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Next steps for clinical translation of adenosine pathway inhibition in cancer immunotherapy

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ABSTRACT

Increasing evidence supports targeting the adenosine pathway in immuno-oncology with several clinical programs directed at adenosine A2 receptor (A2AR, A2BR), CD73 and CD39 in development. Through a cyclic-AMP-mediated intracellular cascade, adenosine shifts the cytokine and cellular profile of the tumor microenvironment away from cytotoxic T cell inflammation toward one of immune tolerance. A perpetuating cycle of tumor cell proliferation, tissue injury, dysregulated angiogenesis, and hypoxia promote adenosine accumulation via ATP catabolism. Adenosine receptor (eg, A2AR, A2BR) stimulation of both the innate and adaptive cellular precursors lead to immunosuppressive phenotypic differentiation. Preclinical work in various tumor models with adenosine receptor inhibition has demonstrated restoration of immune cell function and tumor regression. Given the broad activity but known limitations of anti-programmed cell death protein (PD1) therapy and other checkpoint inhibitors, ongoing studies have sought to augment the successful outcomes of anti-PD1 therapy with combinatorial approaches, particularly adenosine signaling blockade. Preliminary data have demonstrated an optimal safety profile and enhanced overall response rates in several early phase clinical trials with A2AR and more recently CD73 inhibitors. However, beneficial outcomes for both monotherapy and combinations have been mostly lower than expected based on preclinical studies, indicating a need for more nuanced patient selection or biomarker integration that might predict and optimize patient outcomes. In the context of known immuno-oncology biomarkers such as tumor mutational burden and interferon-associated gene expression, a comparison of adenosine-related gene signatures associated with clinical response indicates an underlying biology related to immunosuppression, angiogenesis, and T cell inflammation. Importantly, though, adenosine associated gene expression may point to a unique intratumoral phenotype independent from IFN-γ related pathways. Here, we discuss the cellular and molecular mechanisms of adenosine-mediated immunosuppression, preclinical investigation of adenosine signaling blockade, recent response data from clinical trials with A2AR, CD73, CD39 and PD1/L1 inhibitors, and ongoing development of predictive gene signatures to enhance combinatorial immune-based therapies.

BACKGROUND: THE ADENOSINE PATHWAY AND IMPLICATIONS FOR TUMORIGENESIS

Building on the success, but well known limitations, of immune-checkpoint inhibition across tumor types,[1] rigorous efforts are now underway to identify combinatorial approaches to reverse the immunosuppressive characteristics of the tumor microenvironment (TME).[2] Phase I-III clinical trials are now assessing the safety and efficacy of anti-programmed cell death 1 (PD-1)/programmed cell death ligand 1 (PD-L1) augmentation with other checkpoint inhibitors, costimulatory agonists, antiangiogenesis agents, drugs altering the metabolic milieu of the TME and a variety of tumor-specific targeted therapies, among other approaches.

The adenosine signaling pathway has long been studied as an inhibitory neurotransmitter but has also been observed to have anti-inflammatory properties.[3] The immunomodulatory effects of adenosine were first elucidated nearly 50 years ago while studying cyclic AMP (cAMP) signaling in T cells[4 5]; however, the full impact of adenosine on pro-tumor immunity has only been appreciated more recently. Here, we will discuss the cellular and molecular mechanisms underpinning adenosine-mediated immune suppression, preclinical science investigating the immunomodulatory effects of adenosine blockade in the TME, recent clinical trials evaluating adenosine signaling inhibition in combination with other immune-based therapies, and ongoing efforts to identify biomarkers, including tumor genomic signatures, that can inform patient selection and optimal use of adenosine pathway inhibition.

Under physiological conditions, the nucleoside adenosine is present in relatively small concentrations in the extracellular space (0.05–0.2 μM).[6] In the event of tissue injury, ischemia, or cell lysis/death, intracellular ATP is released, representing an inflammatory "beacon" that heralds the invasion of both innate and adaptive immune cells, cytokines, and other signaling molecules.[7] ATP is rapidly degraded through a set of enzymatic

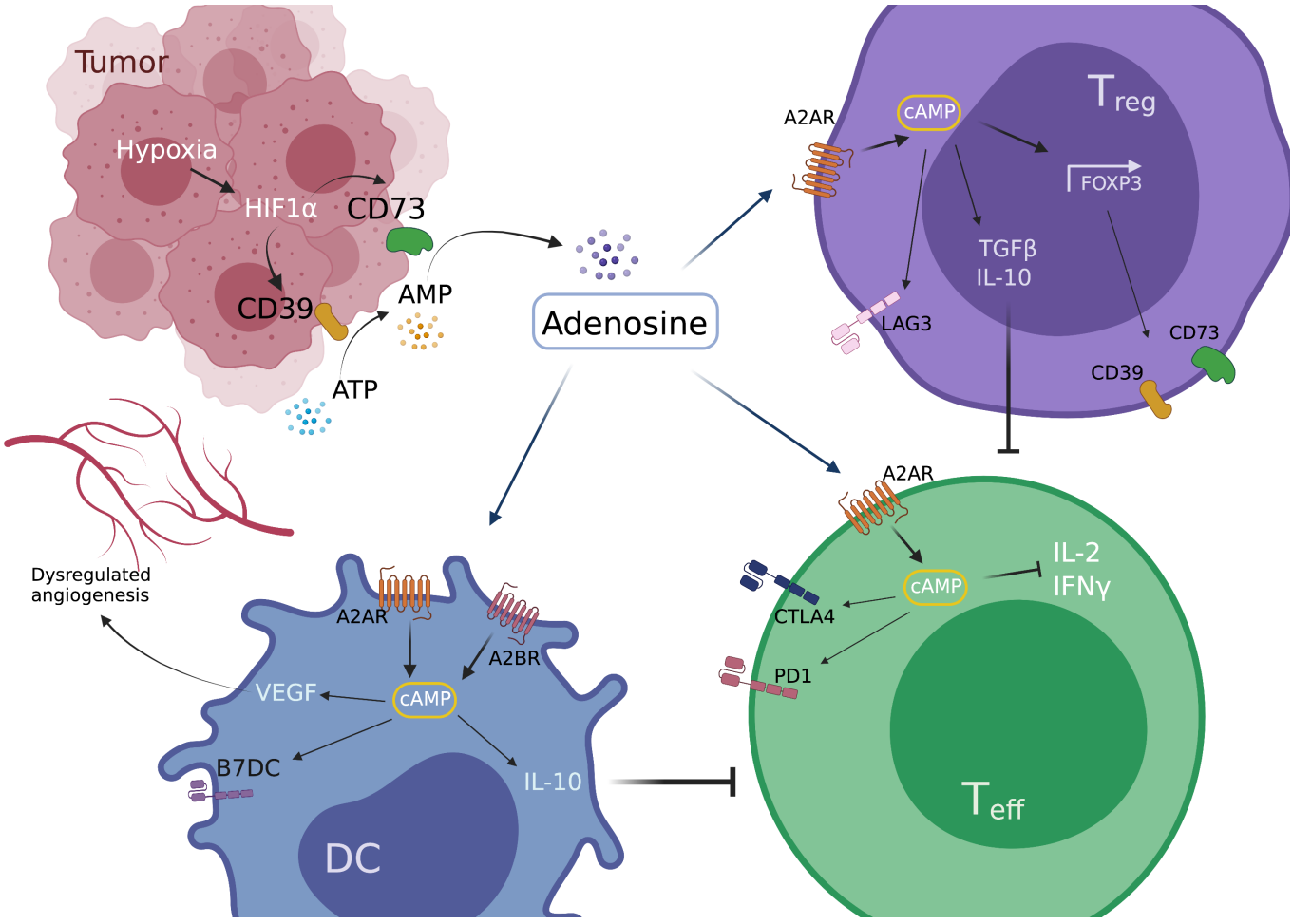

Figure 1 Adenosine-mediated modulation of immune cell function in the tumor microenvironment. IFN, interferon; IL, interleukin.

reactions to adenosine as part of a negative feedback mechanism (figure 1).[8] The ectonucleotidases, CD39 (ectonucleoside triphosphate diphosphohydrolase-1) and CD73 (ecto-5-prime-nucleotidase; NT5E), catabolize ATP to AMP, and AMP to adenosine, respectively, with the overall effect of promoting intracellular signaling through one of four G protein-coupled receptors (GPCR).[7] These GPCRs (A1, A2AR, A2BR, A3) exert their effects primarily via cAMP second messenger signaling which can lead to a host of cellular changes. This process is further heightened through hypoxia itself, an element correlated with tissue ischemia and the dysfunctional vascular supply associated with injury, inflammation and tumorigenesis.[9 10]

Hypoxia, via the hypoxia-inducible factor 1 (HIF1) transcription factor, and other cytokines such as TGF-β, upregulate CD39 and CD73, further enhancing the adenosine effector pathway.[11] Additionally, Ma *et al* showed a significant correlation between HIF1-alpha and A2AR expression in head and neck squamous cell carcinoma (HNSCC tissue). This group also demonstrated the loss of *TGFBR1* and *PTEN* tumor suppressor genes increased both A2AR and CD73 expression.[12] Finally, leukocyte A2AR expression was shown to be upregulated 4–12 hours after tumor necrosis factor (TNF)-alpha or lipopolysaccharide (LPS) exposure.[13] While this negative feedback mechanism is an important adaptation against autoimmunity and counterproductive tissue remodeling, evidence also associates adenosine signaling with a pro-tumor immunophenotype.[14]

The TME comprises a dynamic milieu of tumor, stromal, endothelial, and immune cells. The adenosine GPCRs can be found on all of these cell types with overarching signaling effects leading to immune tolerance and malignant proliferation.[6 15] Among the mononuclear antigen presenting cells (APC), cytotoxic T cells, regulatory T cells (Treg), and natural killer (NK) cells, research has primarily focused on the high affinity A2A receptor and the low affinity A2B receptor, both of which lead to increased cAMP signaling.[16] In the myeloid lineage, adenosine-mediated cAMP signaling promotes altered dendritic cell (DC) differentiation leading to a predominant myeloid DC population.[17] Myeloid DCs promote vascular endothelial growth factor (VEGF)-mediated angiogenesis, matrix remodeling, and immunosuppressive chemokine production.[17] These anomalous tumor associated macrophages (TAM) decrease IFN-γ signaling, inhibit T cell effector function, and potentiate metastatic tumor growth.[18] Additionally, A2B signaling promotes the

expansion of myeloid precursors into immunosuppressive myeloid-derived suppressor cells (MDSC), further potentiating malignant proliferation.[19] A2AR-mediated cAMP signaling in effector T cells inhibits proinflammatory cytokine production (eg, IL6, IL-17, IFN-γ), downregulates costimulatory receptors (eg, TCR, CD28), and upregulates molecular checkpoints (eg, PD1, TIM3).[6] Similar to MDSCs, Treg cells inherently suppress cytotoxicity in the TME; however, Treg cells also upregulate surface CD39, enhancing extracellular adenosine accumulation and further limiting anti-tumor immunity.[20 21] Finally, the innate cytotoxic lymphocytes, NK cells, are also suppressed via A2AR activation, further revealing adenosine's potent and multipronged immunosuppressive effects in the TME.[22] Accordingly, Young *et al* showed that A2AR-deficient NK cells led to enhanced maturity, proliferation, and tumor control in a melanoma murine model.[23]

In addition to immune cells, TME-associated endothelial and neoplastic cells also harbor adenosine receptors with differential downstream signaling effects. Not only do endothelial cells express CD39 and CD73, but multiple experiments have demonstrated adenosine-mediated signaling to evoke angiogenesis in the tumor bed.[24] Additionally, Allard *et al* showed that A2AR-mediated signaling promoted lymphangiogenesis and associated nodal metastases.[25] Given that stimulated angiogenesis in the TME can lead to chaotic and dysregulated vasculature, the pro-angiogenic effects of adenosine signaling can paradoxically promote hypoxia and further suppress the anti-tumor immune response.[26 27] Further, tumor cells themselves can also be affected by adenosine signaling but with a more nuanced, heterogeneous response. On one hand, catabolic enzymes leading to the breakdown of adenosine are downregulated in certain tumors, amplifying extracellular adenosine concentration in the TME.[28 29] Through A1 and A2A receptors, this increased adenosine has been shown to stimulate cellular proliferation in certain breast cancer models.[28] Several groups have also demonstrated a MAPK/ERK-dependent process promoting the growth and invasion of certain cancers (including breast, oral, and urothelial) via A2BR stimulation.[30–32] On the other hand, however, colon cancer and lymphoma models have demonstrated cell cycle arrest when exposed to adenosine via A3 receptor stimulation (the latter exerting its effects through the PI3K pathway as opposed to cAMP).[33] Thus, while adenosine clearly promotes an immune-tolerant environment, its regulation of cellular proliferation and apoptosis of the tumor itself requires further investigation.

Nearly 50 years ago, researchers first discovered a cAMP-dependent mechanism that could potentially explain the elusive "Hellstrom paradox"—the phenomenon describing cytotoxic T cell suppression in the TME leading to malignant immune evasion.[5] The adenosine signaling molecule quickly became one of the etiological culprits and rigorous investigation has now centered on further characterizing the adenosine pathway in the TME.[4 14] Importantly, early research failed to identify other cAMP-elevating GPCR signaling pathways that could compensate for A2AR depletion, further enticing researchers to identify possible targets for inhibition in this non-redundant pathway.[34] These targets included hypoxia and HIF1-alpha, CD39 and CD73 ectonucleotidases, adenosine itself via degrading enzymes, and the A2A/B receptors (the latter already being studied concomitantly in neurological disorders).[35 36] And while early experiments were promising, several overarching questions remained for pre-clinical investigation: (1) what off-target effects could be expected by inhibiting this abundant signaling molecule, (2) are there are other compensatory pathways, (3) could adenosine serve as a master regulator of other anti-inflammatory pathways, (4) how can we ensure that T cells are even infiltrating the TME to potentially respond to adenosine signaling blockade, and (5) what biomarkers could be used to screen tumors for efficacious therapeutic outcomes?

PRECLINICAL INVESTIGATION

Building from the seminal observations surrounding adenosine signaling and the immune response to cancer, preclinical work has focused on reversing the immunosuppressive effects of the adenosine pathway in various cancer models. While a majority of experiments have concentrated on the downstream A2A receptor (A2AR), more recent work has also investigated the blockade of more upstream elements (eg, CD39, CD73, hypoxia) with or without A2AR blockade and other checkpoint inhibitors.[15 37] Given the significant translational potential, initial studies were first directed at better characterizing the immunophenotypic changes to the TME including cytokine profiling and immune cell infiltration in addition to tumor response data. A foundational experiment by Ohta *et al* found that direct A2AR knockout could significantly decrease tumor growth in a T cell lymphoma murine model.[34] Furthermore, decreased IFN-γ levels seen in this malignant model could be rescued and amplified on pharmacological A2AR inhibition. These beneficial results, however, were found to be dependent on a strong CD8 +T cell infiltration; A2AR inhibition in a non-immunogenic B16 melanoma model did not result in a significant change in survival.[34]

Shortly thereafter, Waickman *et al* found similar results in a murine EL4 model with A2AR knockout.[38] This group subsequently combined the A2AR knockout model with a B7-DC/Fc fusion protein (to antagonize the PD1-B7/H1 inhibitory interaction)—the additional PD1-axis checkpoint inhibitor further augmented tumor regression and survival. Combinatorial approaches rapidly escalated across multiple groups to assess the effects of A2AR blockade with anti-PD1 and other agents. In 2015, researchers demonstrated synergistic effects with A2AR knockout and PD1 blockade in not only breast and colon cancer models but also a metastatic murine model.[39] This latter result corroborates prior research showing CD73

playing a critical role in the migration of tumor cells via extracellular matrix (ECM) adhesion.[40 41]

In order to better characterize the relationship between the adenosine and PD1 pathways for more effective coinhibitory options, A2AR expression was measured in T cells exposed to PD1 blockade.[39] While anti-PD1 lead to marked increase in A2AR expression as a compensatory immunosuppressive feedback mechanism, the opposite was not true. Leone *et al* studied the effects of PD1 expression on A2AR blockade which showed an overall decrease in PD1 expression along with other checkpoint pathways (eg, LAG3, FoxP3).[42] Consistently, A2AR blockade lead to a robust increase in IFN-γ, the effective T cell transcriptional factor T-bet, and the costimulatory molecule 4-1BB. Importantly, these results point to the adenosine pathway as a central regulator for other checkpoint processes. They also validate prior work by Ngiow *et al* showing a reduction in CD8 +T cell PD1 levels is necessary to achieve a response to anti-PD1 therapy.[43] Thus, A2AR inhibition may help achieve this therapeutic threshold, unlocking the benefits of PD1 blockade through this synergistic approach.

With the elucidation of the full adenosine pathway, a multitude of combinatorial therapeutic approaches have now been studied in the preclinical setting. As briefly mentioned above, CD39 and CD73 are highly expressed on the surface of Treg cells, increase extracellular adenosine concentration, and potentiate Treg activity.[21] Additionally, Samanta *et al* showed that traditional chemotherapy can induce CD47, CD73, and PDL1 expression in triple-negative breast cancer cells, serving as a potential escape mechanism in this immune-evasive model. Based on this physiology, Stagg *et al* inoculated CD73 deficient mice with colon, breast, melanoma, and lymphoma cell lines.[40] Not only was tumor growth significantly decreased as compared with wild-type mice for each malignancy, but CD73 expressing Treg cells were specifically found to recapture tumor growth, highlighting the important relationship between the adenosine pathway and the immune tolerant Treg activity.[40] Given the success of anti-CD73 monotherapy, combination studies with PD1 and CTLA4 blockade were also investigated.[44] While a reduction in tumor growth was augmented in the combination cohorts, this effect was lost in IFN-gamma deficient mice, further supporting the critical role of an inflamed TME towards successful immune based therapy.[44] CD39 was also targeted in a melanoma murine model resulting in similar, although marginal survival benefits.[45] Even more intriguing, though, was the reduction in endothelial growth along with the lack of Treg-dependent tumor growth and evasion in CD39 deficient mice. This latter effect associated NK-mediated tumor control with Treg-dependent suppression—when wild-type NK cells alone or in combination with CD39-deficient Treg cells were infused, tumor regression was achieved; however when wild-type Treg cells were combined with NK cells the tumors progressed. Overall, this experiment highlights the dual role of CD39 blockade: reducing extracellular adenosine production and counteracting Treg-mediated immunosuppression in the TME.[45] Additionally, CD39 blockade has the added potential of not only limiting downstream adenosine but also accruing ADP and ATP precursors linked to proinflammatory immune activity.[46] Finally, researchers asked whether dual adenosine pathway inhibition could augment tumor control or simply add redundancy or toxicity. Because A2AR deficient mice were shown to upregulate CD73 expression, anti-CD73 antibodies were administered alongside an A2AR inhibitor (SCH58261) in a metastatic B16F10 murine model. Dual CD72/A2AR blockade led to significantly decreased disease burden as compared with monotherapy, with beneficial effects dependent on IFN-gamma, NK, and T cell infiltration.[47]

The addition of adenosine pathway antagonists has not only been studied alongside checkpoint blockade but also targeted therapy. In $BRAF^{V600E}$-mutant melanoma, early phase trials combining vemurafenib with ipilimumab resulted in severe toxicity.[48 49] Additionally, Young *et al* showed that CD73 expression was highly correlated with aggressive disease in patients with $BRAF^{V600E}$-mutant disease.[50] Thus, providing immune stimulation via adenosine antagonism could be a more promising augmentation strategy alongside BRAF/MEK inhibitors. Accordingly, a melanoma murine model treated with both BRAF/MEK and A2AR antagonists showed significantly less metastatic activity as compared with BRAF/MEK blockade alone.[50] Future trials will be needed to evaluate both clinical efficacy and adverse effects with this combinatorial approach.

While a majority of research has thus far focused on discrete, targetable elements of the adenosine pathway, the TME is still often plagued by dysregulated angiogenesis, ischemia, and hypoxia that can further perpetuate adenosine accumulation and immunosuppression.[51] Hatfield *et al* sought to target hypoxia itself in a pulmonary tumor model.[52] On administration of continuous 60% FiO_2, a stark regression in tumors was observed as compared with the normoxic cohort. This differential response was lost, however, on depletion of intratumoral CD4+, CD8+, and NK cells.[52] Furthermore, characterization of the infiltrating T cells found a significant decrease in Treg associated FoxP3, CD39, and CD73. Finally, connecting back to the end of the adenosine pathway, A2AR-deficient models exposed to hyperoxia did not exhibit a significant change in tumor regression as compared with normoxic models. This provides further evidence that the adenosine pathway plays a critical role in potentiating immune tolerance and tumor progression.

As alluded to above, other barriers to efficacious adenosine signaling blockade include the paucity of infiltrating lymphocytes along with the rapid deterioration of an ATP-mediated proinflammatory state in the TME.[46] To address the former, Ohta *et al* studied the effects of adoptive T cell transfer on A2AR blockade in a lung metastasis model.[34] Notably, tumor regression was only seen in the cohort with sufficient

TIL levels exposed to A2AR inhibition, providing evidence for appropriate screening thresholds along with potential adjunctive therapy alongside A2AR blockade. In an effort to potentiate the ATP-mediated proinflammatory state of the TME, Perrot *et al* used traditional chemotherapy in a CD39 knockout fibrosarcoma model.[46] As compared with wild type mice, CD39 deficiency led to significantly reduced tumor growth and improved survival, an effect further augmented by PD1 inhibition. Overall, by blocking the hydrolysis of ATP on release from chemotherapy-induced cell death, an immunogenic TME can be preserved for more effective tumor control.[53 54] Lastly, Wang *et al* recently investigated the role of adenosine deaminase 2 (ADA2) as a therapeutic route towards adenosine depletion. PEGylated ADA2 was shown to stimulate T cell proliferation, promote tumor infiltration, and inhibit tumor growth in both colon and breast cancer murine models.[55]

As we move to translate these concepts into clinical trials, several questions remain. First, what are the effects of adenosine inhibition on the other receptor signaling pathways? While A2AR, and to a lesser extent A2BR, have been studied, the downstream consequences of reduced A1R and A3R signaling are less clear. Further, Cekic *et al* actually showed A2AR deficiency could impair T cell maintenance and memory differentiation, likely via reduced IL-7 signaling.[56] Second, the variety of different immune cells in the TME have a range of both anti-tumor and immune tolerant functionality—parsing out the specific effects of adenosine pathway inhibition on individual cell types will be critical. Notably, recent work has demonstrated tumor regression with DC-specific CD73 blockade, CD73 inhibition in tumors rich with cancer-associated fibroblasts, and APC-specific A2BR blockade.[57–59] This latter finding suggests the possibility of an expanded repertoire of adoptive cellular therapy with immunostimulatory adjuncts. Third, while A2A serves as a non-redundant cAMP-inducing GPCR signaling cascade, other cAMP-mediated pathways (eg, prostaglandin E2, PGE2) should also be investigated.[60] Bottcher *et al* specifically assessed the impact of tumor-derived PGE2 on infiltrative DCs.[61] In this melanoma model, PGE2 led to a decrease in DC-mediated chemoattractants and NK viability, suggesting a therapeutic target in reversing COX-mediated immunosuppression in the TME.[61 62] Finally, the aforementioned mechanism of adenosine generation may be oversimplified. For example, further characterization of the ectoenzymes CD38 and CD203a have uncovered an additional avenue by which AMP is produced via the degradation of NAD+ and ADPR, respectively.[63 64] ENPP1 has been shown to hydrolyze the STING ligand, cGAMP, simultaneously promoting adenosine accumulation and limiting STING-dependent innate immune pathways.[65] Increased CD38 expression has been associated with enhanced MDSC and Treg activity in both hematologic and solid cancers along with PD1/PDL1 resistance through its immunosuppressive adenosinergic signaling.[66–68] Though overall promising, the preclinical data mentioned above will need to be interpreted through appropriate biomarker and genotypic screening modalities to identify efficacious combinatorial regimens for successful patient outcomes in the clinical setting.

CLINICAL DATA AND BIOMARKER INVESTIGATION

The current era of immunotherapy is now focused on the integration of novel agents with standard of care checkpoint blockade with or without chemotherapy or targeted agents. As we discuss specific adenosine pathway inhibitors in the clinical setting, however, it is important to first contextualize the aforementioned biology with current principles of immunotherapy response. The foundational checkpoint inhibitors are designed to prevent T cell anergy in the TME; however, these infiltrating lymphocytes still depend on neoantigenicity to evoke an adaptive immune response.[69] Biomarkers for neoantigenicity include tumor mutational burden (TMB), but more novel genetic signatures associated with a T cell inflamed (Tinfl) TME have provided additional, comprehensive screening tools.[70] Previous studies have found a direct correlation between both increased TMB and Tinfl gene expression scores and anti-PD1 response across most tumor types.[71] While these two biomarkers do not necessarily overlap, indicating a more nuanced connection among mutational burden, neoantigen detection, and T cell infiltration, the predictive utility of a T cell inflamed TME remains strong.[72] Spranger *et al* studied the relationship between intratumoral cytotoxic T cell infiltration and immunoregulatory pathways including PD-L1, IDO and FoxP3 Treg cells.[73] Not only were these suppressive markers dependent on T cell infiltration, but IFN-γ signaling appeared to drive their development, providing a link between lymphocyte invasion, chemokine profiling, and the development of immune tolerant pathways. Using this relationship, Ayers *et al* compared an IFN-γ based signature score with survival outcomes in patients with melanoma undergoing anti-PD1 therapy.[74] Unsurprisingly, significantly improved outcomes were seen in patients with elevated Tinfl expression scores, providing an important predictive tool for checkpoint blockade.

Given the importance of the inflammatory T cell response and the deleterious effect of adenosine signaling described above, a burgeoning collection of adenosine pathway inhibitors have entered clinical trials. Primarily targeting the downstream receptors, A2AR and A2BR (table 1A), more recent trials have expanded to CD73 (table 1B) and CD39 blockade (table 1C). Results from early phase trials point to a modest but consistent ~5% overall response rate (ORR) with A2AR monotherapy and up to ~15% in combination trials. A smaller set of CD73 and CD39 inhibitors have entered clinical trials

Table 1 (A) A2AR antagonists under clinical investigation; (B) CD73 antagonists under clinical investigation; (C) CD39 antagonists under clinical investigation

(A) A2AR antagonists under clinical investigation

Pharmaceutical	Drug	Clinical Trial	Citation	Drug combinations	Indication	Outcomes
Coruvs	Ciforadenant	MORPHEUS phase 1b/2	ESMO poster #1315P	Atezolizumab; SOC	NSCLC	ORR: 18.2% (n=11)
	(CPI-444)	Phase 1/1b: NCT02655822	Fong *et al*	Atezolizumab	Advanced RCC	ORR: 3% (n=33) (mono)
			Cancer Disc. 2020			ORR: 11% (n=35) (combo)
			ASCO 2020 poster #94			Tumor regression observed in 6/10 CD68 +pts (n = 3 mono; n=3 combo)
			AACR 2017 Abstract CT119	Atezolizumab	Advanced cancers	ORR: 6.4% (n=47)
		Phase 1b: NCT04280328		Daratumumab	Relapsed or refractory MM	Est. completion: 7/2025
		Phase 1/1b: NCT03454451		CPI-006 (anti-CD73); pembrolizumab	Advanced cancers	Est. completion: 12/2023
AstraZeneca	Imaradenant (AZD4635)	Phase 1: NCT02740985	JCO abstract #5518	Durvalumab (anti-PD-L1)	mCRPC	ORR 6.1% (n=33) (mono)
						ORR 16.2% (n=37) (combo)
		Phase 1b/2: NCT03381274		Oleclumab (anti-CD73)	NSCLC (EGFRm)	Discontinued
		Phase 1: NCT02740985		Durvalumab, oleclumab, abiraterone, enzalutamide	Advanced cancers	Est. completion: 12/2021
		Phase 2: NCT04089553		Durvalumab, oleclumab	mCRPC	Est. completion: 6/2022
Arcus	Etrumadenant (AB928)*	Phase 1/1b: NCT03720678 (ARC-3)	AACR (2021) Abstract CT129	SOC (mFOLFOX-6)	mCRC	ORR: 9.1% (n=22)
		Phase 1b/2: NCT04660812 (ARC-9)	JCO Abstract #TPS150	Zimberelimab (anti-PD1), bevacizumab (anti-VEGF)	mCRC	Est. completion: 12/2023
		Phase 1: NCT03629756		Zimberelimab	Advanced RCC; mCRPCP	Est. completion: 9/2021
		Phase 2: NCT04262856 (ARC-7)		Zimberelimab, domvanalimab (anti-TIGIT)	NSCLC	Est. completion: 6/2022
		Phase 1/1b: NCT03846310 (ARC-4)		Chemo, pembrolizumab or zimberelimab	EGFRm NSCLC	Est. completion: 2/2023
		Phase 1b/2: NCT04381832 (ARC-6)		Zimberelimab, SOC	mCRPC	Est. completion: 10/2023

Continued

Table 1 Continued

(A) A2AR antagonists under clinical investigation

Pharmaceutical	Drug	Clinical Trial	Citation	Drug combinations	Indication	Outcomes
Novartis	NIR178	Phase 2: NCT03207867		PDR001 (anti-PD1)	Advanced cancers	Est. completion: 6/2022
		Phase 1/2: NCT03207867	EORTC-NCI-AACR Symp (2018)	Spartalizumab (anti-PD1)	Advanced NSCLC	ORR: 6.5% (n=62) (combo)
		Phase1 1/2: NCT02403193	JCO abstract #9089 (2018)	Monotherapy	Advanced NSCLC	ORR: 8.3% (n=24)
		Phase 1/1b: NCT04237649		KAZ954, PDR001, NZV930 (anti-CD73)	Advanced cancers	Est. completion: 2/2022
iTEOS	EOS100850	Phase 1/1b: NCT03873883		Pembrolizumab	Advanced cancers	Est. completion: 12/2022
	Inupadenant	Phase 1: NCT02740985	JCO abstract #2562 (2021)	Monotherapy	Advanced cancers	ORR: 4.8% (n=42)
Cstone Pharma	CS3005	Phase 1: NCT04233060		Monotherapy	Advanced cancers	Est. completion: 12/2021
Palobiofarma	PBF-999	Phase 1: NCT03786484		Monotherapy	Advanced cancers	Est. completion: 2/2022
Incyte	INCB106385*	Phase 1: NCT04580485		INCMGA00012 (anti-PD1)	Advanced cancers	Est. completion: 7/2023

(B) CD73 antagonists under clinical investigation

Pharmaceutical	Drug	Clinical Trial	Citation	Drug combinations	Indication	Outcomes
AstraZeneca	Oleclumab (MEDI9447)	Phase 1b/2: NCT03381274	AACR 2021	Osimertinib	NSCLC (EGFRm)	ORR: 23.1% (combo) (n=26)
		Phase 1/1b: NCT02503774	ASCO 2021	Durvalumab	Advanced cancers	ORR: 5.5% (combo) (n=126)
		Phase 2: NCT03822351	ESMO 2021	Durvalumab	NSCLC	ORR: 38.3% (combo) (n=60)
I-MAB (Tracon)	Uliledlimab	Phase 1: NCT03835949	ASCO 2021	Atezolizumab	Advanced cancers	ORR: 23.1% (combo) (n=13)
Arcus	AB680	Phase 1/1b: NCT04104672	ASCO GI 2021	NP/Gem+zimberelimab	mPDAC	ORR: 41% (combo) (n=17)
Corvus	Mupadolimab (CPI-006)	Phase 1/1b: NCT03454451		Ciforadenant ±pembrolizumab	Advanced cancers	Est completion: 12/2023
BMS	BMS-986179	Phase 1/2a: NCT02754141		Nivolimuab	Advanced cancers	Est completion: 2/2023
Novartis/surface	NZV930 (SRF373)	Phase 1/1b: NCT03549000		Anti-PD1±NIR178	Advanced cancers	Est completion: 2/2022
Akesobio	AK119	Phase 1/1b: NCT04572152		AKI104	Advanced cancers	Est completion: 1/2022
Symphogen	SYM024	Phase 1: NCT04672434		SYM021	Advanced cancers	Est completion: 3/2024
Incyte	INCA00186	Phase 1: NCT04989387		Retifanlimab	Advanced cancers	Est completion: 5/2024
ORIC	ORIC-533	FDA approval for clinical trial launch (6/2021)		TBD	Advanced cancers	TBD

Continued

Table 1 Continued

(C) CD39 antagonists under clinical investigation

Pharmaceutical	Drug	Clinical Trial	Drug combinations	Indication	Outcomes
Tizona	TTX-030	Phase 1/1b: NCT04306900	Budigalimab, pembrolizumab	Advanced cancers	Est. completion: 12/2022
Innate	IPH5201	Phase 1: NCT04261075	Durvalumab±oleclumab	Advanced cancers	Est. completion: 2/2023
Surface	SRF617	Phase 1: NCT04336098	Pembrolizumab	Advanced cancers	Est. completion 11/2022

*, A2AR & A2BR antagonist ; NSCLC, non-small cell lung cancer; ORR, overall response rate; RCC, renal cell carcinoma.

with a wide range of response rates and number of participating patients. While the largest trial of oleclumab (anti-CD73)±durvalumab (NCT02503774) showed only marginally improved ORR,[75] more recent results from the randomized phase 2 COAST trial in stage III non-small cell lung cancer (NSCLC) revealed a promising ORR close to 40% in the dual CD73/PDL1 blockade arm with statistically improved 10-month PFS (64.8 vs 39.2) as compared with anti-PDL1 alone (table 1B).[76] Of note, since EGFR mutant NSCLC has been shown to upregulate CD73 as a putative immune escape mechanism, oleclumab has also been studied alongside the EGFR inhibitor osmiterinib in NSCLC.[77] Additionally, rates of dose-limiting toxicity in these trials have been extremely low, making these drugs even more suitable for combinatorial therapies. Finally, interpretation of the modest benefits mentioned above should be contextualized with the respective patient cohorts. A vast majority of these early phase trials only recruited patients with refractory disease, most progressing past second or third lines of therapy, and many with anti-PD1 resistance. Thus, improved response to dual adenosine plus checkpoint blockade could be expected in certain patients if offered as primary therapy.

The most frequently studied cancers in trials of adenosine pathway inhibitors to date include NSCLC, melanoma, renal cell carcinoma (RCC), and metastatic castrate resistant prostate cancer (mCRPC)—adenosine blockade has at least modest monotherapy activity in each of these malignancies. And while it is tempting to collectively describe the immune-mediated therapy across the group, each cancer type may have a unique set of biological processes that determine responsiveness to adenosine blockade. For example, prostate cancer, which has had limited success with anti-PD1 therapy, has been shown to have an extensive set of adenosine-producing enzymes apart from CD39 and CD73, including prostatic acid phosphatase (PAP), alkaline phosphatase and CD38.[15] This latter enzyme has also been studied in melanoma tumors and, in combination with CD203a and CD73, has been shown to lead to extracellular adenosine generation through NAD+hydrolysis (as opposed to ATP catabolism).[78] Additionally in RCC, hypoxia-induced HIF1 transcriptional regulation (exacerbated by mutant von Hippel-Lindau protein) can induce CD39 and CD73 activity, leading to further adenosine-mediated immune tolerance.[79–81] Finally, differential expression of immunosuppressive cytokines (eg, TGF-ß) along with infiltration of regulatory MDSC and TAM cells can further disrupt the immune landscape.[7] Thus, while the adenosine pathway may be constitutively active in most tumors, identifying cancer-specific pathologic markers will be critical in achieving more effective response rates while targeting this pathway.

The clinical results presented thus far have shown both monotherapy benefit along with combinatorial success alongside PD1, targeted, and dual-adenosine pathway inhibitors. However, the response rates have been relatively modest prompting further investigation to better characterize both the genomic signatures and phenotypic markers linked to therapeutic success. A previous study by Trujillo *et al* found that immunotherapy-relevant genes were positively correlated with PD-L1 levels across the cancer genome atlas (TCGA) cancer types.[82] Recognizable genes in the PD-L1 correlated cluster include *CTLA4, TIGIT, LAG3*, and *CD8A*, while non-PD-L1 correlated genes include *VEGFA* and *TGF-β*. Interestingly, when we integrated genes known to regulate the adenosine pathway into this model, all but one were associated with the non-PD-L1 correlated cluster (figure 2). This is an important finding as most checkpoint inhibitors are primarily effective in PD-L1 expressing tumors at baseline. Conversely, this adenosine expression data may point to a markedly different intratumoral phenotype that no longer depends on IFN-γ related pathways.

To further expand this concept, assessment of genomic signatures from a recent clinical trial of patients with RCC treated with A2AR±PD-L1 blockade could be informative. In this work by Fong *et al* an "AdenoSig" gene signature was identified—a collection of genes with significantly induced expression on administration of an adenosine agonist (*CXCL1, 2, 3, 4, 5, ILB, IL1B, PTGS2*).[83] When comparing this signature with response rates to adenosine blockade, a preliminary correlation was identified, indicating the AdenoSig score may predict response to A2AR inhibitors, at least in RCC. Notably, this signature was found to be a negative predictor within the collective data of The Cancer Genome Atlas, suggesting AdenoSig

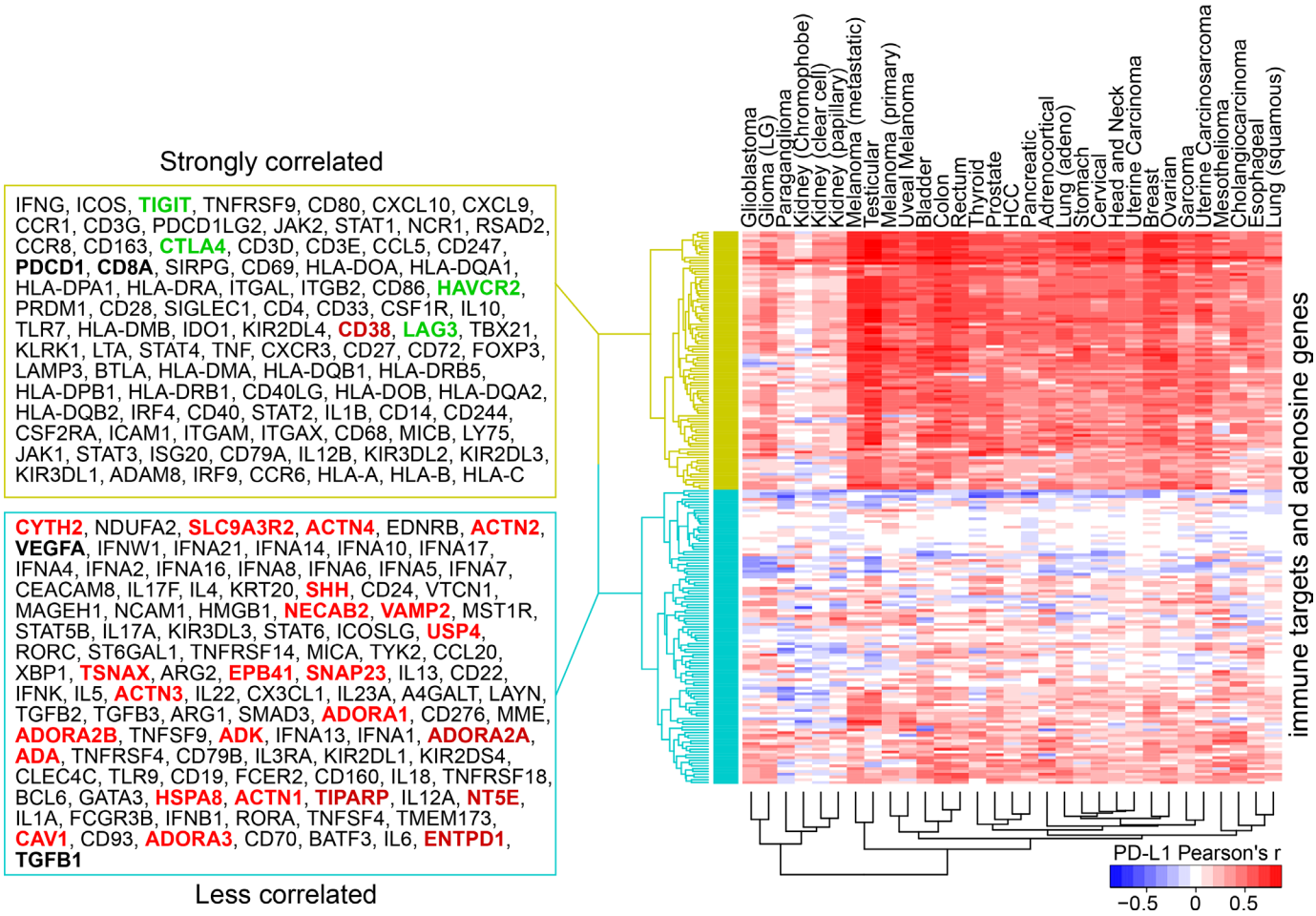

Figure 2 Heatmap of Pearson R coefficients between PD-L1 expression and immune target genes plus adenosine related genes by tumor type. Green = checkpoint targets; Red = adenosine related genes (dark red = targets under clinical investigation); Bold = other immunomodulatory genes.

as a poor risk, immunosuppressive feature, but one that is differentiated from IFN-γ related signatures tied to anti-PD1 response. Finally, in assessing the AdenoSig expression scores among the cancer types with the most clinical trial data thus far (NSCLC, melanoma, RCC, mCRPC), there does not appear to be a consistent pattern among the responding tumor types.[84] This heterogenous data points to additional biologic pathways that will require investigation to more accurately predict response to A2AR blockade.

Supporting the potential biological relevance of the AdenoSig, McDermott *et al* independently assessed a "myeloid inflammatory" gene signature that was associated with immunosuppressive myeloid-related inflammation (*CXCL1, 2, 3, 8, IL-6, PTGS2*) in patients with RCC treated in a clinical trial of anti-VEGF and anti-PD-L1.[85] This signature differed by only one gene as compared with AdenoSig and was associated with overall poor response.[83 85] In tumors with high T cell infiltration and high myeloid inflammatory scores, anti-PD-L1 alone resulted in persistently poor outcomes; it was not until the combination of anti-PD-L1 plus VEGF blockade that outcomes were improved. The authors of the AdenoSig score also recognized this connection but observed that patients with AdenoSig^{hi} tumors had an inverse correlation with angiogenesis-related gene expression. Thus, it is hypothesized that anti-PD1/L1 plus A2AR blockade would be the optimal treatment modality for patients exhibiting elevated AdenoSig or myeloid inflammatory scores as opposed to antiangiogenesis therapy. Regardless, two independent groups identifying very similar genomic signatures associated with immunosuppression and response to immunotherapy is likely relevant. As shown in figure 3A, the tight correlation between the AdenoSig and myeloid inflammatory signatures likely indicates an underlying biology relevant for both VEGF and A2AR blockade.

Another genomic signature, the Adenosine Signaling Score (as opposed to AdenoSig), was developed by Sidders *et al* and comprises genes with expression correlated with A2AR signaling in human cancers (*PPARG, CYBB, COL3A1, FOXP3, LAG3, APP, CD81, GPI, PTGS2, CASP1, FOS, MAPK1, MAPK3, CREB1*).[86] This score was directly correlated with adenosine concentration and significantly reduced in A2AR knockout models. Additionally, the Adenosine Signaling Score was inversely correlated with anti-PD1 response in a conglomerate of patients with NSCLC, HNSCC, and melanoma.[87] As opposed to

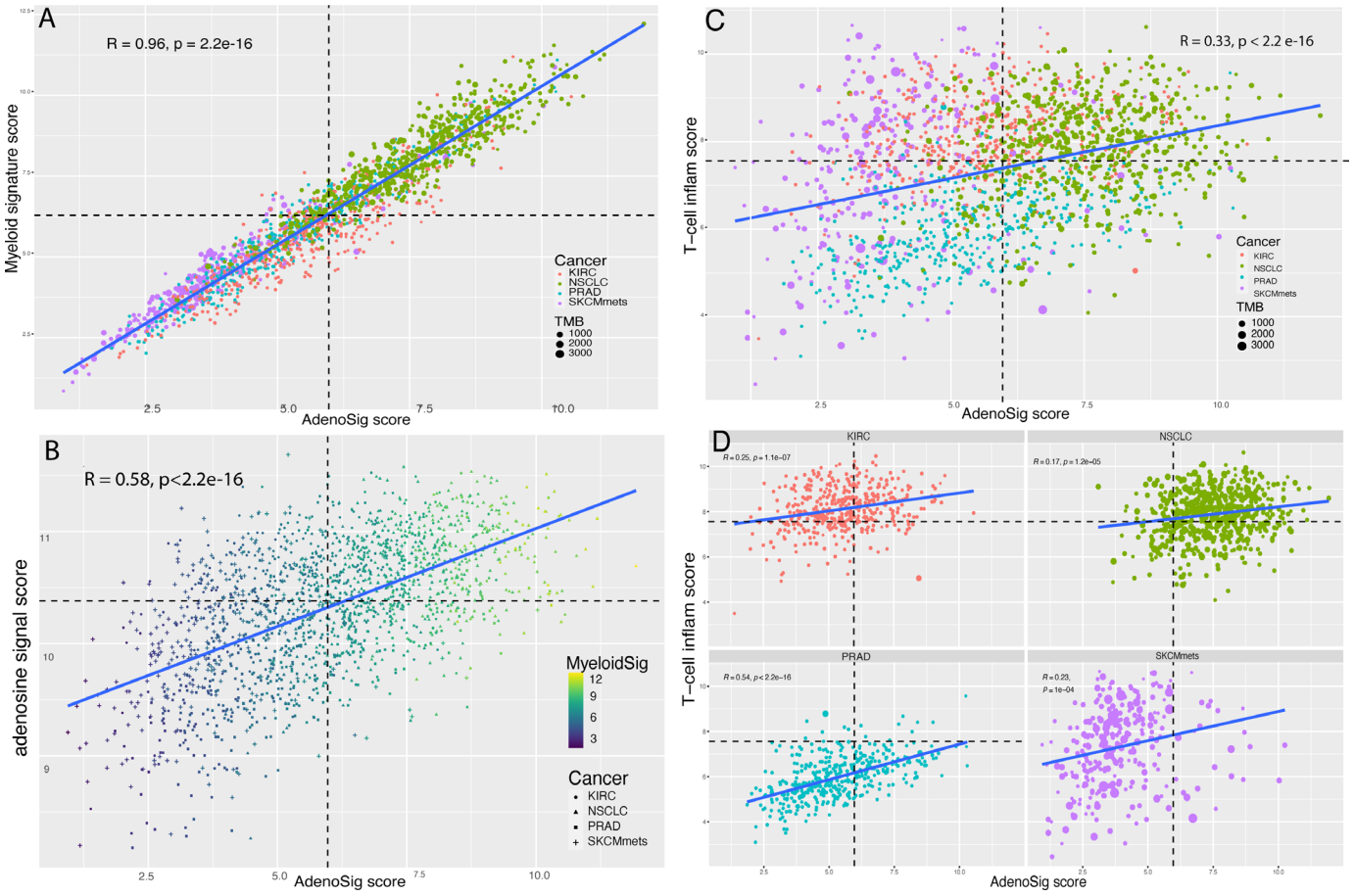

Figure 3 Correlation between the (A) AdenoSig and myeloid signature scores; (B) AdenoSig, adenosine signal, and myeloid signatures; (C & D) AdenoSig and T cell inflamed signatures scores among RCC (KIRC), NSCLC, prostate (PRAD), and melanoma (SKCMmets) tumor samples from the TCGA consortium (see methods). KIRC, kidney renal cell carcinoma; NSCLC, non-small cell lung cancer; PRAD, prostate adenocarcinoma.

the checkpoint inhibitor response, this score was highly predictive of improved outcomes to A2AR blockade in a cohort of patients with mCRPC.[88] In assessing all tumors within TCGA, the adenosine signaling signature has a wide range of expression; and as with AdenoSig, there does not appear to be a consistently elevated score among the cancer types best studied for response to A2AR inhibition (NSCLC, RCC, melanoma, mCRPC).[86] Again, elucidation of additional biomarkers will be crucial for more accurate interpretation of this genomic signature moving forward.

Despite the AdenoSig and adenosine signaling scores only sharing one gene in common, they have a relatively high correlation among the four cancer types previously discussed (figure 3B). Thus, the biology behind the two signatures is likely related, and both scores might offer utility for clinical trial screening. Based on previously mentioned work, however, building a model including T cell-inflamed gene expression with these adenosine signatures will be crucial for optimizing outcomes with A2AR inhibitors. As seen in figure 3C, the T cell-inflamed and AdenoSig gene signatures are positively correlated, although modestly, denoting a subpopulation of Tinfl^{hi}/AdenoSig^{hi} tumors that may be most relevant to target in the clinical setting. In separating these cancer types, nuanced characteristics are revealed (figure 3D). For example, prostate tumors have the highest correlation between Tinfl and AdenoSig scores, melanoma samples have lower overall AdenoSig scores but significantly higher TMB levels, and NSCLC appears to be most abundant in Tinfl^{hi}/AdenoSig^{hi} tumors. Overall, these genetic signatures together with other biomarkers could help select individual tumor samples across various cancer types that may most benefit from adenosine pathway and checkpoint inhibitors.

In looking towards next steps in the field, certain clinical trials have already started to incorporate biomarkers into the patient selection process. For example, the clinical development of INCB106385 as an A2AR antagonist±anti-PD1 therapy will only recruit patients with cytotoxic T cell positive tumors, as measured by immunohistochemistry.[89] Another trial with ciforadenant±atezolizumab reassessed response data based on the presence of tumor-infiltrating CD68 +myeloid cells, an effector cell for adenosine mediated immunosuppression (table 1A). Consistently, a significantly higher number of A2AR responders were found in the CD68+^{hi} cohort as compared with the patients with CD68 +^{lo} tumors.[90] While

prudent use of these biomarkers is encouraging, further incorporation of the aforementioned adenosine-related and T cell-inflamed gene signatures will be an important step forward in developing clinical trials. Other future work will focus on combining multiple targets of the adenosine pathway (eg, HIF1-alpha, CD38, CD39, CD73, A2AR inhibitors), incorporating A2AR antagonists into earlier lines of therapy alongside PD1 or VEGF inhibitors, and combining novel immune-based therapies—as seen in an ongoing phase II clinical trial with PD1, TIGIT, and A2AR inhibition in PD-L1^{hi} NSCLC.[91]

In conclusion, the adenosine pathway is a well-characterized mediator of immunosuppression in the TME. Various aspects of this pathway can be targeted with minimal toxicity, further adding to the clinical repertoire of immunostimulatory agents. While early trials have shown modest benefit with A2AR blockade, considerable work is now underway to assess combinatorial strategies with checkpoint inhibition and other immune-based therapies. Finally, the most successful patient outcomes may depend on advanced biomarkers and genomic signatures associated with both T cell-enriched tumors and the adenosine signaling pathway.

METHODS: GENE EXPRESSION ANALYSIS, HEATMAP, AND CORRELATION PLOTS

RNA-seq gene expression data (release February 4, 2015), preprocessed by the Broad Institute, was downloaded for 31 solid tumor types from TCGA. Note, diffuse large B-cell lymphoma, acute myeloid leukemia, and thymoma data were removed due to disproportionately elevated immune cell transcripts. Gene expression was quantified by RNA-Seq by Expectation Maximization algorithm and raw read counts were subsequently mapped to gene features. The count-based gene expression was normalized across all samples using the upper quartile method with subsequent $\log_2$ transformation. A total of 9508 tumor samples were included in the analysis. The tumor samples were filtered by non-T cell-inflamed, intermediate, and T cell-inflamed tumor groups using a previously defined 160-gene T cell-inflamed signature (Tinfl).[92] A list of 166 immune molecules involved in tumor-immune cell interaction along with 25 adenosine related genes were selected and correlated with *PD-L1* (CD274).[82] Pearson's correlation was computed between the gene expression of each immune molecule and *PD-L1* for each tumor. These correlation values were used for hierarchical unsupervised clustering with Euclidean distance. Two distinct clusters were identified consisting of strongly correlated and less correlated genes used for heatmap construction.

For the correlation plots, a gene set enrichment analysis was performed for the Tinfl, AdenoSig, adenosine signal score, and myeloid gene signatures using expression data from the TCGA tumor types of interest. All analyses were performed using Bioconductor packages in R (V.4.0.3)

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