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Adenosine signaling in human T-cells favors development of IL-17$^+$ cells and induces immunoregulatory characteristics

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Adenosine is an anti-inflammatory agent that accumulates at inflammatory sites and restrains immune responses. It is sequentially converted from ATP by the two ecto-nucleotidases CD39 and CD73 and signals through four adenosine receptors (A1R, A2AR, A2BR, A3R). Adenosine has been extensively researched in immunity since the 1970s and has been found to influence a variety of immune responses, including T-cell differentiation. Signaling through the high-affinity A2AR has been documented to promote T_{regs} in mice while inhibiting Th1, Th2 and Th17 differentiation. Thus, it is considered a potential mechanism for shifting the T_{reg}/Th17 balance towards regulatory fates. In contrast, research on the low-affinity receptor A2BR, relevant only in inflammatory conditions, and other adenosine receptors is sparse. Yet studies in mice indicate potential adenosine-induced Th17 differentiation after A2BR engagement, thus calling into question the narrow focus on the A2A receptors in previous research. Secondly, only few studies explored the relationship between adenosinergic signaling and human T-cell differentiation. Although adenosine inhibited the secretion of pro-inflammatory cytokines in both species, it also inhibited the development and function of T_{regs} in human T-cells in contrast to its effects in mice, thus raising the possibility of species differences. Finally, the discovery of non- and anti-inflammatory Th17 subsets in the last decade has proven the existence of IL-17 producing regulatory subsets with distinct metabolic requirements. This complicates the interpretation of changes in the T_{reg}/Th17 balance and indicates the necessity of additional characterizations of Th17 cells in future studies on adenosine. We thus investigated the effect of pan adenosine receptor stimulation on human T-cell differentiation and hypothesized that adenosine signaling would inhibit human Th17 differentiation while shifting IL-17$^+$ cells to a non- or anti-inflammatory phenotype, possibly through changes in relevant metabolic pathways.

To investigate this hypothesis, we isolated human CD3$^+$ T-cells and cultured them without polarizing cytokines (Th0) or in Th17-inducing conditions (Th17) with and without the addition of the pan adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA). T-cells in Th0 conditions with NECA (Th0/NECA) produced less Th1- and Th2-associated cytokines and more IL-9, IL-10 and IL-17, cytokines associated with non-inflammatory Th17 cells. In Th17 conditions, NECA increased the frequency of IL-17$^+$ cells and the expression of the Th17 master transcription factor RORγt. IL-17$^+$ cells exhibited a CD4$^+$ RORγt$^+$ phenotype with enhanced expression of the Th17-associated surface molecules CD161 and CD196, thus further supporting their identification as Th17 cells. This finding of adenosine-induced Th17 differentiation is in contrast to the majority of previous studies mainly conducted in mice and with a focus on the A2A receptor. Nonetheless, it is supported by five studies reporting IL-17 production in murine T-cells after A2BR stimulation on ACPs. Our study corroborates these findings in the context of more physiological pan adenosine receptor stimulation on human T-cells and without indirect effects through IL-6 up-regulation.

Secondly, NECA-induced IL-17$^+$ cells displayed further changes consistent with non- or anti-inflammatory Th17 cells. They down-regulated IFN-γ co-production and increased surface expression of adenosine-producing CD39. Consequently, T-cells from Th17/NECA cultures were capable of suppressing responder T-cells as was previously described for CD39$^+$ CD73$^+$ IL-17$^+$ cells. Microarray analysis of Th0/NECA cultures compared to Th0 cultures also revealed early transcriptional changes induced by NECA, which were previously described in non- and anti-inflammatory Th17 subsets, e.g. reduced expression of Th1-associated genes and of *IL1R1* and *IL23R*. Thus, Th0/NECA cells exhibited a non-inflammatory and memory- or stem cell-like transcriptional signature. These changes corresponded to the promotion of regulatory features by A2AR signaling and to the role of adenosine in

T-cell quiescence and memory formation. However, our study was amongst the first to further characterize IL-17$^+$ cells as non-inflammatory Th17 cells in the context of adenosinergic signaling.
Lastly, an exploratory analysis of our microarray data of Th0 vs. Th0/NECA cultures revealed changes in metabolic pathways. NECA-treated T-cells exhibited negative enrichment of genes belonging to MYC targets and the mTORC1 pathway, while genes belonging to the FOXO signaling pathway were positively enriched. This corresponded to metabolic changes described in non-pathogenic Th17 cells, while IL-17 induction in general was previously reported in the context of both metabolic activity and quiescence. Genes of the hypoxic signaling pathway, which is tightly interwoven with adenosinergic signaling, were also positively enriched in our microarray data. Hypoxic signaling is implicated in both T_{regs} and Th17 cells and both pathogenic and non-pathogenic Th17 subsets, possibly because of posttranslational modifications and complex interactions with other metabolic pathways. In general, however, metabolic changes in Th0/NECA cells corresponded with those reported for non-pathogenic Th17 cells, making mechanistic links between these changes and the induction of non-inflammatory IL-17$^+$ cells by NECA likely.
Limitations of this study include the choice of CD3$^+$ pan T-cells instead of naïve CD4$^+$ T-cells and of a pan adenosine receptor agonist since these choices complicate mechanistic interpretations and comparisons with previous research. Additionally, numbers of IL-17$^+$ cells in Th17-inducing conditions were low and thus differences in IL-17$^+$ cells between cultures with and without NECA small, even though IL-17 concentration in supernatants was more pronounced. This also complicated isolation of IL-17$^+$ cells for the suppression assays so that suppression assays were performed with all cells from Th17 and Th17/NECA cultures. Further experiments are necessary to clearly identify the suppressor cells and mechanism of suppression. Lastly, while FACS stains were performed on Th17 cultures, microarrays were conducted on Th0 vs. Th0/NECA cells, complicating interpretation of gene expression changes in light of Th17 differentiation.
In conclusion, our study reported the induction of non-inflammatory Th17 cells in human T-cell by the pan adenosine receptor agonist NECA and indicated potential mechanistic links in the form of regulation of metabolic pathways.