (51). We found that IL21 protein recovered in BAL fluid from treated lungs is substantially upregulated in response to SD-101 and anti–PD-1 but not in the absence of CD4+ T cells, suggesting that lack of IL21 might contribute to the severe dysfunctional state of nonhelped CD8+ T cells (Supplementary Fig. S3).

Nonhelped CD8+ T cells from tumor-bearing lungs demonstrated a strong cell proliferation signature consistent with their increased numbers. This strikingly contrasts with numerous reports in infectious disease models, in which loss of proliferation is reported as a characteristic feature of exhaustion (45). A recent study profiling LAG-3−/− 4-1BB−/− CD8+ T cells from murine solid tumors also reported enrichment in cell-cycle GO terms, suggesting exhaustion phenotypes might differ between chronic exposure to tumors or infections (52). It is also possible that the hyperproliferation of CD8+ T cells is due to loss of Treg through CD4+ T-cell depletion. However, CD8+ T-cell expansion did not occur in saline-treated tumor-bearing lungs depleted of CD4+ T cells (Supplementary Fig. S4), demonstrating that Treg absence alone in the context of lung tumors does not increase CD8+ T-cell expansion. In addition, in the absence of CD4+ T cells, there is a complete absence of TLS formation in response to SD-101 plus anti–PD-1. A crucial role of CD4+ T cells in the formation of TLSs has been demonstrated in the context of thyroid tissue inflammation mediated by overexpression of CCL21 (53), but similar findings in tumor models have not been reported. Thus, CD4+ T cells may constitute the nucleating events for the assembly of lymphoid aggregates.

The absence of CD4+ T-cell help also results in the abrogation of the SD-101–mediated increase of germinal center B cells, a result consistent with the lack of TLSs and the well-described role of CD4+ T-cell help in promoting B-cell activation and germinal center B-cell development. An important outstanding question is whether B-cell activation and antibody production mediated by SD-101 play a functional role in the generation of the CD8+ T-cell response. One limitation of this study is that only orthotopically transplanted metastatic tumor models but not genetically modified autochthonous models were studied. In particular, the rapid growth of transplanted models and the absence of a well-developed stroma may lead to differences in responses to therapy, compared with slower, spontaneous tumors. In summary, we show that localized delivery into the whole lung of the TLR9 agonist SD-101 combined with systemic PD-1 blockade results in substantial and durable reduction in tumors, both in the lung and in metastatic sites outside the lung. This demonstrated that treatment of a tumor-bearing organ can achieve results comparable with direct SD-101 injection into subcutaneous tumors. However, there are significant differences in the mechanism by which inhaled SD-101 plus anti–PD-1 achieves effective antitumor T-cell responses. These include the prominent generation of paratumoral TLSs by SD-101 itself and the requirement for CD4+ T cells in the generation of these TLSs, and in preventing exhaustion and maintaining functionality of tumor-reactive CD8+ T cells. Our findings support the use of inhaled immunostimulatory TLR9 agonists in combination with systemic anti–PD-1 for the treatment of lung cancer and other cancer types that frequently metastasize to the lung.

Disclosure of Potential Conflicts of Interest
M. Gallotta has ownership interest (including stock, patents, etc.) in a stock. E. Degagne has ownership interest (including stock, patents, etc.) in a stock. R.L. Coffman is the VP and chief scientific officer at Dynavax Technologies. C. Guiducci has ownership interest (including stock, patents, etc.) in a stock. No potential conflicts of interest were disclosed by the other authors.

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Inhaled TLR9 Agonist Unlocks Anti–PD-1 Therapeutic Potential

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