

## Background

FOXP3-expressing regulatory T cells (Treg) are crucial for immune balance and tolerance, but they can lose stability, transforming into impaired Foxp3<sup>lo</sup> Tregs (exTregs), which exacerbate inflammation. Purine metabolites like adenosine and ATP are believed to play critical roles in cellular metabolism and immune responses. We hypothesized their involvement in regulating Treg stability, suggesting that disruptions in purine metabolism might lead to decreased Foxp3 expression.

## Materials and Methods

**Mice and Cells:** Foxp3GFP mice on a C57BL/6 background were used to isolate CD4<sup>+</sup>Foxp3GFP<sup>+</sup> nTregs. Primary dermal lymphatic endothelial cells (LECs) of C57BL/6 mice (C57-6064L) were cultured according to the manufacturer's instructions in provided mouse endothelial cell medium. FACS Aria II used cell sorting, and cell purity was always >98%.

**Treg conversion to exTreg assay:** The inverted transwell (TW) insert (24-well) with 5 μm pore size was coated with 0.2% gelatin for 1 hour at 37°C before seeding with 1.0x10<sup>5</sup> LECs in 100 μl mouse endothelial cell medium. Two days later, 2x10<sup>5</sup> Tregs in 100 ul were loaded into the upper well of the Boyden chamber while the lower chamber contained mCCL19 (50ng/ml) (Figure 1). All cells were prepared in IMDM containing transferrin/insulin and 0.5% fatty acid-free BSA. After 16-hour incubation, Foxp3 expression was assessed in cells from the upper and lower chambers by flow cytometry.

**Quantification of cellular metabolites by Mass Spectrometry:** We sorted 2x 10<sup>5</sup> Tregs (CD25<sup>hi</sup>Foxp3<sup>hi</sup>) and exTregs (CD25<sup>lo</sup>Foxp3<sup>lo</sup>) from upper and lower wells following a 16-hour incubation with LEC in a Boyden chamber and analyzed cellular metabolites using the capillary electrophoresis Fourier - transform mass spectrometry (CE-FTMS) (Human Metabolome Technologies America, Inc.).

**Perturbation of purine metabolism:** We employed pharmacological antagonists targeting intracellular de novo purine synthesis, salvage pathway, degradation, and the extracellular purine metabolic pathway (Table 1).

**Assessment of the impact of purine metabolism perturbation on gene expression:** Treg markers, including GIRT, Helios, T-bet, and RORγT, were quantified using qRT-PCR. LEC markers, including Lyve-1, VCAM-1, VLA-4, P-, E-, L-selectins, CXCR4, CCR7, VE-Cadherin, ICAM-1, LFA-1, were assessed using flow cytometry.

**Statistical Analysis:** Each experiment included duplicates, with a total of 2-3 experiments conducted. Statistical significance was determined using either unpaired t-tests or one-way ANOVA tests. \* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001

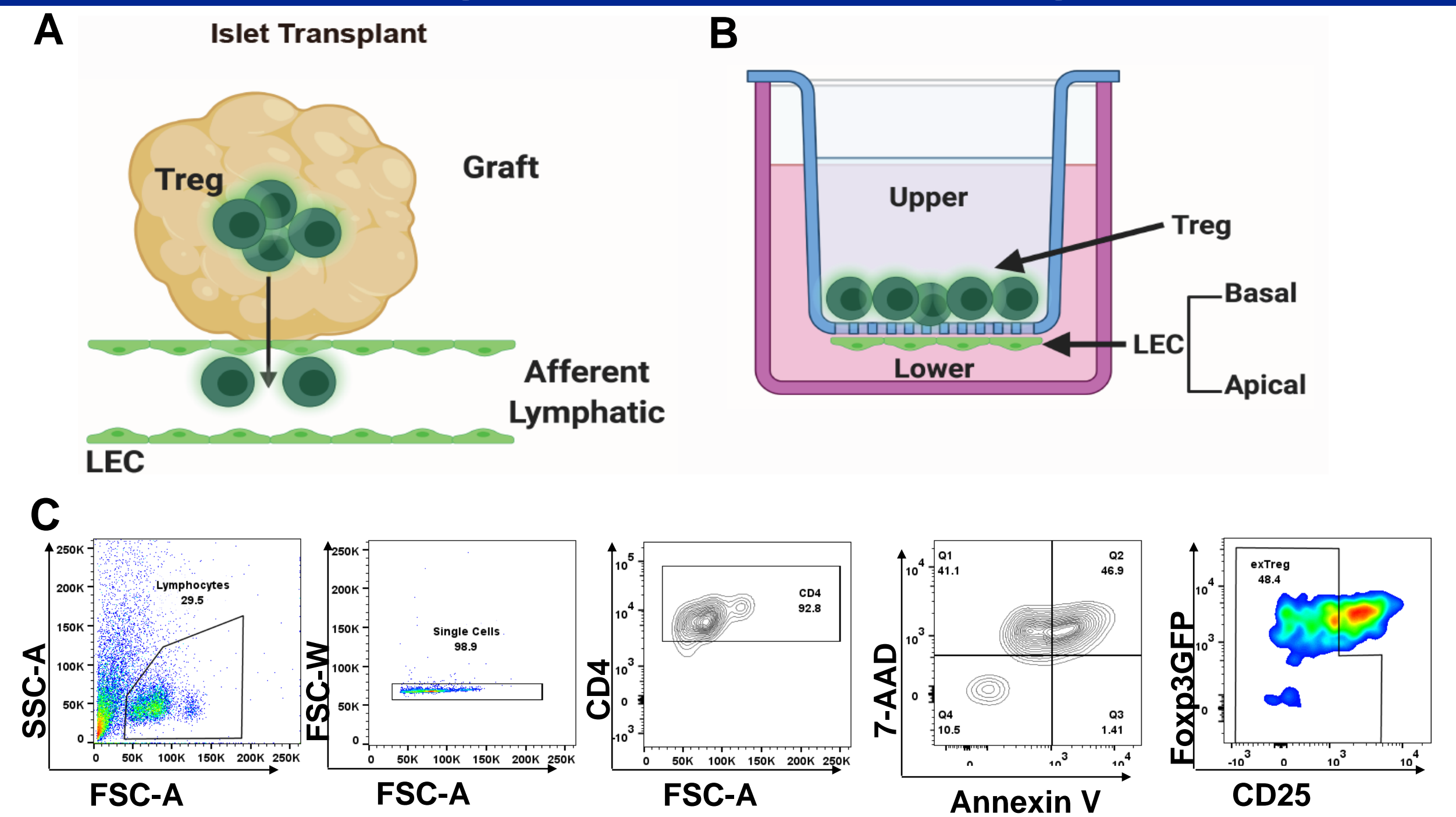
Table 1 Purine metabolic enzyme antagonists

Purine Metabolic Pathways	Antagonists	Targets
De Novo Synthesis	6-Thioguanine (6-TG)	DNA synthesis Hypoxanthine-guanine phosphoribosyltransferase (GPRT/HPRT)
	6-Mercaptopurine (6-MP)	DNA synthesis Hypoxanthine-guanine phosphoribosyltransferase (GPRT/HPRT)
Salvage Pathway	Azathioprine Fludarabine	5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR)
	2'-Deoxycytosine (DCF) ABT 702 hydrochloride (ABT-702)	Adenosine deaminase (ADA) Adenosine kinase (ADK)
Extracellular Pathway	Sodium Polyoxotungstate (POM1) Adenosine 5'-(α,β-methylene)diphosphate sodium salt (AMPCP)	PD39 CD73
	78c	CD38
Degradation	Allopurinol	Xanthine oxidase (XO)
	9-Deazaguanine (9-DG)	Purine nucleoside phosphorylase (PNP)

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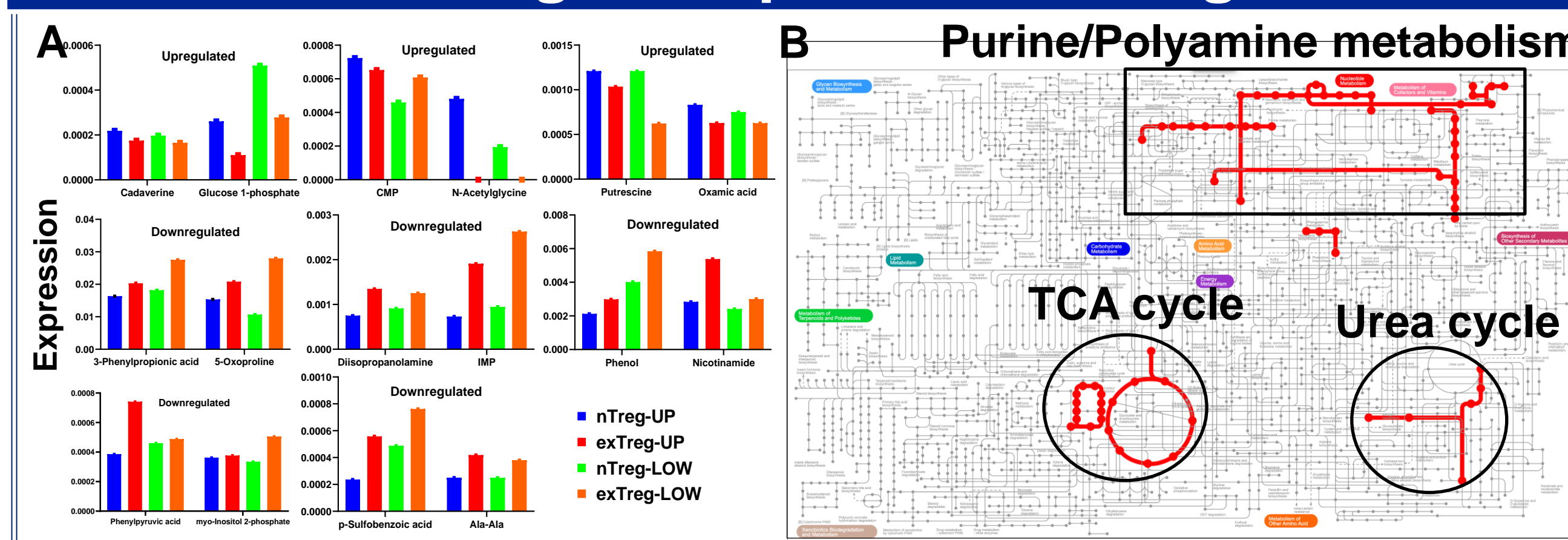
## Results

### Development of an in vitro assay to assess Treg transition to exTregs



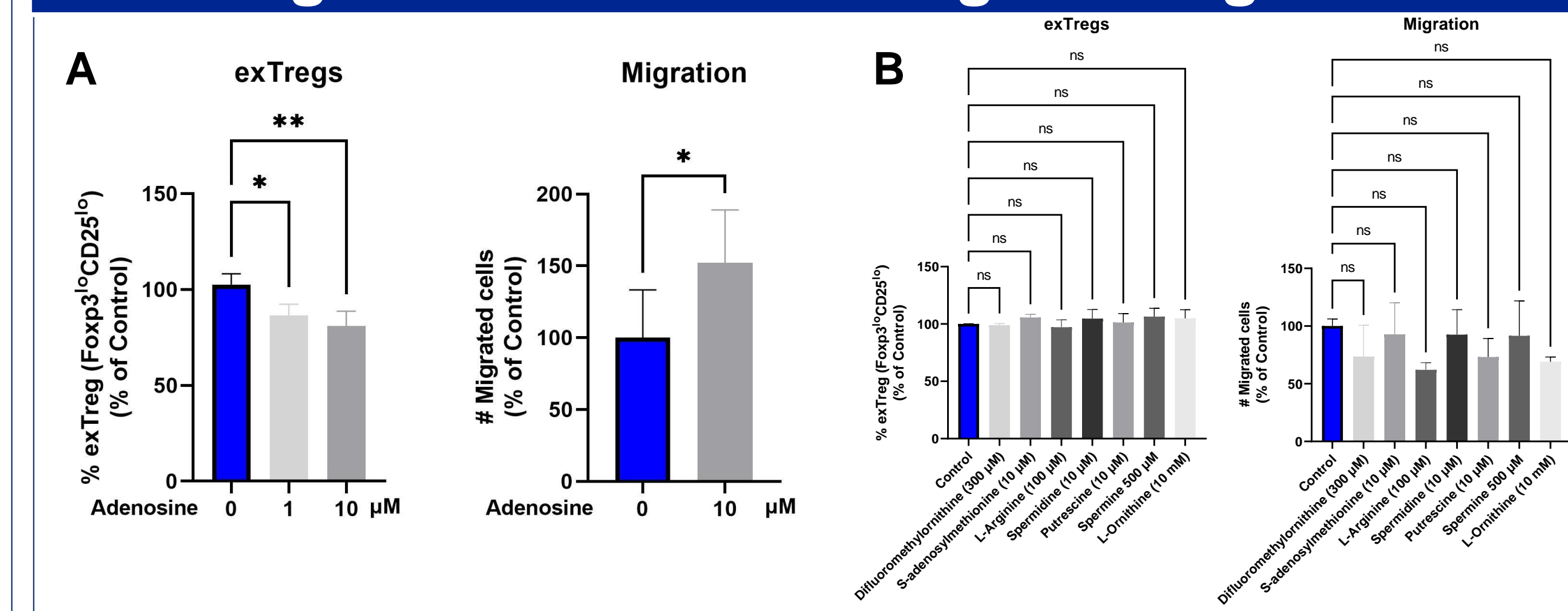
**Figure 1.** (A) In an islet transplant model, Tregs migrate from graft tissue to draining lymph node (dLN) via afferent lymphatics. During migration, Tregs contact the basal surface of LEC. (B) An in vitro assay was constructed to simulate the arrangement and interaction of Treg with LEC using a Boyden chamber transwell (TW)-based assay. Tregs are loaded in the upper well and migrate across LEC grown on an inverted TW membrane. Non-migrated Tregs from the upper well and migrated cells from lower well are harvested and analyzed by flow cytometry. (C) Gating Strategy for exTreg assay. Following harvesting from upper well and lower wells, Tregs were stained with CD4, CD25 and then stained with Annexin V and 7-AAD. Live Treg (CD25<sup>hi</sup>Foxp3<sup>hi</sup>) and exTreg (CD25<sup>lo</sup>Foxp3<sup>lo</sup>) were assessed.

### Purine metabolites differentially expressed in Tregs compared to exTregs



**Figure 3.** (A) Expression of representative 16 metabolites differentially expressed in Tregs compared to exTregs. (B) Functional module analysis showing that the 16 candidates are involved in three metabolic pathways including tricarboxylic acid cycle (TCA cycle), urea cycle, and purine/polyamine metabolism.

### The effect of purine and polyamine metabolites on Treg conversion to exTreg and migration

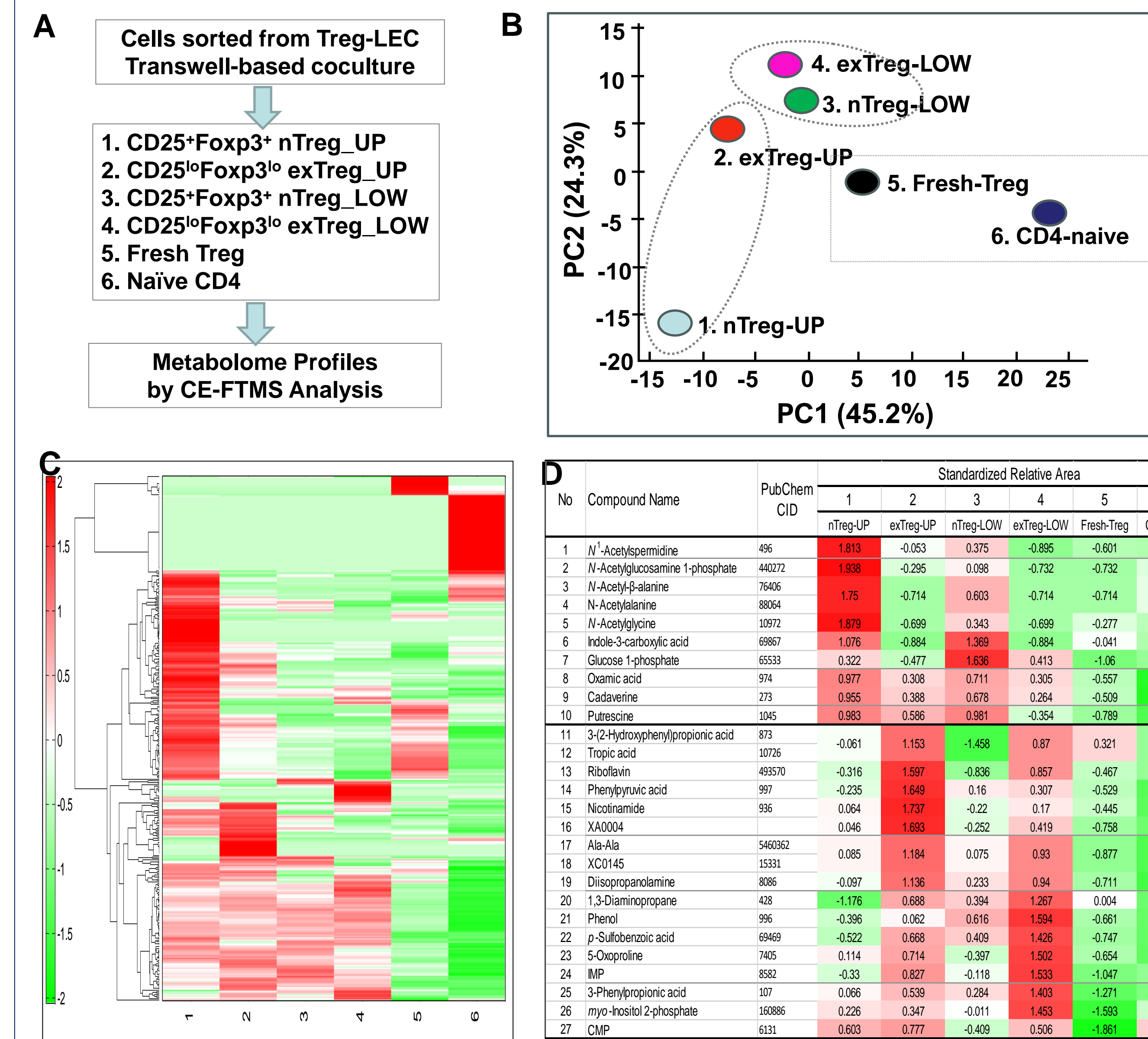


**Figure 4.** The effect of a purine metabolite, adenosine (A) and polyamine metabolites (B) on Treg conversion to exTregs and Treg migration across LEC. Polyamine metabolites include Difluoroethylornithine, S-adenosylmethionine, L-Arginine, Spemidine, Putrescine, Spermine, and L-Ornithine.

## References

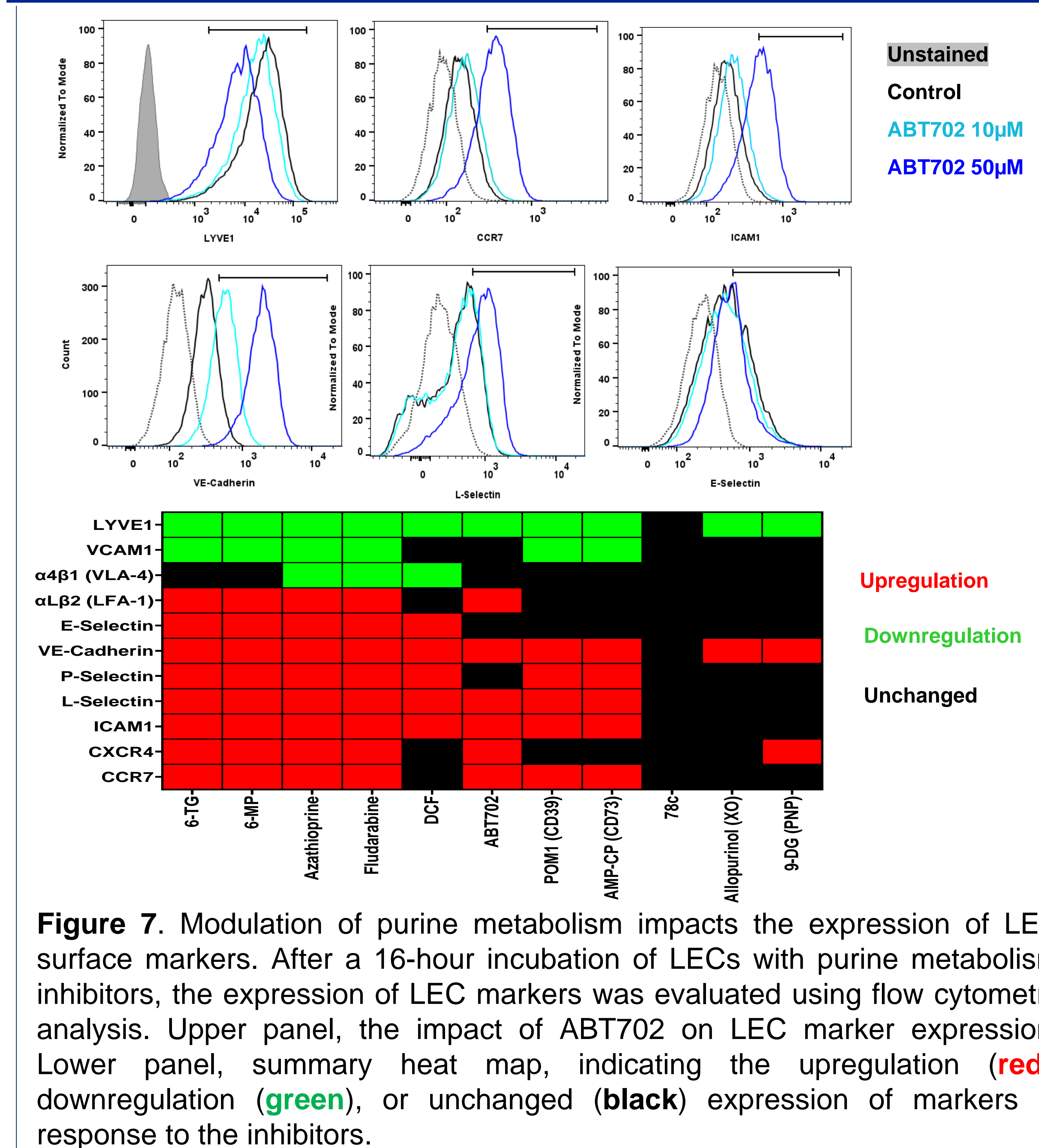
- Saxena, V. et al. (2022) Treg tissue stability depends on lymphotoxin beta receptor and adenosine-receptor-driven lymphatic endothelial cell responses. *Cell Reports* 19:39(3):110727.
- Piao, W. et al. (2020) Regulatory T Cells Condition Lymphatic Endothelia for Enhanced Transendothelial Migration. *Cell Reports* 30(3):458

### Metabolome profiles were changed for Treg conversion to exTregs



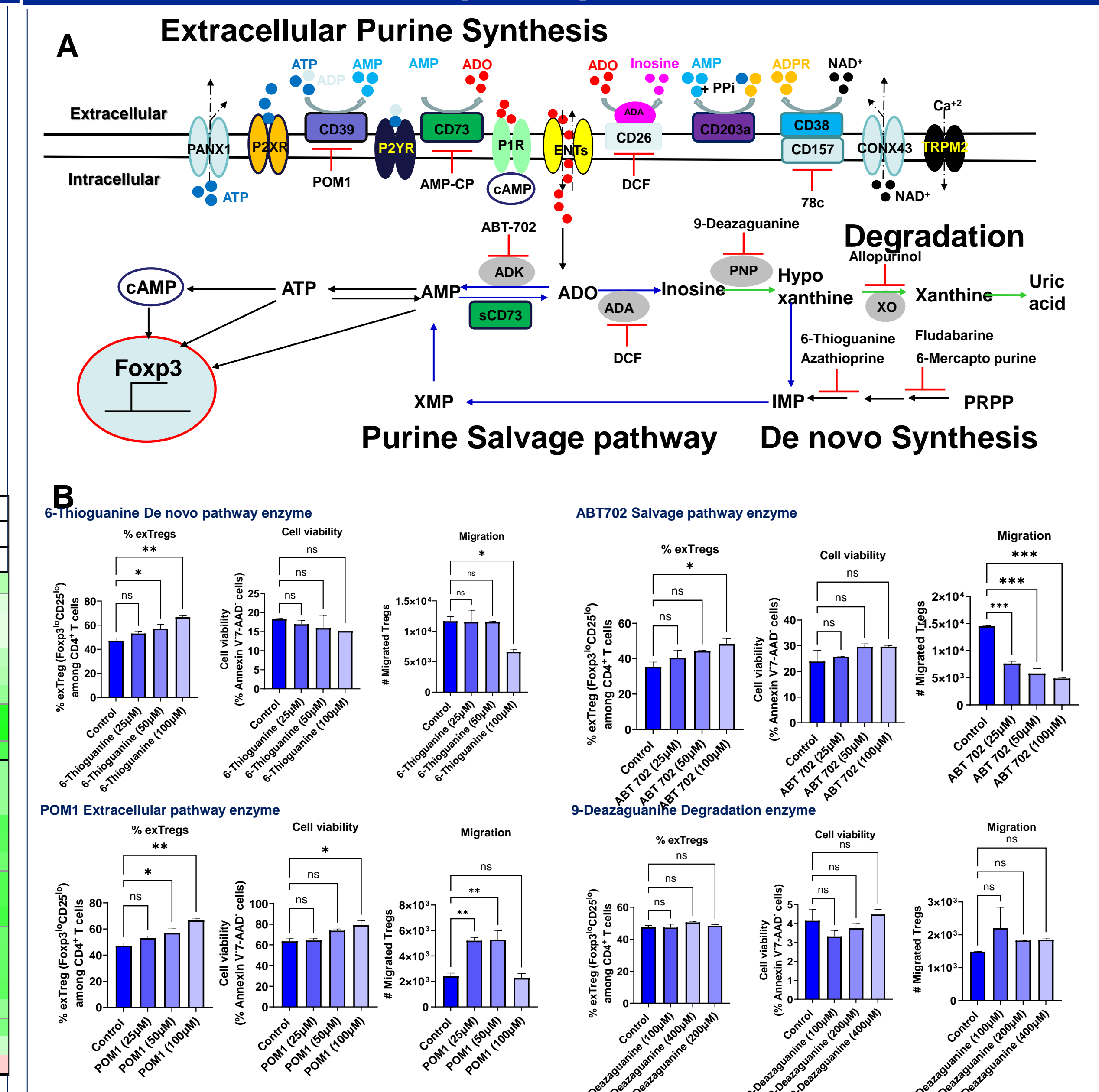
**Figure 2.** Treg conversion to exTregs induces discrete changes in metabolome profiles indicative for cellular metabolic reprogramming. (A) Treg (CD25<sup>hi</sup>Foxp3<sup>hi</sup>) and exTreg (CD25<sup>lo</sup>Foxp3<sup>lo</sup>) sorted from upper and lower wells in Boyden-chamber Treg-LEC coculture. Freshly sorted nTregs and naïve CD4 T cells used as controls. (B) Principal component analysis (PCA). (C) Heatmap showing changes in expression of 378 metabolites between groups. (D) 27 candidate metabolites with similar differential expression between up- and low-Tregs vs exTregs.

### Purine metabolism alters the expression of LEC markers



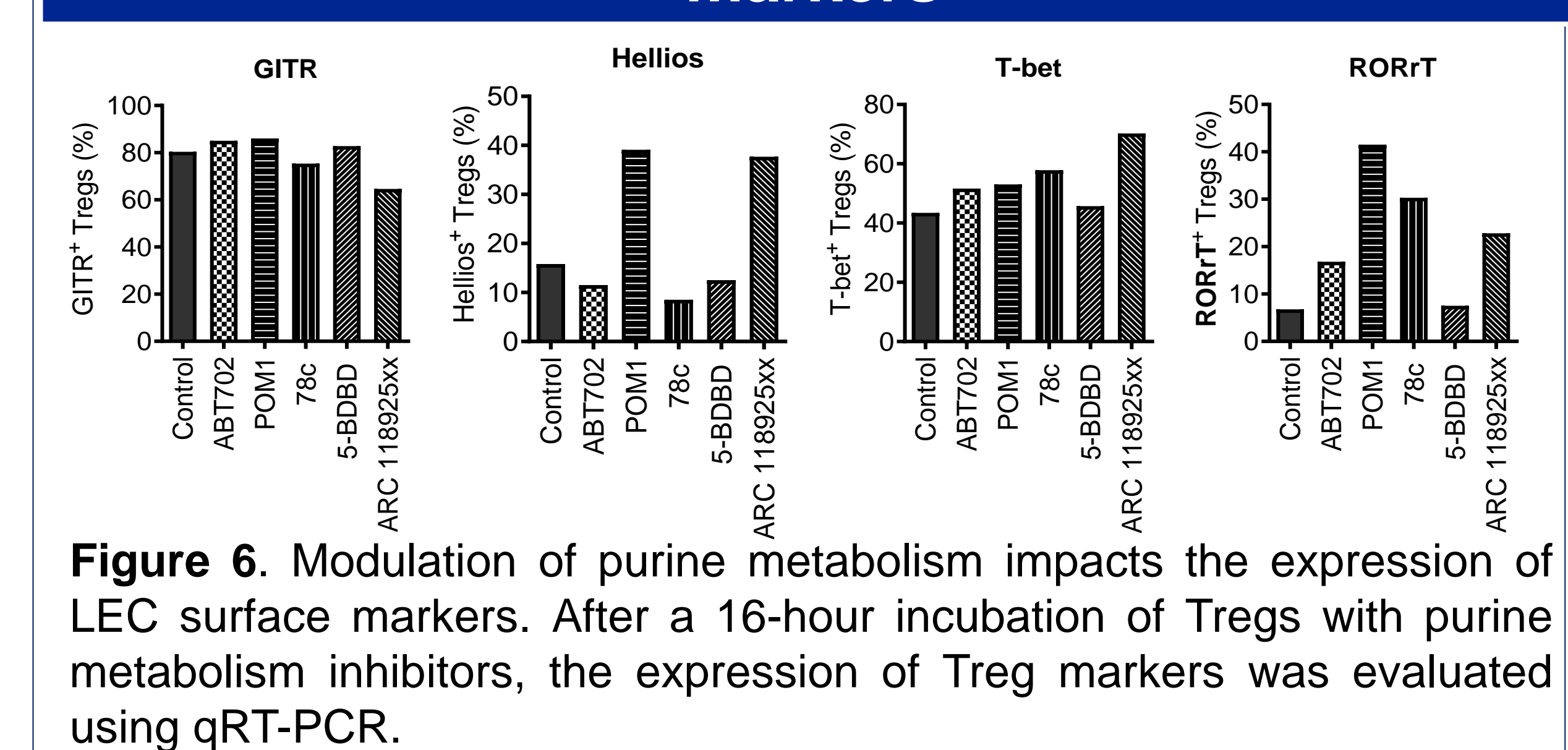
**Figure 7.** Modulation of purine metabolism impacts the expression of LEC surface markers. After a 16-hour incubation of LECs with purine metabolism inhibitors, the expression of LEC markers was evaluated using flow cytometry analysis. Upper panel, the impact of ABT702 on LEC marker expression. Lower panel, summary heatmap, indicating the upregulation (red), downregulation (green), or unchanged (black) expression of markers in response to the inhibitors.

### Modulation of purine metabolism changes Treg Foxp3 expression



**Figure 5.** The purine metabolic inhibitors impact on Treg conversion to exTregs. (A) Purine metabolic pathways and inhibitors targeting the metabolic enzymes. PRPP, phosphoribosyl diphosphate; IMP, inosinic monophosphate; XMP, Xanthosine monophosphate; ADO, Adenosine; AK, adenosine kinase; ADA, adenosine deaminase; PNP, purine nucleoside phosphorylase; IMPDH, IMP dehydrogenase (B) exTreg assays under indicated conditions including Adenosine 5'-(α,β-methylene)diphosphate sodium salt (AMPCP), Adenosine Kinase Inhibitor (ABT 702), Pentostatin, 6-Mercaptopurine, 6-Thioguanine, Azathioprine, 9-Deazaguanine, and Fludarabine.

### Purine metabolism alters the expression of Treg markers



**Figure 6.** Modulation of purine metabolism impacts the expression of LEC surface markers. After a 16-hour incubation of Tregs with purine metabolism inhibitors, the expression of Treg markers was evaluated using qRT-PCR.

## Conclusions

- Treg-LEC interactions during transendothelial migration regulate Treg stability.
- Treg conversion to exTregs is associated with cellular metabolic reprogramming.
- Modulation of purine metabolism influences the expression of Treg Foxp3 and LEC markers.
- Purine metabolism and Treg stability are closely related and sustaining purinergic homeostasis could be a potent therapeutic targets to improve immune suppression in transplant.