A1 and A2A adenosine receptors play a protective role to reduce prevalence of autoimmunity following tissue damage

Reut Riff[1], Oshri Naamani[1], julia mazar[2], Yosef Haviv[2], Cidio Chaimovitz[2], and Amos Douvdevani[1]

[1]Ben-Gurion University of the Negev
[2]Soroka Medical Center

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Abstract

Tissue damage associated with trauma might release a sufficient autoantigen substrate to break immune tolerance. In a previous study, we showed that the leukopenia observed following severe inflammation is related to adenosine A1-receptor (A1R) desensitization and A2AR upregulation. We hypothesized that, under destructive pathological conditions this mechanism is beneficial in reducing prevalence of autoimmunity. In this study, we aim to evaluate the protective role of A1R and A2AR in prevention of autoimmune diseases. We used two murine models of autoimmune diseases: type 1 diabetes (T1D) induced by low-dose streptozotocin and pristane-induced lupus (PIL) and on neutrophils we studied NETosis regulation by adenosine. In both the T1D and PIL models, A1R-KO mice were predisposed to the development of autoimmunity. In the PIL model, in WT mice, parallel to the decline of A1R mRNA levels, lymphocytes number dropped (-85%) 6h after pristane injection. WT mice remained without any sign of disease at 36 weeks. In contrast, following pristane 43% of A1R-KO mice suffered from lupus-like disease. Compared to A1R-KO, in WT mice at 10 days A2AR mRNA levels were significantly higher. Similar to PIL, in T1D model the presence of A1R and A2AR was protective. In addition, we found that A1R increases and A2AR suppresses NETosis. We suggest that adenosine-dependent immune suppression and reduction in neutrophil extracellular traps (NETs) limits the reactive T-cells and development of anti-double strand DNA (dsDNA) antibodies that promote autoimmunity.

A_1 and A_{2A}adenosine receptors play a protective role to reduce prevalence of autoimmunity following tissue damage

Short Title: Protective role of adenosine against autoimmunity

Reut Riff*, Oshri Naamani* §, Julia Mazar+, Yosef S. Haviv++, Cidio Chaimovitz++, and Amos Douvdevani*++

*Departments of Clinical Biochemistry and Pharmacology, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel

SSDepartment of Science, Hemdat Hadarom, College of Education, Netivot, Israel

+ Laboratory of Hematology, Soroka University Medical Center and, Ben-Gurion University of the Negev, Beer-Sheva, Israel.

++ Department of Nephrology, Soroka University Medical Center and Ben-Gurion University of the Negev, Beer-Sheva, Israel.

reutriff@gmail.com naamanio7@gmail.com julia.mazar@gmail.com havivy@bgu.ac.il cidio@bgu.ac.il amosd@bgu.ac.il

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Corresponding author:

Reut Riff, reutriff@gmail.com.

Departments of Clinical Biochemistry and Pharmacology,

Faculty of Health Sciences, Ben-Gurion University of the Negev,

Beer-Sheva 84105, Israel.

Telephone: 972-8-6403214

List of abbreviations:

A_1R – A_1 receptor

$A_{2A}R$ – A_{2A} receptor

$A_{2B}R$ – A_{2B} receptor

A_3R – A_3 receptor

BM – Bone marrow

cAMP – Cyclic adenosine mono phosphate

CCPA – 2-Chloro-N^6-cyclopentyladenosine

cfDNA – cell-free DNA

CGS or CGS21680– phenethylamino-5'-N-ethylcarboxamideadenosine hydrochloride

DCPCX – 8-cyclopentyl-1,3-dipropylxanthin

dsDNA – double-strand DNA

FCS – Fetal calf serum

HL-60 – Human leukemia

KO – Knock-out

NETs – Neutrophils extracellular traps

PIL– Pristane induced lupus

qPCR – quantitative polymerase chain reaction

RBC – Red blood cells

SIRS – Systemic inflammatory response syndrome

ssDNA – single-strand DNA

STING – Stimulator of interferon genes

STZ – Streptozotocin

T1D – Type 1 diabetes

WT – Wild type

Abstract

Tissue damage associated with trauma might release a sufficient autoantigen substrate to break immune tolerance. In a previous study, we showed that the leukopenia observed following severe inflammation is related to adenosine A_1-receptor (A_1R) desensitization and $A_{2A}R$ upregulation. We hypothesized that, under destructive pathological conditions this mechanism is beneficial in reducing prevalence of autoimmunity. In this study, we aim to evaluate the protective role of A_1R and $A_{2A}R$ in prevention of autoimmune diseases. We used two murine models of autoimmune diseases: type 1 diabetes (T1D) induced by low-dose streptozotocin and pristane-induced lupus (PIL) and on neutrophils we studied NETosis regulation by adenosine. In both the T1D and PIL models, A_1R-KO mice were predisposed to the development of autoimmunity. In the PIL model, in WT mice, parallel to the decline of A_1R mRNA levels, lymphocytes number dropped (-85%) 6h after pristane injection. WT mice remained without any sign of disease at 36 weeks. In contrast, following pristane

43% of A_1R-KO mice suffered from lupus-like disease. Compared to A_1R-KO, in WT mice at 10 days $A_{2A}R$ mRNA levels were significantly higher. Similar to PIL, in T1D model the presence of A_1R and $A_{2A}R$ was protective. In addition, we found that A_1R increases and $A_{2A}R$ suppresses NETosis. We suggest that adenosine-dependent immune suppression and reduction in neutrophil extracellular traps (NETs) limits the reactive T-cells and development of anti-double strand DNA (dsDNA) antibodies that promote autoimmunity.

Scientific Background

Autoimmune diseases are chronic conditions initiated by the loss of immunological tolerance to self-antigens, leading to self-attack of the immune system on one's organs. Although differing from each other, autoimmune diseases present several shared common phenotypes: the presence of nonspecific autoantibodies [i.e., antinuclear antibodies and double-strand DNA (dsDNA)], high levels of cytokines, and the presence of infiltrating immune cells. Anti-dsDNA is a known hallmark of lupus and other autoimmune diseases, but it is not only a disease marker, it also promotes autoimmunity. The presence of dsDNA in the cytoplasm has been described as a potent danger signal that activates stimulator of interferon genes (STING), a regulator of the immune response (1). Activating STING leads to a signaling cascade that eventually alters pro-inflammatory molecule production. A defect or unnecessary alert in this mechanism has been described as underpinning the auto-inflammatory process (2). One source of dsDNA is related to the production of neutrophil extracellular traps (NETs). Activated neutrophils extrude their DNA and bactericidal molecules, creating NETs in a unique type of cell death called NETosis (3). Neutrophils from patients with various autoimmune diseases are more likely to undergo NETosis than those of healthy donors (4). Moreover, the presence of autoantibodies promotes the release of NETs.

Adenosine is a potent modulator of lymphocyte development, proliferation, and activity (5-7). There are four types of adenosine receptors, all of which are members of the G protein-coupled receptor family: A_1, A_{2A}, A_{2B}, and A_3. The A_1 (A_1R) and A_3 receptors (A_3R) activate Gi, which inhibits adenylyl cyclase activity and decreases cAMP levels and promote pro-inflammatory response. A_{2A} receptor ($A_{2A}R$) interacts with Gs, and the A_{2B}receptor ($A_{2B}R$) interacts with Gs/Gq to induce adenylyl cyclase activity and elevates cAMP levels, thus, promoting anti-inflammatory responses (3). A_1R exerts the highest affinity for adenosine, and it is the first to react in the early phase of inflammation, while Gs-coupled $A_{2A}R$ is related to immunosuppression and resolution of inflammation.

Adenosine receptors expressed on a wide variety of both non-immune and immune cells (8) and all four adenosine receptors have been described on neutrophils (9). A_1R stimulation enhances their adherence to endothelium, chemotaxis (8), and their activity (10), while $A_{2A}R$ inhibits neutrophil trafficking and effector functions such as oxidative burst, inflammatory mediator production, and granule release (reviewed in (11, 12)). Multiple reports suggest that the onset of autoimmune disorders is at least in part related to a partial or

complete loss of function in the purinergic pathways and to local defective production of adenosine (reviewed in (9)).

In a model of SIRS, we have previously shown that a surge of adenosine desensitizes G_i-coupled adenosine A_1R and upregulates G_s-coupled $A_{2A}R$, an effect that provokes a cAMP-dependent lymphotoxic response. The depletion of lymphocytes and their functional impairment has been thought to be part of the pathology in SIRS that worsens recovery and decreases chances of survival (13). We now suggest that adenosine-dependent lymphocyte depletion observed in SIRS might be a normal physiological mechanism that minimizes lymphocyte exposure to self-antigens to reduce the prevalence of autoimmunity.

In the current study, we aimed to evaluate the protective role A_1R and $A_{2A}R$ have in the prevention of autoimmune diseases.

Materials and Methods

Mice

Experiments were conducted after obtaining permission from the Israel Committee for Animal Experiments (IL-32-06-2013, IL-25-5-2016, IL-39-8-2017). BALB/c and C57BL/6 mice were purchased from Harlan (Jerusalem, Israel), A_1R knock-out (KO) mice ($A_1R^{-/-}$ on C57BL/6 background) and $A_{2A}R$ knock-out mice ($A_{2A}R^{-/-}$ on BALB/c background) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed under specific pathogen-free conditions and maintained in the vivarium of Ben-Gurion University. All experiments were approved by the Ben-Gurion University Committee for Ethical Care and Use of Animals in Experiments.

Agonists, Antagonists, and Inhibitors

A_1R agonist 2-chloro-N^6-cyclopentyladenosine (CCPA), $A_{2A}R$ agonist 2-*p* -(carboxyethyl) phenethylamino-5'-N-ethylcarboxamideadenosine hydrochloride (CGS21680), and A_1R antagonist 8-cyclopentyl-1,3- dipropylxanthine (DPCPX) were purchased from Sigma-Aldrich (Rehovot, Israel).

Lupus Model

Pristane, a natural saturated terpenoid alkane obtained primarily from shark liver oil, was shown to induce a lupus-like disease in mice (14). Injection of pristane into the peritoneal cavity results in a chronic peritonitis associated with high tissue levels of interleukin 6 (IL-6) (15), that leads in a slow process to lupus-like disease (16).

Mice were injected i.p. with 0.5 ml of pristane (Sigma Aldrich) and followed weekly for external signs of lupus such as alopecia, chronic wounds, or death. Spleen, blood, and peritoneal lavage were collected at sacrifice (6h, 24h, 48h, 6 days, 10 days, or 8 months after injection) (17).

Anti-Double-Strand DNA (dsDNA)

Disease activity was considered according to anti-dsDNA antibodies (18). Serum was separated by centrifugation at 4degC at 3000 rpm for 10 min, and serum anti-dsDNA levels were analyzed using a murine ds-DNA standard enzyme-linked immunosorbent assay (ELISA) kit (Alpha Diagnostics Inc.; San Antonio, TX, USA).

Differential blood cell counts

Blood samples of 200 μl in heparin-coated tubes were counted with an ADIVA 2120 blood count device (Siemens; Munich, Germany).

Cell-free DNA assay

Peritoneal lavage was performed with 5 mL of PBS at the experiment endpoint. cfDNA was quantified, as previously described, using our rapid SYBR® Gold fluorometric assay (19).

Splenocytes production

For mRNA levels, spleens were harvested, and cells were collected and treated with RBC lysis solution (5 Prime Inc.). Cells were incubated in a petri dish at 37^{o}C with medium for 1h. They were then washed, adhered cells were collected, and RNA was extracted using a PerfectPure RNA Tissue Kit (5 Prime Inc.).

mRNA Analysis by Quantitative PCR

RNA was extracted using a Perfect Pure RNA Tissue Kit (5 Prime Inc.). cDNA was prepared using a high capacity cDNA reverse transcription kit (Applied Biosystems; Foster City, CA, USA).

Quantitative real-time polymerase chain reaction (qPCR) assays were performed with a Fast SYBR Green Master Mix (Applied Biosystems) on a StepOne Plus real-time PCR machine (Applied Biosystems) with the following mouse-specific primers: **RPL-12** sense 5'-ATG ACA TTG CCA AGG CTA CC-3', anti-sense 5'-CAA GAC CGG TGT CTC ATC TGC -3';$\mathbf{A_1R}$ sense 5'-TAC ATC TCG GCC TTC CAG GTC G-3', anti-sense 5'-AAG GAT GGC CAG TGG GAT GAC CAG-3';$\mathbf{A_{2A}}$ sense 5'- CGC AGG TCT TTG TGG AGT TC-3', anti-sense 5'-TGG CTT GGT GAC GGG TATG-3';

Type 1 Diabetes Model (TID)

We employed the model of low-dose streptozotocin (STZ, Sigma-Aldrich) to induced TID in all of our experiments. In this model, diabetes develops only when STZ induces both β-cell toxicity and T-cell-dependent immune reactions (20). We employed a regimen involving multiple administrations of low-dose STZ in mice (21). Diabetes was induced in 8-week-old C57BL/6 mice of both sexes by i.p. injection of STZ (50 mg/kg in citrate buffer) on five consecutive days. Blood glucose levels were measured using a glucometer (Accu-Chek Aviva, Roche Diagnostics; Indianapolis, IN, USA). Regularly, in all STZ-injected mice throughout the experiment, animals with glucose levels >200mg/dl for two consecutive days were considered to be diabetic (22).

Regulation of NETosis by Adenosine

Differentiated HL-60 cells

HL-60 cells (CCL240; American Type Culture Collection) were grown in RPMI 1640 and supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/l L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Biological Industries; Bet Haemek, Israel). HL-60 cells were differentiated into neutrophils by culturing the cells in medium containing 5μM retinoic acid (RA, Sigma-Aldrich) for 72h. Differentiation was confirmed by detection of Surface CD11b, which is an early marker of neutrophil differentiation in HL-60 (23). Differentiation was confirmed when CD11b expression levels were at least 90%. Untreated HL60 cells stained with the isotype control were used as background for undifferentiated cells.

Mice neutrophils ex-vivo

Bone marrow (BM) cells from the os femoris and tibia were collected and treated with red blood cell (RBC) lysis solution (5 Prime Inc.; Gaithersburg, MD, USA). Neutrophils were then isolated via density gradient according to Swamydas et al. (24) using centrifugation with Histopaque 1077 and 1199 (Sigma-Aldrich). Cells were washed twice, and neutrophils were counted after trypan blue staining using a Neubaur hemocytometer. Cells were grown in RPMI 1640 and supplemented with 10% heat-inactivated FCS, 2 mmol/l L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Biological Industries; Bet Haemek, Israel).

NETs assay

RA-differentiated HL-60 neutrophils or BM-isolated cells were seeded ($2x10^5$ per well) in 96-well plates. Cells were pre-incubated with (or without) adenosine agonists (A_1R specific agonist CCPA, 1nM. or $A_{2A}R$ agonist GCS, 30nM) for 30 min. Then they were treated with 200nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) and 0.03% H_2O_2 for 3h (25, 26). For DNA detection, Sytox green dye (Molecular Probes, Invitrogen AG; Basel, Switzerland) was used.

2.8 Statistical Analysis

All comparisons between groups were carried out by a Mann–Whitney nonparametric t-test or by a one-way ANOVA followed by a Tukey post-test using Prism 6 software (GraphPad; San Diego, CA, USA). p values below 0.05 were considered significant. Data are presented as mean ± SD, unless mentioned otherwise.

Results

Autoimmunity

Pristane induced lupus (PIL) model

Kinetics of disease development

We have previously shown that elevated adenosine in severe inflammation causes A_1R depletion associated leukopenia (13). To study the role of A_1R in autoimmunity, we compared the appearance of pristane-induced lupus-like disease between a group of A_1R-KO mice and a group of WT mice. In contrast to WT mice that remained without any sign of disease, KO mice started to exhibit the classic pathological signs of lupus: alopecia, chronic skin wounds, and death starting at 10 weeks from induction. At 36 weeks following pristane injection, 43% of A_1R-KO mice suffered from lupus-like disease; five died, three suffered from alopecia, and one suffered from chronic wounds, while WT mice had no physical signs of disease (Figure 1A, p <0.05), although they did develop Anti-dsDNA, which is a hallmark of lupus. Anti-dsDNA levels in vehicle-treated WT mice were 12670.15±4712.31 ng/ml compared to 39775.08±19352.7 ng/ml in pristane-treated WT mice (Figure 1B, p <0.05). Spleens were removed and measured at the experience endpoint and were significantly larger following pristane injection in WT mice 0.136±0.016 vs. 0.29±0.099 gr. (Figure 1C, p <0.01). Differences in Anti-dsDNA and in spleen size did not reach significance between WT and A_1R-KO mice following pristane injection, as the mice with the most severe illness died before the experience endpoint and therefore are not included in these results.

As expected, the basal WBC counts were lower in A_1R-KO mice compared to WT mice (Figure 2A, p <0.05). In blood counts of WT mice, following pristane injection, we observed a deep reduction in WBC number, and the main leukocyte population to be affected was lymphocyte (Figure 2B). At 6h, lymphocyte counts were reduced by 85% from 7.44±3.48 to 1.05±0.73 cells $x10^3/\mu l$, (Figure 2B,p <0.05). All other cell populations were not significantly affected by pristane injection (Figure 2C, 2D). Partial recovery in lymphocyte counts was observed in the WT group 48h after injection.

We followed the A_1R and $A_{2A}R$ mRNA levels normalized to RPL-12), at several time points, for 10 days (Figure 3). In WT mice, parallel to the decline of WBC, A_1R mRNA levels dropped 6h after pristane injection (Figure 3A,p <0.05), and returned to basal level 24h after treatment. In these mice, $A_{2A}R$ was induced following the injection, reaching significance at 10 days (240h, Figure 3B,p <0.05). In contrast, A_1R-deficient mice failed to upregulate the immunosuppressive receptor $A_{2A}R$ and stayed low for 10 days (Figure 3B,p <0.05).

Type-1 diabetes model

In accordance with the lupus model, the absence of both A_1 and A_{2A} receptors was found to accelerate the induction of type-1 diabetes (T1D). At 15 days, after the first STZ injection, all A_1R-KO mice were diabetic, while the WT group at this time point remained free of disease (Figure 4A). Similarly, A_{2A}R-KO mice (BALB/c background) were also more susceptible than WT mice to early development of T1D. At day 20, all A_{2A}R-KO mice had elevated blood glucose levels, while only 40% of WT mice propagated T1D (Figure 4B).

Cell-free DNA (cfDNA) underpins the progression of autoimmune diseases (18). In the PIL model, in both groups, we observed the elevation of cfDNA levels in peritoneal lavage with a peak at 24h, (significant in the WT group, p <0.01; Figure 4A). A decline in cfDNA levels was observed at 48h. Ten days (240h) after injection, cfDNA levels stabilized, cfDNA levels in A_1R-KO were significantly higher compared to WT mice (531±120 vs. 267±60 ng/ml, respectively,p <0.01; Figure 5A). Similarly, in the T1D model at day ten, cfDNA levels in A_1R-KO mice were elevated compared to basal WT cfDNA levels (360.04±107.64 vs. 99.62±62.43 ng/ml, respectively, p <0.01; Figure 5B).

Regulation of NETosis by Adenosine Receptors

DNA released from neutrophils that undergo NETosis is a major source of cfDNA. We explored the regulation of NETosis by adenosine receptors using differentiated HL-60 cells stimulated for NETosis by PMA+H_2O_2. Stimulation of neutrophil-like HL-60 cells with CCPA (1nM), a specific A_1R agonist prior to induction of NETosis, increased NETs production by 18% compared to untreated control cells. In contrast, pre-treatment with the A_{2A}R agonist CGS21680 (30nM) diminished NETs production by 30% (*p <0.05, **p <0.001; Figure 6A). In accordance with these results, neutrophils isolated from A_1R-KO mice produced 35% less NETs compared to WT mice (p <0.05, Figure 6B), and neutrophils from A_{2A}R-KO mice without the suppressive effect of A_{2A}R produced 70% more NETs (p <0.01, Figure 6B).

Discussion

In previous studies, we found that elevated adenosine either from uncontrolled systemic inflammation or by pharmacological treatment, downregulates and desensitize the adenosine A_1R and upregulates the immunosuppressive A_{2A}R (27). So what is the benefit of this acute immunosuppressive mechanism for recovery from a severe inflammatory damage? We hypothesized that, under destructive pathological conditions, normal immune response is probably unwanted, and such induction of lymphopenia and immunosuppression is a beneficial physiological mechanism to reduce the prevalence of autoimmunity.

Our data from mice with PIL-like disease and T1D show that without the presence of A_1R these autoimmune diseases exacerbate. Similar to our study, Tsutsui et al. showed in experimental allergic encephalomyelitis that compared to WT mice, A_1R-KO mice developed a severe progressive-relapsing form of the disease (30). We suggest that early response of A_1R is the trigger of a protective mechanism, and without this receptor, mice are prone to severe autoimmunity.

Following pristane injection, in WT animals, we observed an acute reduction of A_1R and leukocyte counts. Pristane in the peritoneum is known to cause inflammation and damage (reviewed in (35)) and rapid desensitization of A_1R observed immediately after pristane injection is probably due to elevated levels of adenosine.

We believe that the fast depletion of A_1R in the presence of elevated adenosine removes its anti-apoptotic protection and by reduction of G_i activity enables an early lymphotoxic effect by elevation of cAMP (13). The effect of A_1R depletion is transient, and after 48h, lymphocyte counts begin to recover. The long-term suppression of immunity probably mediated by the elevated A_{2A}R (36), which is induced by A_1R and peaks at 48h and remains high in WT animals even 10 days after pristane injection. These findings are consistence with the findings that following adjuvant-induced arthritis, adenosine concentration in plasma

stays high for weeks (37). The critical role of early A_1R stimulation in upregulation of $A_{2A}R$ was shown by our group previously (38). Accordingly, in the current study in A_1R-KO mice, pristane injection fails to upregulate $A_{2A}R$, and mRNA level of $A_{2A}R$ is lower in A_1R-KO also at T=0, which probably enables stronger lymphocyte reactivity in A_1R-depleted animals.

Additional support for the role of low $A_{2A}R$ in the development of autoimmunity is our observation that $A_{2A}R$-KO mice were predisposed to TID development. Similar to our findings, Deaglio et al. showed that $A_{2A}R$-KO mice were more susceptible to STZ-induced diabetes with the presence of hyper-proliferative T cells (45), and Zhang et al. showed that $A_{2A}R$ activation suppressed inflammation in the progression of lupus nephritis (46).

In the present study, we suggest that adenosine regulates the release of DNA by NETosis and that the same $A_1R/A_{2A}R$ dependent immunosuppressive mechanism reduces cfDNA levels. The presence of double-stranded DNA (dsDNA) was shown to be more than a distinct marker for illness severity; dsDNA is also a STING activator (47) and recent studies show a clear association between elevated cfDNA levels and autoimmunity (48).

We used differentiated HL-60 cells to study regulation of NETosis by agonists of adenosine receptors. Similar to the effect on other neutrophil functions, Such as adherence to endothelium, chemotaxis, activation and trafficking (8-12), the A_1R agonist enhanced NETs production (118%). $A_{2A}R$ is a negative regulator of NETosis- stimulation of cells with a specific $A_{2A}R$ agonist decreased NETs production to 70% of untreated cells. In accordance, neutrophils isolated from A_1R–KO mice produced fewer NETs (65%) compared to those isolated from WT mice, and vice versa: neutrophils isolated from $A_{2A}R$–KO were stronger producers of NETs (170%). In support of our data, Liu et al. showed that activation of $A_{2A}R$ inhibits neutrophil cell death, and suggest that this finding is part of the anti-inflammatory role of $A_{2A}R$ in modulating neutrophil survival during SIRS (49). In addition a recent study by Ali et al. has shown that A_{2A} adenosine receptor agonist attenuates NETosis (50).

In the T1D model, cfDNA levels in A_1R-KO mice were elevated compared to basal levels of WT mice. In both models, the higher levels of cfDNA were in accordance with the proportion of animals' sickness. In the PIL model, we followed the levels of cfDNA in detail during the first 10 days after pristane injection. At day 10, $A_{2A}R$ levels were low in A_1R-KO mice, compared to WT mice while cfDNA levels were significantly higher than the WT mice, which might contributed to the severe development of the disease in this group.

To conclude, adenosine initiates diverse cellular responses directed to prevent excessive inflammation in order to restore immune homeostasis. Our data from PIL and T1D propose that A_1 and A_{2A} receptors have a protective role in autoimmunity development. The acute elimination of lymphocytes and reduction of DNA release from NETosis depends on A_1R desensitization and long-term suppression maintained by A_1R-dependent elevation of $A_{2A}R$. We believe that severe traumatic events trigger adenosine mediated protective mechanism in order to reduce reaction against self-antigens.

Disclosure

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Author contributions

R.R. performed all experiments and, with the help of O.N., prepared the manuscript. J.M. was responsible for all hematological measurements and also helped in preparing the manuscript. Y.S.H. and C.C. helped in designing the study and acted as medical advisors. A.D. conceptualized the experiments, supervised all members of the research team, and helped in manuscript preparation. All authors contributed, in part, to the writing and editing of the final version.

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Figure 1. Susceptibility of A_1R-KO mice to PIL.C57BL/6 WT and A_1R-KO mice were injected with pristane and monitored for alopecia, chronic wounds, or death for 36 weeks. (A) Rate of disease appearance * p <0.05 (n=8-14). Mantel-Cox test sighs of disease graphs. After 36 weeks, mice were sacrificed and analyzed for (B) anti-dsDNA levels in serum of surviving mice by ELISA, and (C) spleen size of surviving mice was measured. *p <0.05, ** p <0.01 (n=3-8). Values are mean ± SE.

Figure 2. Lymphopenia following pristane injection. Following pristane injection to C57BL/6, WT mice, and A_1R-KO mice (n=3-6). Blood counts were performed at the indicated time points. (A) WBC, (B) lymphocyte, (C) neutrophils, and (D) monocytes. *p <0.05, ** p <0.01, compared to control (WT at T=0). Values are mean ± SE.

Figure 3. A_1R and A_{2A}R mRNA levels in PIL. Pristane was injected into C57BL/6 WT and A_1R-KO mice. To examine the dynamic expression of the two high-affinity adenosine receptors, A_1R and A_{2A}R, the spleen was removed at indicated time points (6h, 24h, 48h, and 10 days). A_1R and A_{2A}R mRNA levels in adherent splenocytes were analyzed by real-time PCR and normalized by housekeeping RPL-12 levels. Results are median + interquartile range, (n=3-6) * p <0.05, between expression levels of each receptor to expression at time 0. least two independent experiments performed

Figure 4. Adenosine receptors and susceptibility to induced autoimmune type-1 diabetes. Mice were injected with low dose streptozotocin (STZ, 50mg/kg) for five consecutive days. Mice were considered diabetic when glucose remained above 200mg/dl. The experiment was ended when, in one of the groups, all animals were sick. (A) C57BL/6 WT and A_1R-KO mice, (B) Balb/C WT and A_{2A}R-KO mice. Mantel-Cox test sighs of disease graphs. (n=5-7).

Figure 5. cfDNA in autoimmune diseases. Peritoneal lavage was collected to examine the levels of cfDNA in (A) PIL model at several time points after pristane injection, and in (B) T1D model at day 10 after first STZ injection. Analyzed for cfDNA levels by a direct rapid fluorometric assay with the fluorochrome SYBR Gold (lower panel). Results are median + interquartile range, (n=3-6) *p <0.05, ** p <0.01, between expression levels of each receptor to expression at time 0. ^^p <0.01, ^^^ p <0.001 compared to WT at the same time point (n=5-7). At least two independent experiments performed

Figure 6. Adenosine receptors regulate NET production. (A) HL-60 cells differentiated by retinoic acid (RA) to neutrophil-like cells (2x10^5 cells/well) in triplicates were pre-exposed to A_1 adenosine receptor agonist (CCPA, 1nM) or A_{2A} adenosine receptor agonist (GCS, 30nM), and then cells were stimulated with PMA (200nM) and H_20_2 (0.03%) in the presence of DNA fluorescent dye (5 μM SYTOX Green) for 3h. Relative NETs production was measured by fluorescence in 96-well plates and normalized to NETs production by cells treated with only H_2O_2+PMA. (B) Neutrophils (2x10^5 cells/well) in triplicates were isolated from bone marrow of WT and A_1R-KO mice (C57BL/6 background) and WT and A_{2A}R-KO mice (BALB/c). Relative NETs production was measured by fluorescence and normalized to NETs production by WT neutrophils treated with H_2O_2+PMA.*p < 0.05; **p < 0.01. Values are mean ± SE. At least two independent experiments performed

Authorship

R.R. performed all experiments and, with the help of O.N., prepared the manuscript. J.M. was responsible for all hematological measurements and also helped in preparing the manuscript. Y.S.H. and C.C. helped in designing the study and acted as medical advisors. A.D. conceptualized the experiments, supervised all

members of the research team, and helped in manuscript preparation. All authors contributed, in part, to the writing and editing of the final version.

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