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ORIGINAL ARTICLE

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

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Summary

Adenosine is a potent modulator that has a tremendous effect on the immune system. Adenosine affects T cell activity, and is necessary in maintaining the T helper/regulatory T cell (T_{reg}) ratio. Adenosine signalling is also involved in activating neutrophils and the formation of neutrophil extracellular traps (NETs), which has been linked to autoimmune disorders. Therefore, adenosine, through its receptors, is extremely important in maintaining homeostasis and involved in the development of autoimmune diseases. In this study, we aim to evaluate the role of adenosine A_1 and A_{2A} receptors in involvement of autoimmune diseases. We studied adenosine regulation by NETosis *in vitro*, and used two murine models of autoimmune diseases: type I diabetes mellitus (T1DM) induced by low-dose streptozotocin and pristane-induced systemic lupus erythematosus (SLE). We have found that A_1R enhances and $A_{2A}R$ suppresses NETosis. In addition, in both models, A_1R-knock-out (KO) mice were predisposed to the development of autoimmunity. In the SLE model in wild-type (WT) mice we observed a decline of A_1R mRNA levels 6 h after pristane injection that was parallel to lymphocyte reduction. Following pristane, 43% of A_1R-KO mice suffered from lupus-like disease while WT mice remained without any sign of disease at 36 weeks. In WT mice, at 10 days $A_{2A}R$ mRNA levels were significantly higher compared to A1R-KO mice. Similar to SLE, in the T1DM model the presence of A_1R and $A_{2A}R$ was protective. Our data suggest that, in autoimmune diseases, the acute elimination of lymphocytes and reduction of DNA release due to NETosis depends upon A_1R desensitization and long-term suppression of $A_{2A}R$.

KEYWORDS

cell-free DNA, dsDNA, lupus, NETs, type 1 diabetes

INTRODUCTION

Autoimmune diseases are chronic conditions characterized by the loss of immunological tolerance to self-antigens, leading to self-attack of the immune system on the organs. Genetic, environmental, hormonal and immunological factors are considered important in the etiology of autoimmune diseases such as systemic lupus erythematosus (SLE) (1), rheumatoid arthritis (RA) (2), type I diabetes mellitus (T1DM), etc. (3–5). In the course of these diseases we are witnessing the involvement of various immune cell types. For instance, T cells are capable not only serving as inducers of autoimmune disease but also to inhibit such disease. Naive $CD4^+$ cells can differentiate into T helper cells that secrete cytokines, but in the presence of tumor necrosis factor (TNF)-β will differentiate into T regulatory cells (T_{regs}) (reviewed in (6)), a special population of T cells that modulate the immune system and maintain tolerance to self-antigens. Therefore, T

cells are extremely important in maintaining homeostasis and are involved in the development of autoimmune diseases (7). Neutrophils are also significant regulators of immune responses, which are found to be key players in various autoimmune diseases (8). Defective development, growth and death of neutrophils may lead to the initiation of autoimmune diseases (reviewed in (8)). Another potent immune-modulator that has a tremendous effect on the immune cells during autoimmunity is adenosine (9–11). Adenosine can affect T cell development, proliferation and activity (12–14). Adenosine acts on four receptors, all of which are members of the G protein-coupled receptor family: A_1 receptor (A_1R), A_{2A} receptor ($A_{2A}R$), A_{2B} receptor ($A_{2B}R$) and A_3 receptor (A_3R). The A_1R and A_3R activate Gi protein, which inhibits its adenylyl cyclase activity and decreases cyclic adenosine monophosphate (cAMP) levels and promotes a proinflammatory response. $A_{2A}R$ interacts with Gs and the $A_{2B}R$ interacts with Gs/Gq to induce adenylyl cyclase activity and elevates cAMP levels, thus promoting anti-inflammatory responses (15). Adenosine can be generated from adenosine triphosphate (ATP) degradation by ecto-nucleoside triphosphate diphosphohydrolase 1 (CD39), which catalyzes the sequential transition of ATP to adenosine diphosphate ADP and adenosine monophosphate (AMP) and by the ecto-5′-nucleotidase (CD73), which dephosphorylates AMP. Both CD39 and CD73 are highly expressed on T_{regs} and regulatory B cells (B_{regs}), and are therefore important for shifting the balance between inflammation and immunosuppressive environment (16,17). The adenosine signaling pathway is also necessary for T_{regs} to exert their suppressive function. $A_{2A}R$ has a critical role in maintaining T helper/T_{regs} ratio, as well as the overall T/B lymphocyte ratio within the germinal centers in secondary lymphoid organs (18).

Adenosine also regulates the activation of neutrophils (19). A_1R promotes neutrophil chemotaxis, whereas $A_{2A}R$ and $A_{2B}R$ inhibit neutrophil activation (20). Adenosine signaling is also involved in the formation of the so-called neutrophil extracellular traps (NETs), which are chromatin filaments coated with proinflammatory and effector molecules released by neutrophils into the extracellular space in response to inflammatory triggers. NETs formation has the main role of containing pathogen spreading, but it has been also linked to autoimmune disorders. Activation of $A_{2A}R$ reduces NETs formation, thus contributing to down-regulating NET proinflammatory activity and counteracting the development of autoimmunity (21).

The critical involvement of adenosine in autoimmune diseases is supported by many studies (reviewed in (22)). For example, in a murine model of lupus, it has been shown that $A_{2A}R$ agonist results in significant improvements in renal function (23). In children with T1DM it has been observed that low CD39 levels correlate with disease activity, suggesting a potential compromise in T_{reg} function (24). Abnormal regulatory immune modulators are critical to the pathogenesis of autoimmune diseases, therefore adenosine and the dynamics of its receptors are important to understand during such diseases. We have previously shown in a model of systemic inflammatory response syndrome (SIRS) that a surge of adenosine desensitizes Gi-coupled adenosine A_1R and up-regulates Gs-coupled $A_{2A}R$, which is an important part of the pathology in SIRS and probably needs to be taken into consideration while treating (25). We hypothesize that following traumatic events the suppression of the immune system associated with elevation of $A_{2A}R$ prevents autoimmunity. Therefore, in the current study, we aimed to assess the roles that A_1R and $A_{2A}R$ have in the course of autoimmune diseases in two important murine autoimmunity models (lupus and T1DM).

MATERIALS AND METHODS

Mice

Experiments were conducted after obtaining permission from the Israel Committee for Animal Experiments [interleukin (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 a C57BL/6 background) and $A_{2A}R$ knock-out mice ($A_{2A}R^{-/-}$ on a 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) and $A_{2A}R$ agonist 2-*p*-(carboxyethyl) phenethylamino-5'-N-ethylcarboxamideadenosine hydrochloride (CGS21680), were purchased from Sigma-Aldrich (Rehovot, Israel).

Regulation of NETosis by adenosine

Differentiated human leukemia (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 5 μM retinoic acid (RA; Sigma-Aldrich) for 72 h. Differentiation was confirmed by detection of surface CD11b, which is an early marker

of neutrophil differentiation in HL-60 (26). Differentiation was confirmed when CD11b expression levels were at least 90%. Untreated HL-60 cells stained with the isotype control were used as background for undifferentiated cells.

NETs assay

RA-differentiated HL-60 neutrophils or bone marrow (BM)-isolated cells were seeded (2×10^5 per well) in 96-well plates. Cells were preincubated with or without adenosine agonists (A_1R-specific agonist CCPA, 1 nM or A_{2A}R agonist GCS, 30 nM) for 30 min. They were then treated with 200 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 0.03% H_2O_2 for 3 h (27,28). For DNA detection, Sytox green dye (Molecular Probes, Invitrogen AG; Basel, Switzerland) was used.

Type 1 diabetes model (TID)

We employed the model of low-dose streptozotocin (STZ; Sigma-Aldrich) to induced T1DM in all our experiments. In this model, diabetes develops only when STZ induces both β cell toxicity and T cell-dependent immune reactions (29). We employed a regimen involving multiple administrations of low-dose STZ in mice (30). Diabetes was induced in 8-week-old C57BL/6 mice of both sexes by intraperitoneal (i.p.) injection of STZ (50 mg/kg in citrate buffer) on 5 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 >200 mg/dl for 2 consecutive days were considered to be diabetic (31).

Lupus model

Pristane, a natural saturated terpenoid alkane obtained primarily from shark liver oil, was shown to induce a lupus-like disease in mice (32). Injection of pristane into the peritoneal cavity results in chronic peritonitis associated with high tissue levels of IL-6 (33), which leads to a slow process to lupus-like disease (34).

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 euthanasia (6, 24, 48, 6 and 10 days or 8 months after injection) (35).

Anti-double-strand DNA (dsDNA)

Disease activity was considered according to anti-dsDNA antibodies (36). Serum was separated by centrifugation at 4°C at 1000 ***g*** 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 phosphate-buffered saline (PBS) at the experiment end-point. cfDNA was quantified, as previously described, using our rapid SYBR® Gold fluorometric assay (37).

mRNA analysis by quantitative polymerase chain reaction(qPCR)

Splenocytes production

For mRNA levels, spleens were harvested, and cells were collected and treated with red blood cell (RBC) lysis solution (5 Prime, Inc., San Francisco, CA, USA). Cells were incubated in a Petri dish at 37ºC with medium for 1 h. They were then washed, adhered cells were collected and RNA was extracted using a PerfectPure RNA Tissue Kit (5 Prime, Inc.).

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).

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′; 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; A_{2A} sense 5′- CGC AGG TCT TTG TGG AGT TC-3, anti-sense 5′-TGG CTT GGT GAC GGG TATG-3′.

Statistical analysis

All comparisons between groups were carried out using a Mann–Whitney non-parametric *t*-test or one-way analysis of variance (ANOVA) followed by a Tukey post-test using Prism version 6 software (GraphPad, San Diego, CA, USA). *P*-values below 0.05 were considered significant. Data are presented as mean ± standard deviation (s.d.) unless mentioned otherwise.

RESULTS

DNA released from neutrophils that undergo NETosis is a major source of cfDNA and underpins the progression of autoimmune diseases (36). To explore the regulation of NETosis by adenosine receptors, we used differentiated HL-60 cells stimulated for NETosis by PMA + H_2O_2. Stimulation of neutrophil-like HL-60 cells with CCPA (1 nM), a specific A_1R agonist before induction of NETosis, increased NETs production while pretreatment with the A_{2A}R agonist CGS21680 (30 nM) diminished NETs production (Figure 1).

A_1 and A_{2A} adenosine receptors have a different and opposite role in inflammation progress (38); nevertheless, absence of both receptors were found to accelerate the induction of T1DM. At 15 days after the first STZ injection, all A_1R-KO mice were diabetic (glucose > 200 mg/dl), while the wild-type (WT) (C57 black background) group at this time-point remained free of disease (Figure 2a). Similarly, A_{2A}R-KO mice (BALB/c background) were also more susceptible than WT mice to the early development of T1DM. On day 20 all A_{2A}R-KO mice had elevated blood glucose levels, while only 40% of WT mice propagated T1DM (Figure 2b). In agreement with our results, murine models of A_{2A}R-KO develop stronger autoimmune disease in a shorter time (23,36,39). However, to investigate whether A_1R has a role in autoimmunity or specifically in the development of T1DM, we performed another model. Similarly to our T1DM observation, we observed the appearance of pristane-induced lupus-like disease only in A_1R-KO mice compared to WT mice. A_1R-KO mice began to exhibit the classic pathological signs of lupus: alopecia, chronic skin wounds and death starting long before the WT mice. 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 3a), although they developed anti-dsDNA (Figure 3b), which is a hallmark of lupus. Spleens were removed and measured at the experiment end-point and were significantly larger following pristane injection in WT mice (Figure 3c).

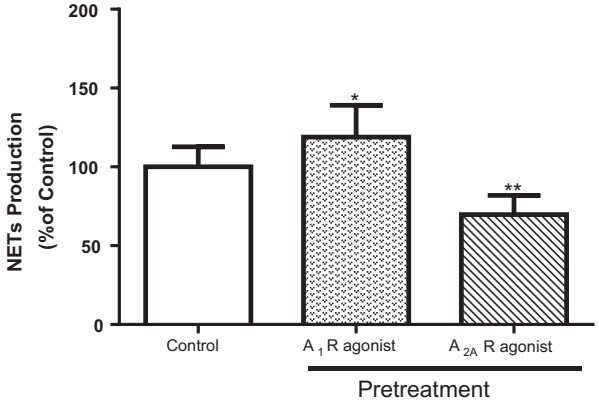

FIGURE 1 Adenosine receptors regulate neutrophil extracellular traps (NET) production. (a) Human leukemia (HL)-60 cells differentiated by retinoic acid (RA) to neutrophil-like cells (2×10^5 cells/well) in triplicate were pre-exposed to A_1 adenosine receptor agonist [2-chloro-N^6-cyclopentyladenosine (CCPA), 1 nM] or A_{2A} adenosine receptor agonist [phenethylamino-5′-N-ethylcarboxamideadenosine hydrochloride (GCS), 30 nM], and cells were then stimulated with phorbol myristate acetate (PMA) (200 nM) and H_20_2 (0.03%) in the presence of DNA fluorescent dye (5 μM SYTOX green) for 3 h. 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.*$P < 0.05$, **$P < 0.01$. Values are mean ± standard error (s.e.). At least two independent experiments were performed

These results, although unexpected, follow our results in the T1DM model. Thus, we evaluated the short-term period after pristane injection using complete blood counts. The basal white blood cell (WBC) counts were lower in A_1R-KO mice compared to WT mice (Figure 4a). In blood counts of WT mice following pristane injection, we observed a sharp reduction in WBC count, where the main leukocyte population to be affected was the lymphocyte count (Figure 4b). At first, lymphocyte counts were reduced and partial recovery was observed 48 h after injection. Neutrophil counts reduced significantly in A_1R-KO mice following pristane injection compare to T = 0 (Figure 4c).

We followed the A_1R and A_{2A}R mRNA levels (normalized to RPL-12) at several time-points for 10 days (Figure 5). In WT mice, parallel to the decline of WBC, A_1R mRNA levels dropped 6 h after pristane injection (Figure 5a) and returned to basal levels 24 h after treatment. In these mice A_{2A}R was induced following injection, reaching significance at 10 days (240 h, Figure 5b). In contrast, A_1R-deficient mice failed to up-regulate the immunosuppressive receptor A_{2A}R and stayed low for 10 days (Figure 5b).

As mentioned above, cell-free DNA (cfDNA) is a product of neutrophils undergoing NETosis, and is considered to be a marker of the progression of autoimmune diseases (36). In both models, baseline cfDNA levels were elevated in A1R-KO mice compared to WT mice. Also, in both models, cfDNA levels were elevated in the autoimmune group compared to untreated mice (Figure 6).

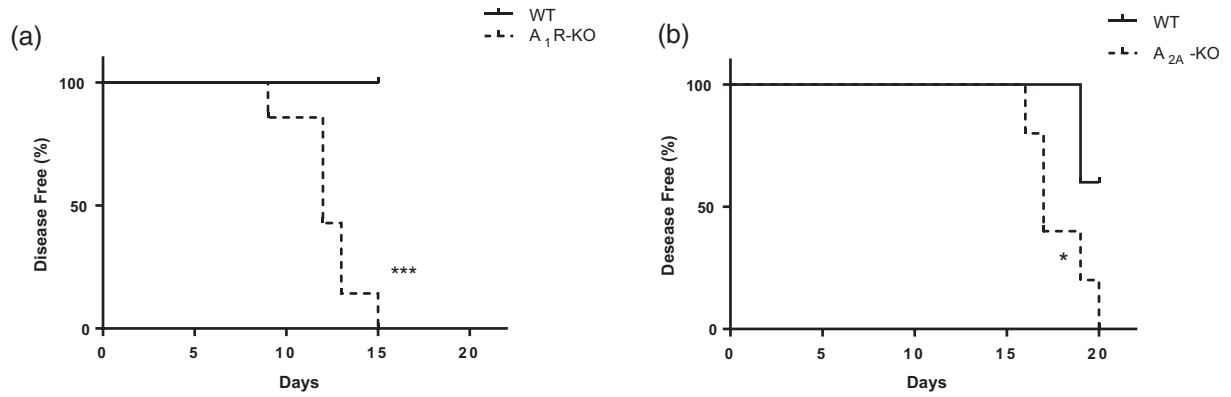

FIGURE 2 Adenosine receptors and susceptibility to induced autoimmune type I diabetes. Mice were injected with low-dose streptozotocin (STZ, 50 mg/kg) for 5 consecutive days. Mice were considered diabetic when glucose remained above 200 mg/dl. The experiment was ended when, in one of the groups, all animals were sick. (a) C57BL/6 wild-type (WT) and A_1 receptor-knock-out (A_1R-KO) mice, (b) Balb/C WT and A_{2A}R-KO mice. Mantel–Cox test signs of disease graphs ($n = 5–7$)

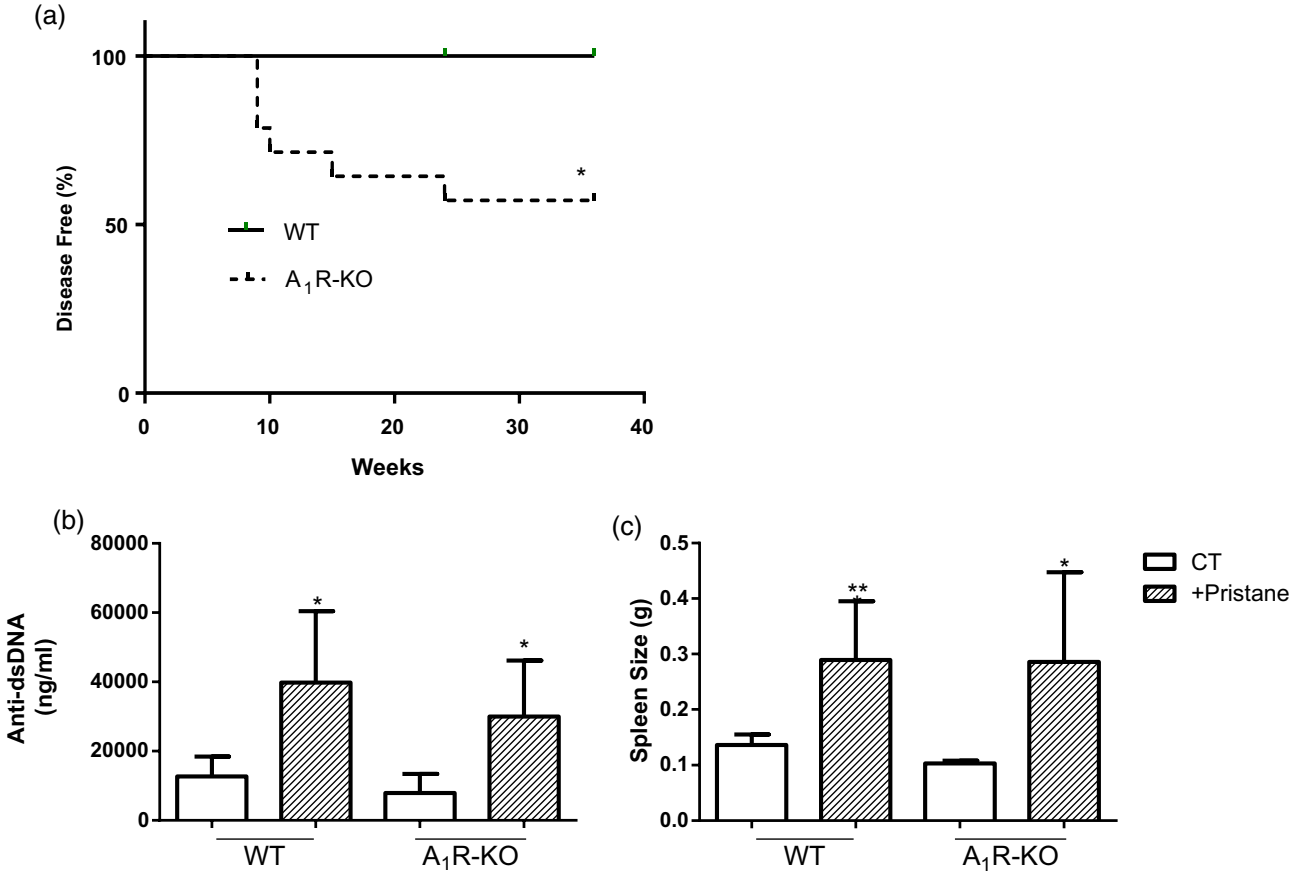

FIGURE 3 Susceptibility of A_1 receptor-knock-out (A_1R-KO) mice to pristane-induced lupus (PIL). C57BL/6 wild-type (WT) and A_1R-KO mice were injected with pristane and monitored for alopecia, chronic wounds or death for 36 weeks. (a) Rate of disease appearance. $^*P < 0.05$ ($n = 8–14$). Mantel–Cox test signs of disease graphs. After 36 weeks, mice were euthanized and analyzed for (b) anti-dsDNA levels in serum of surviving mice by enzyme-linked immunosorbent assay (ELISA) and (c) spleen size of surviving mice was measured. $^*P < 0.05$, $^{**}P < 0.01$ ($n = 3–8$). Values are mean ± standard error (s.e.)

DISCUSSION

Multiple reports suggest that the onset of autoimmune disorders is related at least in part to a partial or complete loss of function in the purinergic pathways and local defective production of adenosine (reviewed in (22)).

In the present study, we suggest that adenosine regulates the release of DNA by NETosis and that the same A_1R/

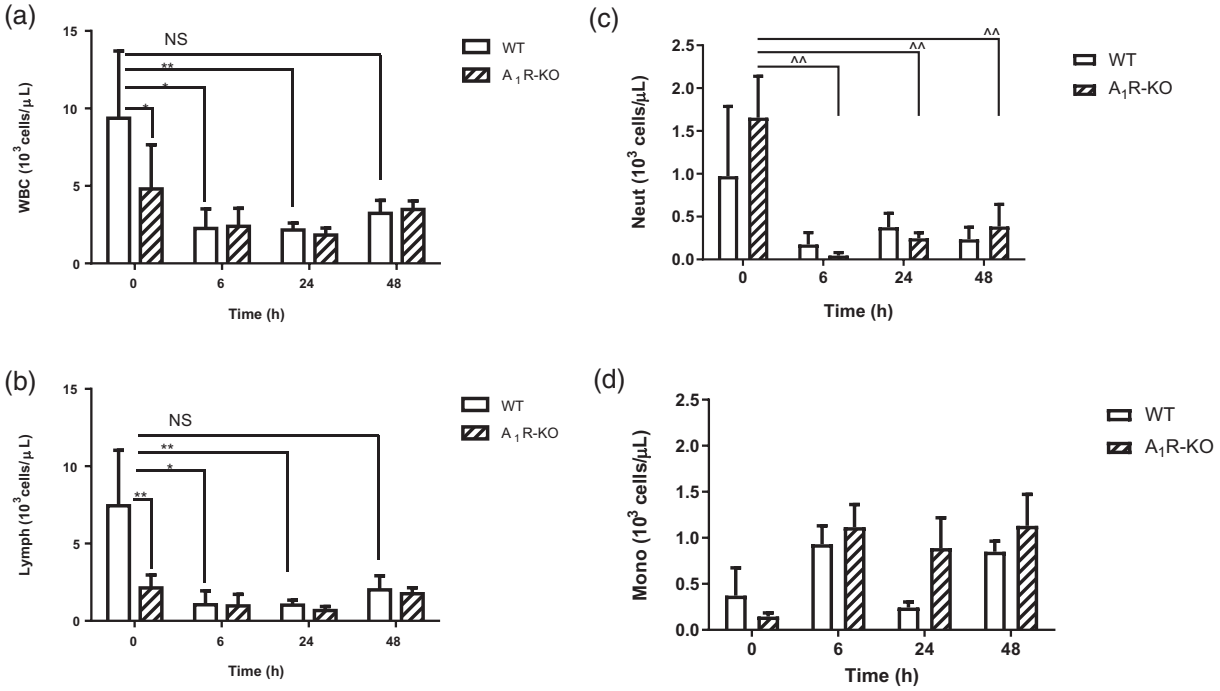

FIGURE 4 Lymphopenia following pristane injection. Following pristane injection to C57BL/6, wild-type (WT) mice and A_1 receptor-knockout (A_1R-KO) mice ($n = 3$–6). Blood counts were performed at the indicated time-points. (a) White blood cells (WBC), (b) lymphocytes, (c) neutrophils and (d) monocytes. $^*P < 0.05$, $^{**}P < 0.01$, compared to control (WT at $t = 0$). Values are mean ± standard error (s.e.)

A_{2A}R-dependent immunosuppressive mechanism reduces cfDNA levels.

Although differing from each other, autoimmune diseases present several shared common phenotypes: high levels of cytokines, the presence of infiltrating immune cells and the presence of non-specific autoantibodies; i.e. anti-nuclear antibodies and dsDNA. The latter 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 the stimulator of interferon genes (STING), a regulator of the immune response (40). Activating STING leads to a signaling cascade that eventually alters proinflammatory molecule production. A defect or unnecessary alert in this mechanism has been described as underpinning the autoinflammatory process (41). One source of dsDNA is related to the production of NETs: activated neutrophils that extrude their DNA and bactericidal molecules, creating NETs in a unique type of cell death called NETosis (15). Neutrophils from patients with various autoimmune diseases are more likely to undergo NETosis than those of healthy donors (42). Moreover, the presence of autoantibodies promotes the release of NETs.

We studied the 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 also enhanced NETs production. A_{2A}R was found to be a negative regulator of NETosis when stimulation of cells with a specific A_{2A}R agonist decreased NETs production of untreated cells. 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 (43). Moreover, a recent study by Ali *et al*. has shown that the A_{2A}R agonist attenuates NETosis in anti-phospholipid syndrome (44).

As A_{2A}R is known for its anti-inflammatory activity, A_{2A}R-KO mice are expected to develop autoimmune disease faster and worsen compared to WT mice. In accordance with our expectations, mice were rapidly ill, as shown in our T1DM model and as shown in the work by Zhang *et al*. in mice with lupus nephritis (23). In recent studies, Patinha *et al*. used the model of STZ in rats and also showed that agonist for A_{2A}R has a protective role in kidneys in hypertensive diabetic nephropathy (45,46). T1DM was shown to modify the expression of adenosine receptors in the brain and alteration in the balance of A_1R and A_{2A}R also effects locomotor activity (47). A_{2A}R seems to up-regulate in coronary flow during diabetes development (48), and the activity of A_{2A}R is needed for the therapeutic activity of $Mg2^+$ (49). While the role of A_{2A}R is not completely understood it is undoubtedly important, and requires further study.

Surprisingly, our data from mice with lupus-like disease and T1DM show that autoimmune diseases are also exacerbated without the presence of A_1R. Similar to our study, Tsutsui *et al*. showed in experimental allergic

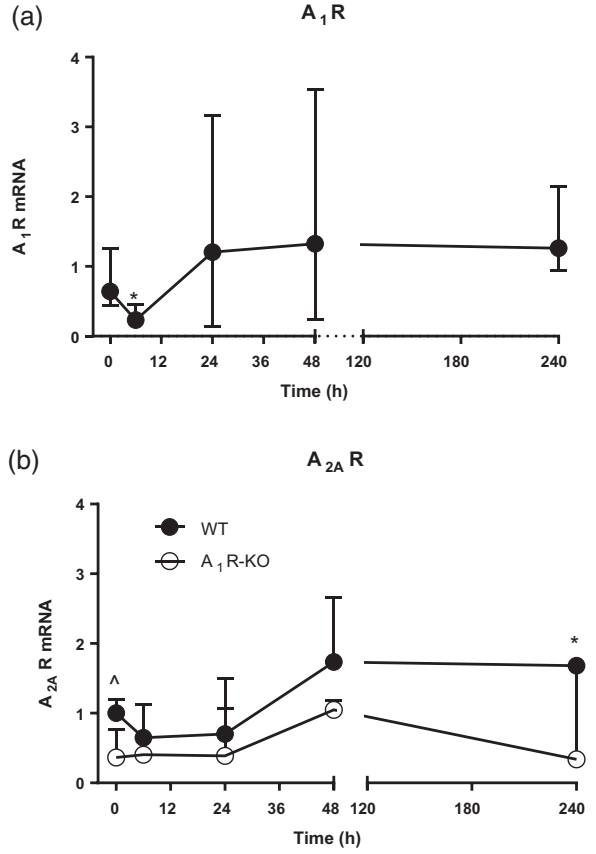

FIGURE 5 A_1 receptor (A_1R), A_1R and $A_{2A}R$ mRNA levels in pristane-induced lupus (PIL). Pristane was injected into C57BL/6 wild-type (WT) and A_1R-knock-out (KO) mice. To examine the dynamic expression of the two high-affinity adenosine receptors, A_1R and $A_{2A}R$, the spleen was removed at the indicated time-points (6, 24 and 48 h and 10 days). A_1R and $A_{2A}R$ mRNA levels in adherent splenocytes were analyzed by real-time polymerase chain reaction (PCR) and normalized by housekeeping ribosomal protein L12 (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. ^$P < 0.5$ compared to WT at the same time-point. At least two independent experiments were performed

encephalomyelitis that, compared to WT mice, A_1R-KO mice developed a severe progressive–relapsing form of the disease (50). In our lupus model, we observed that differences following pristane injection in anti-dsDNA and spleen size did not reach significance between WT and A_1R-KO mice, as the mice with the most severe illness died before the experiment end-point and therefore are not included in these results. Following pristane injection, we observed an acute reduction of A_1R and leukocyte counts in WT animals. Pristane in the peritoneum is known to cause inflammation and damage (reviewed in (51)), and rapid desensitization of A_1R observed immediately after pristane injection is probably due to elevated levels of adenosine. The role of $A_{2A}R$ in regulating lymphocyte depletion is controversial (38). It has been shown that elevations of cAMP following $A_{2A}R$ activation can cause lymphocyte apoptosis (52–54). There are also studies showing the opposite – activation of $A_{2A}R$ can protect lymphocytes from cell death (55,56) We have previously published that the activation of A_1R and $A_{2A}R$ are interdependent: 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 (25,57,58) it is possible that differences in A_1R expression or activation change $A_{2A}R$ activity. In this study, we found that the effect of A_1R depletion is transient, and after 48 h lymphocyte counts begin to recover. We believe that the long-term suppression of immunity that was observed is probably mediated by the elevated $A_{2A}R$ (59), which is induced by A_1R, peaks at 48 h and remains high in WT animals even 10 days after pristane injection. The significant phenomenon of early A_1R stimulation following by up-regulation of $A_{2A}R$ was previously shown by our group (60). These findings also correspond with another work showing that following adjuvant-induced arthritis, adenosine concentration in plasma remains high for weeks (61). In this study, pristane injection failed to up-regulate $A_{2A}R$ in A_1R-KO mice, and the mRNA level of $A_{2A}R$ was also lower in A_1R-KO at $T = 0$. Hence, starting with low $A_{2A}R$ levels leads to stronger lymphocyte reactivity in A_1R-depleted animals and severe autoimmunity disease. A recent study shows a clear association between elevated cfDNA levels and autoimmunity (62).

In the T1DM 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 lupus model, we followed the levels of cfDNA in detail during the first 10 days after pristane injection. On 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 contribute to the severe development of the disease in this group (63).

Adenosine receptors are expressed on a wide variety of immune cells (19). Several studies have shown that $A_{2A}R$ has a role in inhibits TNF-α secretion by macrophages (64–66) as well as an important role in alteration of macrophages from M1 macrophages to M2 macrophages (67, 68) which also contribute to autoimmunity development (69). All four adenosine receptors have been previously described in neutrophils (70). A_1R stimulation enhances neutrophils' adherence to endothelium, chemotaxis (19) and their activity (71), while $A_{2A}R$ inhibits neutrophil trafficking and effector functions such as oxidative burst, inflammatory mediator production and granule release (reviewed in (20,72)).

In conclusion, adenosine initiates diverse cellular responses to prevent excessive inflammation and restoring

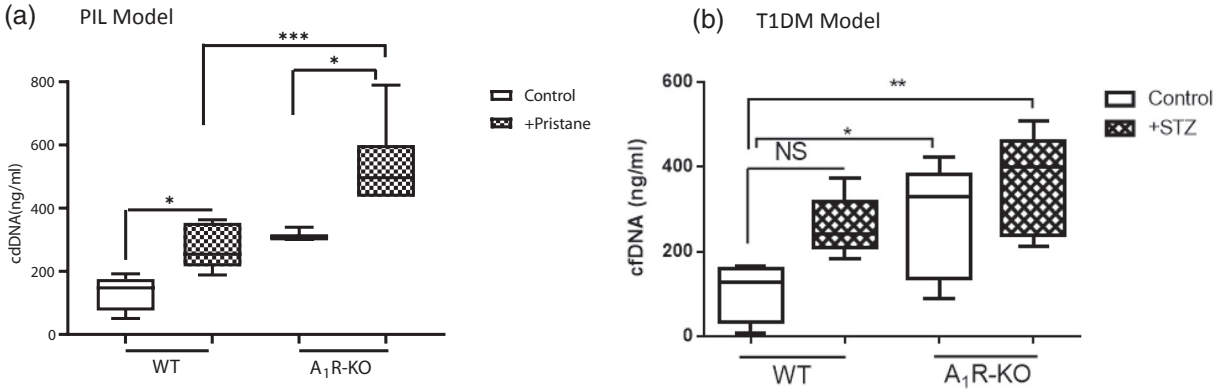

FIGURE 6 cfDNA in autoimmune diseases. Peritoneal lavage was collected to examine the levels of cfDNA in (a) the pristane-induced lupus (PIL) model and (b) the type I diabetes mellitus (T1DM) model at day 10. After the first injection, analyzed for cfDNA levels by a direct rapid fluorometric assay with the fluorochrome SYBR Gold (lower panel). Results are median + interquartile range, ($n = 3$–6). $P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, between expression levels of each receptor to expression at time 0. $^{\wedge\wedge}P < 0.01$, $^{\wedge\wedge\wedge}P < 0.001$ compared to WT at the same time-point ($n = 5$–7)

immune homeostasis. Our data from the lupus model and T1DM suggest that A_1 and A_{2A} receptors have a protective role in autoimmunity development. The acute elimination of lymphocytes and reduction of DNA release due to NETosis depends upon A_1R desensitization and long-term suppression maintained by A_1R-dependent elevation of $A_{2A}R$. We believe that, based on these findings, severe traumatic events trigger a protective mechanism conducted by adenosine to reduce reaction against self-antigens.

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CONFLICT OF INTEREST

The authors have declared that no competing interests exist.

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.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article or available from the corresponding author (R. R.) upon reasonable request.

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