Introduction
High levels of extracellular adenosine generated in the tumor microenvironment (TME) engage A2aR and A2bR adenosine receptors on immune cells, resulting in immunosuppression (Figures 1-3). Expression of A2aR and A2bR can vary by cell type with T cells predominantly expressing A2aR, while macrophages express both A2aR and A2bR and some cancer cells primarily expressing A2bR.

We describe the transcriptional effects of adenosine on immune cells and provide a mechanistic rationale for restoration of anti-tumor immune responses with the dual adenosine receptor antagonist etrumadenant.

Methods
Isolated immune cell experiments: CSFF T cells and dendritic cells (DC) were isolated from healthy human blood by negative selection. DC were matured with LPS/IFNγ for 24 hours in the presence of NECA (sythetic recombinant receptor agonist) or antagonists, then analyzed by RNA sequencing and cytometric bead array.

PBMC CRES production: PBMC were freshly isolated from healthy human blood and incubated with anti-CD3/anti-CD28 beads in the presence of adenosine receptor agonists and antagonists for 24 hours. Supernatants were collected and analyzed for CRES production by ELISA.

TAM and MDSC experiments: TAM and MDSC were isolated from tumor tissues of tumors expressing B180F101 or tumors by sequential magnetic bead isolation TME (FluoroMax+, magneticMSC IgGγ, mMSC IgGβ-1-4). Cells were stimulated with NECA + antagonist for 24 hours and their RNA was extracted for Nanostring gene expression analysis.

Without data analysis: Tumor-Strained Total RNA fractions from DC were sequenced on 50M 150bp PE reads. Genes were quantified using STAR aligner and false-negative quantification with Genelex 36. Differential expression analysis was performed using a moderated t-test. Pathway analysis was performed using webGAST and the sprenne package "tumors" which accounts for inter-gene correlations. Pathway gene sets were defined from MGI.org. A CIT score using was obtained from McDavid et al., and adenosine response was defined experimentally using monocline derived cells analyzed by Nanostring.

Table 1: Potency and Selectivity of Adenosine Receptor Agonists and Antagonists

<table>
<thead>
<tr>
<th>Potency</th>
<th>Selectivity</th>
<th>A2aR agonist</th>
<th>A2aR antagonist</th>
<th>A2bR agonist</th>
<th>A2bR antagonist</th>
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<tr>
<td>5 µM</td>
<td>95%</td>
<td>10</td>
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<td>90%</td>
<td>10</td>
<td>4.4</td>
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</tr>
<tr>
<td>50 µM</td>
<td>75%</td>
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Conclusions

In tumor cells, epidermal and CD8+ T cells expressing cancer cells, dual A2aR/A2bR antagonist with etrumadenant (preclinical candidate) demonstrated immunosuppression and gene expression changes greater than A2a-selective antagonists.

These studies build upon the established rationale for targeting A2aR in T and NK cells, demonstrating the importance of A2bR in adenosine-mediated immune cell immunosuppression, and provide a mechanistic rationale for restoration of anti-tumor immune responses with the dual adenosine receptor antagonist etrumadenant.

Figure 1A: Adenosine production from RFP-infused mice vs TME. Levels of ATP, ADP, and ATP were quantified in TME of adenosine receptor agonists and antagonists for 24 hours. Supernatants were collected and analyzed for CRES production by ELISA.

Figure 1B: PBMC CRES production: PBMC were freshly isolated from healthy human blood and incubated with anti-CD3/anti-CD28 beads in the presence of adenosine receptor agonists and antagonists for 24 hours. Supernatants were collected and analyzed for CRES production by ELISA.

Figure 1C: Tumor-Strained Total RNA fractions from DC were sequenced on 50M 150bp PE reads. Genes were quantified using STAR aligner and false-negative quantification with Genelex 36. Differential expression analysis was performed using a moderated t-test. Pathway analysis was performed using webGAST and the sprenne package "tumors" which accounts for inter-gene correlations. Pathway gene sets were defined from MGI.org. A CIT score using was obtained from McDavid et al., and adenosine response was defined experimentally using monocline derived cells analyzed by Nanostring.

Figure 2: Adenosine Signaling Drives Immunosuppression in T Cells Through A2aR Which is Reversed by Etruma.

Figure 3: Etruma Provides Greater Suppression of Adenosine-Mediated Changes than A2a-selective Antagonist in Isolated Human DCs.

Figure 4: A2aR Signaling in Cancer Cell Lines Drives Gene Expression Changes That Are Blocked by Etruma.

Figure 5: Etruma in Combination with Doxorubicin Reduces 4T1 Syngeneic Tumor Growth and Lung Metastases.