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Involvement of the CD39/CD73/adenosine pathway on T cell proliferation and NK cell-mediated ADCC in Sézary syndrome

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Involvement of the CD39/CD73/adenosine pathway on T cell proliferation and NK cell-mediated ADCC in Sézary syndrome

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Running title: CD39/CD73/adenosine pathway in Sézary syndrome

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Extracellular adenosine (eAdo) has been identified as a potent inhibitor of anti-tumor immune responses and enhancer of tumor survival. Two ectonucleotidases, CD39 and CD73, are involved in the generation of eAdo from ATP. Numerous studies have demonstrated their active role in promoting solid tumor outgrowth and spreading but also in inhibiting the anti-tumor immune response. Indeed, accumulation of eAdo was detected in solid tumors, and binding of eAdo to its receptors (A2AR) expressed at the surface of immune cells provides an immunosuppressive signal on effector T, NK and NKT cells, macrophages/dendritic cells and neutrophils¹. In addition, signaling through A2AR upregulates a number of anti-inflammatory molecules and the activity of regulatory T-cells, leading to a long-lasting immunosuppressive environment^{2,3}. Accordingly, the use of A2AR-antagonists has demonstrated promising efficacy in the treatment of cancer⁴. Anti-CD39 or -CD73 antibodies were also developed that efficiently block the hydrolysis of ATP into immune-suppressive eAdo and unleash anti-tumor immunity by stimulating DCs and macrophages and restoring T-cell activation^{5,6}.

Sézary syndrome (SS) is an aggressive form of cutaneous T-cell lymphoma characterized by the presence of a malignant CD4⁺ T-cell clone in skin and blood, and a global immunodeficiency⁷. We previously demonstrated an abnormal expression of CD39 by SS patients' circulating tumor T-cells⁸, questioning the functional consequences of this overexpression in terms of anti-tumor immune response suppression and tumor cell growth. To gain insights into the potential role of the CD39/CD73/adenosine pathway in tumor escape, we further evaluated both CD39 and CD73 expression by SS patients' circulating tumor CD4⁺ T-cell clone and non-malignant T/B/NK lymphocytes. Multicolor flow cytometry analysis was performed on the blood of healthy donors (HD, n=49) and SS patients whose malignant CD4⁺ T-cell clone can be distinguished from the non-malignant CD4⁺ T-cells using an available anti-TCRV β mAb (n=37). With this larger cohort, we confirmed the previously described overexpression of CD39 by Sézary patients' malignant CD4⁺ T-cell clone when compared to

HD CD4⁺ T-cell population (Figure 1A; means±SEM in supplemental Table 1). Remarkably, addition of CD73 marker allowed to distinguish 3 groups of patients according to CD39 and/or CD73 positivity of their tumor clone (Figure 1B): 51.3% (n=19/37) and 27% (n=10/37) of the patients had a tumor clone with a CD39⁺CD73⁻ or CD39⁻CD73⁺ phenotype respectively, while 8.1% (n=3/37) were characterized by a mainly CD39⁺CD73⁺ malignant clone (representative labelings shown in supplemental Figure 1A). No overexpression of either marker was detected on the malignant cells of 5/37 tested patients (13.5%). Significant overexpression of CD39 or CD73 by non-malignant CD4⁺ T-cells was also observed in SS compared to HD (Figure 1A and 1B; Table 1). However, it could be noted that, in a given patient, the non-malignant CD4⁺ T-cells and the malignant clone showed distinct CD39/CD73 expression profiles (Figure 1B), re-enforcing the previously reported notion of genetic and phenotypic disease heterogeneity⁹⁻¹². Further comparison of the non-malignant lymphocyte populations of SS patients *vs.* HD revealed: i) an up-modulation of CD39 and a down-modulation of CD73 on CD8⁺ T-cells, ii) higher expression of CD39 or CD73 by NK cells and iii) a similar CD39/CD73 phenotype on B cells (Figure 1C-E and Table S1). Altogether, these data highlight a strong bias in the expression profile of CD39 and/or CD73, not only on the tumor clone but also on the non-malignant CD4⁺ and CD8⁺ T-cells and NK cells, in the context of SS.

Despite high expression level of CD39 by SS patients' malignant cells, no significant increased release of soluble CD39 was detected in SS patients' plasma samples when compared to HD (supplemental Figure 1B). However higher levels of circulating AMP were observed (Figure 2A), as well as an increased ability of SS patients' CD4⁺ T-cells to promote ATP hydrolysis (Figure 2B). Since eAdo overproduction might occur due to CD39/CD73 overexpression, we tested its possible impact on SS patients' T-cell functions. Proliferations assays were conducted on peripheral blood mononuclear cells (PBMC) activated with

CD3/CD28 beads. As reported previously¹³, the CD3/CD28-induced proliferation of HD CD4⁺ and CD8⁺ T-cells was strongly inhibited in the presence of ATP (Figure 2C; n=6). This ATP-induced inhibition was completely abolished when istradefylline, an antagonist inhibitor of the eAdo A2AR, or an anti-CD73 mAb previously identified as blocking CD73 nucleotidase activity¹⁴, was added. In contrast A438079, an antagonist of the ATP receptors P2X7R, remained ineffective in preventing the effects of ATP (Figure 2C). Similar experiments performed on SS patients' PBMC (n=6) revealed an identical CD8⁺ T-cell response as in HD, namely an ATP-mediated inhibition of proliferation that was abolished by addition of istradefylline or anti-CD73 mAb, but not A438079 (Figure 2C). Regarding CD4⁺ T-cells, although malignant cells proliferated less than non-malignant cells upon CD3/CD28 activation, both populations' proliferation was affected by ATP. However, abolition of the ATP-mediated inhibition of proliferation by istradefylline or the anti-CD73 mAb appeared fully effective on non-malignant CD4⁺ T-cells but only partial on the malignant clone which proliferation remained altered (Figure 2C). There again, A438079 did not prevent the inhibitory effects of ATP regardless of the CD4⁺ T-cell subtype. It therefore seemed that the ATP immunosuppressive function relied on the same mechanism on SS patients' non-malignant CD4⁺ and CD8⁺ T-cells that on HD normal T-cells, involving the conversion of ATP into eAdo by CD39/CD73 and eAdo binding to A2AR on T-cells. In contrast, our data suggest that another mechanism prevailed in malignant CD4⁺ T-cells as inhibition of eAdo production or eAdo/A2AR interaction was not sufficient by itself to fully prevent their ATP-induced inhibition of proliferation. As mentioned above, malignant cells developed lower proliferation levels than their non-malignant counterparts in response to CD3/CD28 activation (Figure 2A), a phenomenon previously attributed to antibody-induced cell death resistance¹⁵. One could therefore hypothesize that part of the malignant cells are biased towards apoptosis resistance, remaining unresponsive to proliferative signals.

New therapeutic approaches in CTCL include depleting antibodies such as the recently developed anti-CCR4 (Mogamulizumab)¹⁶ and anti-KIR3DL2 (IPH4102/Lacutamab)¹⁷ antibodies. It has been demonstrated that NK cells actively participated in malignant cell depletion by exerting antibody-dependent cell cytotoxicity (ADCC)^{18,19}. Inhibition of ADCC would therefore be detrimental for the obtention of maximal anti-tumor responses. We thus explored the impact of ATP/eAdo generation on NK cell ADCC function. Assays were conducted using IL15-treated PBMC as source of activated NK cells, the CD20⁺ B cell line Raji as target cells, and the anti-CD20 antibody Rituximab as therapeutic antibody. Efficient killing of Raji cells was promoted by HD PBMC, that was not significantly affected by addition of ATP (Figure 2D, left). Consequently, addition of istradefylline or A438 did not interfere in the process leading to target cell depletion. Identical results were obtained with SS patients' PBMC (Figure 2D, right).

We here established that CD39 and/or CD73 cell surface expression is dysregulated on SS patients' tumor clone but also on the non-malignant T and NK cell populations, resulting in increased ATP hydrolysis. We further demonstrated that, in an ATP-enriched cellular environment, inhibition of the CD39/CD73/adenosine pathway could completely restore non-malignant CD4⁺ and CD8⁺ T-cell proliferation but not malignant T-cell proliferation. Finally, the ADCC activity of NK cells did not seem affected by high ATP levels. In light of these data, the combined use of ADCC-driving therapeutic antibodies and antagonist inhibitors of CD39/CD73/adenosine pathway could represent an interesting option for improving Ab-dependent anti-tumor immune responses in the context of SS.

Conflict of interest disclosure

The authors declare no competing financial interests.

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Authorship contributions

G.S, M.D, A.Bo and N.T performed the experiments; A.M-C designed the research; A.M-C, A.Be, A.DM and M.B analyzed the data; M.B, C.R-W and A.DM were attending doctors for the patients; A.M-C, M.B, A.DM and A.B wrote or critically reviewed the manuscript.

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Figures Legends

Figure 1: Abnormal expression of CD39 and CD73 on Sézary patients malignant and non-malignant circulating lymphocytes. Expression of CD39 and CD73 was assessed on total blood of Sézary patients (SS; n=37) or healthy donors (HD; n=49) by flow cytometry. After acquisition on a flow cytometer, the percentage of CD39- and/or CD73-positive cells was estimated on (A) HD (CD3⁺CD4⁺) or SS total (CD3⁺CD4⁺), malignant (CD3⁺TCRVβ⁺CD4⁺) or non-malignant (CD3⁺TCRVβ⁻CD4⁺) CD4⁺ T cells, (D) NK cells (CD3⁻CD56⁺) and (E) B cells (CD3⁻CD19⁺). (B) Histograms showing CD39 and CD73 single (SP) or double (DP) expression by normal (HD) or malignant/non-malignant (SS) CD4⁺ T cells of each donor tested in each group. (A, C-E) Statistical analysis was performed using a Mann-Whitney *t* test. ** *p* < 0.01, *** *p* < 0.001, **** *p* < 0.0001.

Figure 2: Impact of CD39/CD73 over-expression on malignant and non-malignant T and NK cell functions. (A) Quantification of AMP in the plasma of HD and Sézary patients (n=10 per group). (B) Quantification of cell-associated CD39/CD73 enzymatic activity using purified CD4⁺ T cells from HD or SS patients (n=4 per group). (C) T cell proliferation assay. CFSE- or CTV-labeled PBMC from HD or SS patients (n=6 per group) were either left untreated or activated for 4 days with CD3/CD28 beads in the presence of ATP alone or combined to istradefylline (50 mM), A438079 (A438; 20 μM) or a blocking anti-CD73 mAb (10 μg/ml). Following immunolabeling, proliferation of the CD4⁺ and CD8⁺ T cells was assessed by flow cytometry. (D) ADCC assay. HD or SS patients PBMC were incubated with IL15 in the presence of ATP alone or combined to istradefylline or A438. After 4 days, cells were mixed with Raji target cells at an E/T ratio of 20/1 and Rituximab (10 μg/ml). Target cells apoptosis was monitored by 7-AAD labeling. Results are expressed as the mean ± SD of the % of 7AAD⁺ Raji cells obtained for the indicated conditions with HD (left panel) and SS

(right panel) PBMC (n=4 per group). **(A-D)** Statistical analysis was performed using a Mann-Whitney t test. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns: not significant.

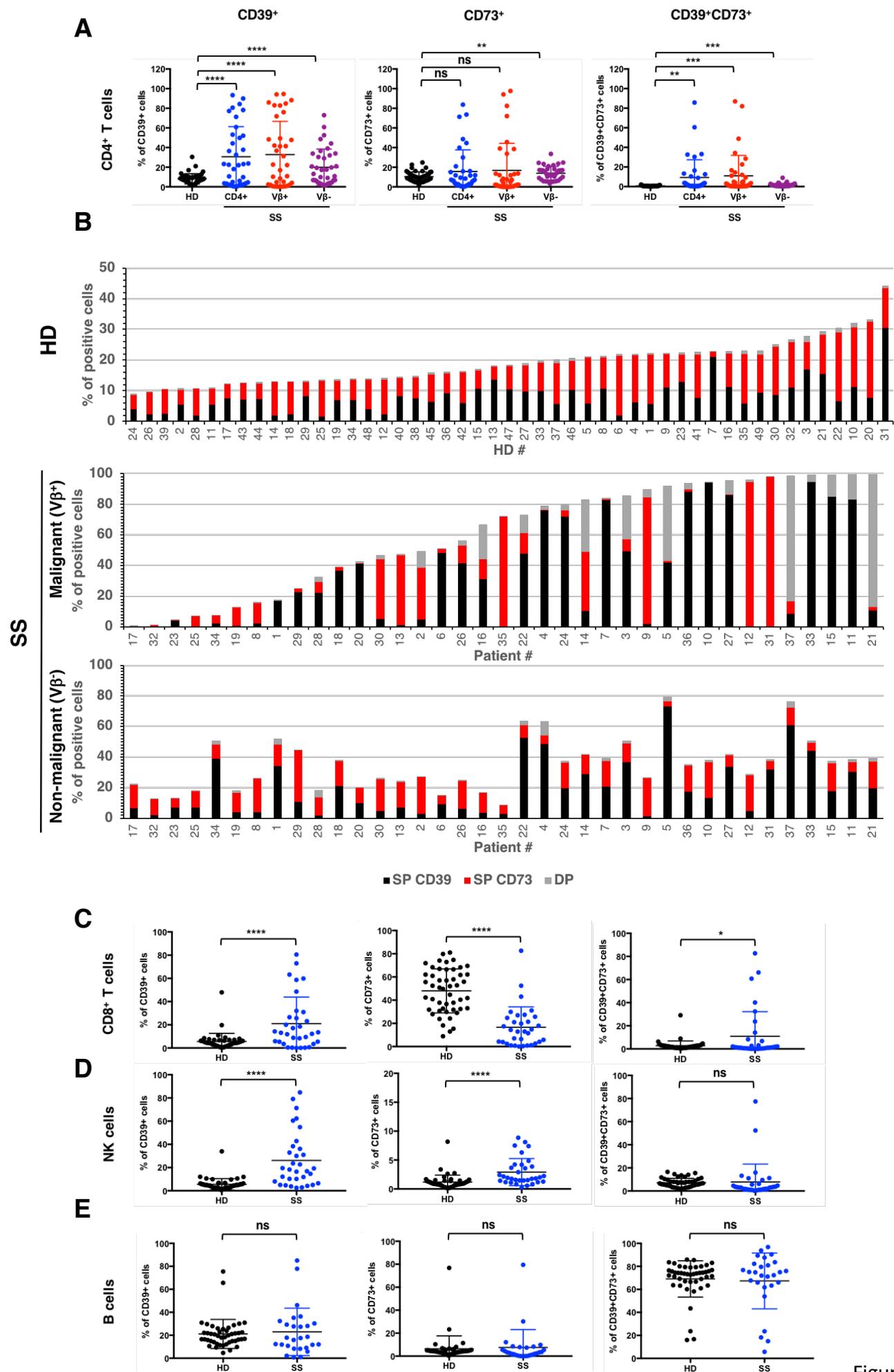


Figure 1

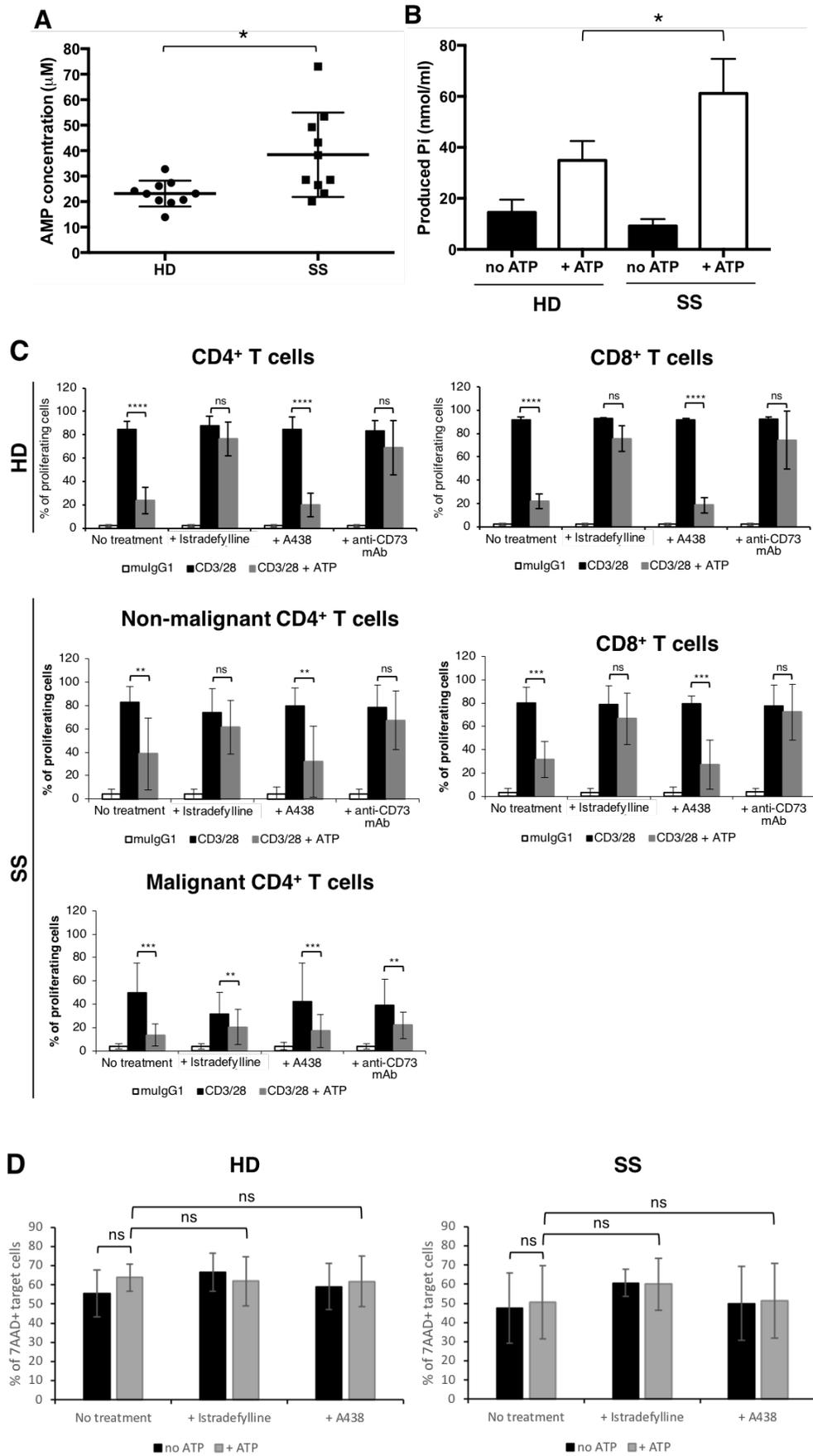


Figure 2