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J Immunol 2011; 186:6798-6806; Prepublished online 18 May 2011; doi: 10.4049/jimmunol.1004222
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High Levels of Adenosine Deaminase on Dendritic Cells Promote Autoreactive T Cell Activation and Diabetes in Nonobese Diabetic Mice

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Adenosine has been established as an important regulator of immune activation. It signals through P1 adenosine receptors to suppress activation of T cells and professional APCs. Adenosine deaminase (ADA) counters this effect by catabolizing adenosine. This regulatory mechanism has not been tested in a disease model in vivo. Questions also remain as to which cell types are most sensitive to this regulation and whether its dysregulation contributes to any autoimmune conditions. We approached this issue using the NOD model. We report that ADA is upregulated in NOD dendritic cells, which results in their exuberant and spontaneous activation. This, in turn, triggers autoimmune T cell activation. NOD DCs deficient in ADA expression have a greatly reduced capacity to trigger type I diabetes. We also provide evidence that although many cell types, particularly T cells, have been implicated as the suppression targets by adenosine in an in vitro setting, DCs also seem to be affected by this regulatory mechanism. Therefore, this report illustrates a role of ADA in autoimmunity and suggests a potential target for therapeutic intervention. The Journal of Immunology, 2011, 186: 6798–6806.

The role of dendritic cells (DCs) in immune induction extends to both protective responses and autoimmunity. The activation of DCs is driven by microbial products (pathogen-associated molecular patterns [PAMPs]), as well as by host factors (danger signals) (1). As in all controlled biological events, these stimulatory signals are offset and balanced by negative regulation. Those negative regulators include cytokines, such as TGF-β and IL-10, and surface receptors, such as programmed death ligand-1, as well as intercellular suppression by regulatory T cells (Tregs). Overall, a balance among these signals is essential to set the boundary between protection and self-inflicted harm. In this regard, one particular regulation, the role of adenosine in immune activation, is an important topic (2).

Adenosine is produced by all cells and signals through P1 adenosine receptors (P1R), which are widely distributed in all tissues. In most cases, P1R engagement triggers adenylyl cyclase and leads to the production of cAMP and downregulation of cellular activation (3). Adenosine production is significantly elevated in stressed tissues in order for the host protection. Adenosine may be secreted or converted from ATP in the extracellular space by a series of ectoenzymes, including CD39 and CD73 (4, 5). It is established that adenosine-mediated suppression downregulates T cell activation in response to TCR/CD3 ligation by blocking CD25 expression and inhibiting IL-2 production (6, 7). This pathway is also important in controlling NKT cell-mediated liver inflammation (8). Tregs use CD39 and CD73 to actively remove ATP, an immune activator, and convert it into adenosine for the suppression of other T cells. Similar suppressive effects are seen with macrophages and DCs (9–12). However, how adenosine is handled in immune activation is rather complex. Although high extracellular adenosine is present in inflamed tissues, it is also subject to the catabolic degradation by adenosine deaminase (ADA). This enzyme converts adenosine into inosine, for further processing into xanthine/hypoxanthine and uric acid. In fact, because adenosine-mediated suppression is strong, the presence of ADA is essential for some immune activation to take place (9, 10). On T cells, the presence of surface ADA enhanced their activation (13–15). In a simplistic extrapolation, high ADA on immune cells effectively behaves like an immune adjuvant.

Type 1 diabetes (T1D) results from autoimmune destruction of insulin-producing pancreatic β cells, leading to hyperglycemia and vascular complications (16, 17). The relevance of ADA/adenosine-mediated immune regulation to T1D rests on the role of DCs in the induction of this autoimmune disorder. The induction of autoimmune T cells is believed to be dependent on the cross-presentation of autologous Ags by professional APCs, mainly DCs (18–20). This leads to exuberant autoimmune CD8 T cell activation. The question arises whether ADA plays an important role in regulating DC activation in vivo and whether it is involved in the cross-presentation of autologous host Ags by DCs to autoimmune T cells.

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Received for publication January 4, 2011. Accepted for publication April 16, 2011.

This work was supported by funding from Canadian Institutes of Health Research (Grant MOP-86468) and National Institutes of Health (Grant R21AI089963) (to Y.S.). P.S.’s contribution was supported by the Canadian Institutes of Health Research and the Natural Sciences and Engineering Research Council of Canada. A.D.M. is supported by a Canadian Institutes of Health Research studentship.

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Abbreviations used in this article: ADA, adenosine deaminase; BMDC, bone marrow dendritic cell; DC, dendritic cell; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; KO, knockout; LN, lymph node; PAMP, pathogen-associated molecular pattern; PEG, polyethylene glycol; P1R, P1 adenosine receptor; T1D, type I diabetes; Treg, regulatory T cell; WT, wild-type.

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In characterizing DCs from NOD mice, we made a surprising finding that ADA levels on these cells are higher than in other strains, implying that NOD DCs are not afforded the same calming effect by regular levels of adenosine. These DCs are hyperresponsive to PAMP stimulation and have an enhanced ability to activate Ag-specific T cells. Therefore, we produced ADA-deficient NOD mice (D2.0 NOD) to study this phenomenon in detail. We report in this article that, despite the relatively normal immune development in these knockout (KO) mice, D2.0 NOD DCs are generally incapable of triggering autoimmune diabetes in adoptively transferred hosts. Our results suggested that intrinsic ADA is an important factor for DCs to trigger T cell activation in vivo. In addition, we obtained evidence that ADA-deficient T cells are less affected in promoting T1D development as long as Ag-presenting DCs are ADA+. Overall, this report suggests that adenosine regulation may be a potential method of intervention for T1D.

Materials and Methods

Mice, cells, and reagents

Female C57BL/6, B10, NOD, and SJL/J mice were purchased from The Jackson Laboratory and used between 7 and 12 wk of age. Those mice, 8.3 CD8+ TCR transgenic NOD mice (8–10 wk of age), and D2.0 and D2.0 NOD mice were maintained at the University of Calgary animal facilities. All mice were maintained in a specific pathogen-free facility, according to the Institutional Animal Care and Use Committee guideline. ADA-deficient mice were obtained with weekly injections of polyethylene glycol (PEG)-ADA, as previously described (21). The D2.0 NOD mice were generated by crossing D2.0 FVB/N founder strain to NOD. ADA KO and wild-type (WT) alleles and ADA minigene were identified by the following primers: Tg forward: 5′-AGCCAACGCAGACGACGACA-3′, Tg reverse: 5′-GCAGGCCTCCTTATCAAGA-3′; Ko forward: 5′-AC-AACAGACAAATCGGCTGCTCTGATG-3′; and Ko reverse: 5′-TTCTGATGTCCTGTCTTGGAT-3′. The KO and minigene alleles were further crossed to NOD mice separately for TTCTGA-3′; TTGAGCCTGGCGAAC-3′; and minigene+ offspring. The further intercross among ADA−/− mice was generated by crossing D2.0 FVB/N founder strain to NOD. ADA KO and wild-type (WT) alleles and ADA minigene were identified by the following primers: Tg forward: 5′-AGCCAACGCAGACGACGACA-3′, Tg reverse: 5′-GCAGGCCTCCTTATCAAGA-3′; Ko forward: 5′-AC-AACAGACAAATCGGCTGCTCTGATG-3′; and Ko reverse: 5′-TTCTGATGTCCTGTCTTGGAT-3′. The KO and minigene alleles were further crossed to NOD mice separately for at least nine generations before they were intercrossed to produce ADA−/− and minigene−/− offspring. The further intercross among ADA−/− mice produced a pool of mice that were maintained from birth with weekly i.p. PEG-ADA injection and screened for ADA−/− and minigene−/− population. Only identified KO mice were used for further weekly PEG-ADA injections.

All reagents were purchased from Sigma-Aldrich, unless otherwise indicated. ADA Abs were purchased from Rockland (100-401-140) and Santa Cruz Biotechnology (H-300). Secondary Abs were purchased from Jackson ImmunoResearch Laboratories. All other Abs and reagents were from eBioscience. Killed Escherichia coli was produced by adding 250 μg/ml ampicillin at 16 and 20 h after inoculation in Luria-Bertani broth. The culture was stopped at 24 h, and the bacteria pellet was subjected to freeze thaw twice. This pellet was tested to be sterile in mammalian cell culture. Cptg oligonucleotide 1826 was obtained from Coley.

NOD or D2.0 NOD bone marrow (BM) DCs were produced, as previously described, with 3 ng/ml rGM-CSF and 3 ng/ml rIL-4 (9). In all BMDC assays, 6- or 7-d DC cultures were gently washed twice with warm PBS, and the supernatants were measured for IFN-γ by ELISA. ADA-depletion assays

A total of 5 ml of protein A beads (RepliGen) was washed in PBS and incubated with 2 mg Rockland ADA Ab, in buffer (pH 8.5), for 20 min with tumbling. The Ab was then cross-linked to the matrix with freshly opened 20 mM dimethyl pimelimidate dry powder (Pierce), and the reaction was terminated by adding glycine to the mixture. FBS was passed through a free-flow column filled with the protein A/ADA Ab beads. The beads were regenerated by acid wash (0.1 M glycine [pH 2.6]), and the FBS elute was further depleted for at least one round. The depletion was confirmed by HPLC analysis of adenosine-to-inosine conversion (9).

8.3 CD8+ T cell activation

NOD or D2.0 NOD DCs were washed twice and replenished with fresh warm medium. They were then incubated with indicated treatments overnight, followed by pulsing with 10−5 M NRP-V7 or A7 or a control TUM peptide for an additional 1 h. Cells were washed off the plate and washed twice. Twenty thousand DCs pulsed with Ag peptides were mixed with 105 T cells in a total volume of 200 μl in 96-well plates. The mixture was incubated for 48 h, and the supernatants were measured for TNF-α by ELISA.

Adoptive transfers

A total of 106 purified NOD or D2.0 NOD BMDCs, pulsed with 1 μg/ml NRP-V7 peptide, was injected i.v. into 8–10-wk-old female 8.3 TCR transgenic NOD mice. ADA−/− mice were purified in a total volume of 200 μl in 96-well plates. The mixture was incubated for 48 h, and the supernatants were measured for TNF-γ by ELISA.

FACS analysis

Cells were washed, and FcRs were blocked using anti-CD16/CD32 Ab for 30 min. For surface staining, cells were incubated with FITC and PE/Cy5-labeled appropriate Abs in different combinations for 30 min. For intracellular staining, surface-stained cells were fixed, permeabilized using a fixation/permeabilization kit (eBioscience), and incubated with fluorochrome-labeled isotype control or marker-specific Abs at room temperature. For some assays, in vitro-stimulated cells were restimulated using PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (1 mg/ml) for 4 h, preincubating for intracellular cytokines. Stained cells were analyzed using FACS-Calibur (BD Biosciences) or LSR analyzer (BD Biosciences), and the data were analyzed using FlowJo (TreeStar).

Real-time PCR

The method was published previously (15). In short, RNA was isolated from DCs obtained from C57BL/6 and NOD mice. The QIAGEN QIAshredder and the RNeasy mini kit were used for RNA isolation, according to the manufacturers’ protocols. The quality and quantity of RNA were analyzed using the Nanodrop 1000. CDNA was synthesized using the QIAGEN QuantiTect Reverse Transcription kit. Real-time PCR was performed according to the protocol from QIAGEN QuantiTeq SYBR Green PCR kit using the ABI Prism 7000. The following primers were designed specifically for real-time PCR with product sizes ~150 bp: ADA forward primer, 5′-GAACAGACGACTGAGGGAGC-3′; ADA reverse primer, 5′-CCACAGAGGACCCGACCTTTG-3′; and GAPDH forward primer, 5′-TTCCACACCAGGAGAACGC-3′; GAPDH reverse primer, 5′-GCGATGGACTGTGGTCATGA-3′. The data were analyzed using the delta Ct method to determine the relative expression of the gene of interest ADA in NOD mice compared with B6 mice.

Statistical analysis

Student t tests were shown in all bar graphs, with p values indicated. The error bars represent SEs. Log-rank assay (Prism) was used for survival analysis.

Results

NOD DCs express high levels of ADA

In our previous work, we found that DC-associated ADA removes the suppressive effect of adenosine and is absolutely required for...
DC activation. Addition of ADA to the DC-stimulation culture enhanced proinflammatory cytokine production (9) (data not shown). This suggested that ADA is linked to hyperresponsiveness of DCs. To study the state of DCs in NOD mice, we stained their BMDCs for surface ADA expression, compared with non-NOD mice, such as BALB/c, C57BL/6, and B10. ADA levels on NOD CD11c+ BMDCs were higher compared with the controls (Fig. 1A, 1D). Because BMDCs differentiate from their progenitors and have not been subjected to the in vivo environment of the NOD strain, such a high expression of ADA is likely intrinsic to NOD DCs rather than being a result of the inflammatory milieu in NOD mice. ADA does not have a transmembrane domain; its association with the plasma membrane in mouse is reportedly via binding to PIR A2B and A1 receptors (22, 23). In addition, the higher levels of ADA on NOD DCs could be from endogenous, as well as external, serum (bovine) sources in the culture media (9). Therefore, we isolated DCs from NOD mice with MACS beads and stained them for surface ADA. Higher ADA was also seen on primary DCs from splenic, mesenteric, and pancreatic LNs (Fig. 1B). The high expression level is likely contributed by high ADA mRNA (normalized to GAPDH), as indicated by real-time PCR (Fig. 1A). NOD DCs stimulated with CpG showed enhanced ADA expression; the magnitude of upregulation was smaller than for other DCs with similar treatment (Fig. 1C). The absolute amounts of ADA on all DCs were similar after the stimulation. This suggested that ADA upregulation is a stimulated response to reduce adenosine in the vicinity; however, NOD DCs had a higher basal ADA expression without any stimulation. With regard to the cause of this high expression, we studied ADA levels on B10 mice. ADA expression B10 BMDCs is lower than NOD. Therefore, MHC haplotype and Idd 1 locus, which B10 mice obtained from NOD, have not been subjected to the in vivo environment of the NOD strain, such a high expression of ADA is likely intrinsic to NOD mice. Interestingly, the maximal CD86 expression (stimulated by killed bacteria) was similar. However, NOD DCs expressed it at higher levels without any stimulation.

Because autoimmune responses are characterized by the presence of self-recognizing T cells, we stimulated IGRP206-214 (islet-specific glucose-6-phosphatase catalytic subunit-related protein)-specific 8.3 CD8+ T cells (24, 25) with NOD DCs loaded with peptide NRP-V7, a superagonistic mimotope of IGRP206-214. Compared with B10 DCs, which share the same MHC background with NOD strain (H2g7), NOD DCs induced greater IL-2 and IFN-γ production by 8.3 CD8+ T cells or total 8.3 splenocytes after 48 h, suggesting that the hyperresponsiveness of NOD DCs can enhance autoreactive T cell activation (Fig. 2C). This result suggested that the excessive ADA on NOD DCs is responsible for the hyperactivation of T cells that they stimulate.

**T cell activation is suppressed without endogenous ADA**

From an immunological vantage point, a large amount of evidence has been collected that suggests that T cells are sensitive to

**NOD DCs are hyperresponsive**

We reasoned that the high ADA on NOD cell surface may result in a hyperresponsive DC activation, because the negative inhibition of adenosine is diminished with an abundance of ADA. NOD BMDCs were stimulated with PAMPs, as indicated. IL-12 and TNF-α production were measured by ELISA. Fig. 2A shows that, compared with control, NOD DCs produced more inflammatory cytokines, such as TNF-α, in support of our reasoning. When EHNA, an ADA inhibitor, was added to the culture, cytokine production was reduced, confirming the role of ADA in this process (Fig. 2A). We also assessed the expression of CD86 following the same treatment. Similar to the cytokines, CD86 expression on NOD DCs was greater than the controls (Fig. 2B). Interestingly, the maximal CD86 expression (stimulated by killed bacteria) was similar. However, NOD DCs expressed it at higher levels without any stimulation.

![Figure 1](https://example.com/figure1.png)

**FIGURE 1.** NOD DCs express high levels of surface ADA. A, GM-CSF/IL-4–cultured day 6 BMDCs from NOD or non-NOD strains (BALB/c shown, similar results with C57BL/6 DCs) were stained with CD11c (PECy5) and Santa Cruz ADA (FITC) Abs. ADA levels on CD11c+ cells are shown. The gray graphs represent the second Ab alone. B, Cells from spleen and mesenteric and pancreatic LNs from NOD and BALB/c mice were stained with CD11c and ADA Abs, as in A. ADA expression on CD11c+ cells is shown. C, As in A, DCs were treated with CpG for 24 h prior to FACS analysis. Mean fluorescence intensities minus background are 13.45 and 10.85 for NOD and 6.06 and 2.55 for B6 DCs, with or without CpG stimulation, respectively. D, Top row, As in A, except that B10 and NOD DCs were analyzed. Bottom row, Comparison of ADA expression on BMDCs from B6, NOD, and SJL/J mice.
adenosine suppression. Unlike for DCs, we did not consistently see enhanced surface ADA expression on NOD T cells. Thus, the question becomes “Is intrinsic ADA expression essential for T cell activation, although it is not abundant on the cell surface?”

We analyzed T cell activation with FVB/N ADA KO (D2.0) mice. Because ADA KO is perinatally lethal, the mice are maintained with a weekly injection of PEG-ADA. CD4 and CD8 T cells from the founder strain of D2.0 (21, 26) were purified and stimulated with anti-CD3, and IL-2 production was measured by ELISA. To rule out any role of serum ADA, we produced ADA-deficient FBS by double-affinity immunodepletion. The resulting FBS showed no ADA activity (9), and the depleted serum was used in place of regular FBS. Fig. 3A shows that, upon plate-bound anti-CD3 stimulation, ADA-deficient T cells produced less IL-2 than did controls. The presence of serum ADA rescued IL-2 production to some extent, but it never approached the level of control T cells. Adding adenosine or EHNA further inhibited IL-2 production, which indicated that intrinsic ADA is an important regulator in T cell activation.

To approach the same issue with an in vivo model, we immunized D2.0 mice (H-2b) with B6 (H-2b) splenocytes initially, followed by a challenge with EL4 cells (from B6, H-2b, allogenic to FVB/N H-2b), which led to an alloreactivity by the T cells in the recipients, and intracellular IFN-γ production from those infused CD4 T cells was determined. Fig. 3D shows that, even with regular serum ADA levels, ADA−/− T cells mounted little response to allogenic targets. Overall, Fig. 3 shows that T cell-associated ADA is important for T cell activation in vivo.

Charaterization of D2.0 NOD mice
To test the role of ADA on T1D, it was necessary to generate an ADA deficiency in a suitable mouse strain. ADA KO mice were available under FVB/N founder background (21, 26). To transfer the KO phenotype into NOD mice, a two-step rescue scheme is necessary to produce the adult mice. First, a minigene with restricted placenta ADA expression must be present to ensure the birth of homozygous KO. ADA-deficient newborns are further rescued by weekly i.p injection of PEG-conjugated ADA. Previous reports on these strains showed a largely normal phenotype in the peripheral and central immune cells (21, 26). To produce ADA KO NOD mice from the current FVB/N background, the minigene and the ADA-deficient allele were bred separately into NOD mice for 10 generations. Then, the hemizygous minigene and the ADA-deficient allele were bred separately into ADA KO NOD mice from the current FVB/N background, the resulting D2.0 NOD (D2.0 NOD) mice show no gross abnormalities, although they are severely lethargic and become moribund upon PEG-ADA withdrawal. In the initial article by Apasov et al. (27), ADA deficiency without PEG-ADA injection showed greater

![FIGURE 2. NOD DCs are hyperreactive. A, Day-6 NOD or B6 DCs were not treated or were treated with Cpg Oa or a combination of Cpg (5 μg/ml) and EHNA (10 μM + 5 mM adenosine) for 24 h, and TNF-α and IL-12p70 production were measured by ELISA. B, Top row, Similar to A, except that DCs were stimulated with killed E. coli (0.0025% total volume) for 24 h, and CD86 expression was determined by FACS. Mean fluorescence intensities minus background are 27.87 and 15.77 for NOD and 27.48 and 1.98 for B6 DCs, with or without stimulation, respectively. Bottom row, Comparison of surface CD86 expression on the surface of unstimulated BMDCs from B6, NOD, and SJL/J mice. C, Left panel, CD8+ T cells were purified from 8.3 CD8+ TCR transgenic mice and cultured with killed B6 splenocytes. In this setup, MHC class II I-Aq and I-Eq of FVB/N strain allogenically stimulated B6 CD4 T cells was determined. Fig. 3B that D2.0 T cells had a reduced response to stimulation, suggesting that T cell activation in vivo is also regulated by endogenous ADA. To test whether T cell-intrinsic ADA is important under the regular serum ADA background, we depleted CD4 T cells from ADA-proficient FVB/N mice (Fig. 3C). These mice were injected i.v. with D2.0 CD4 T cells obtained after two rounds of immunization (as in Fig. 3B). B6 splenocytes were instead infused into the recipients, and intracellular IFN-γ production from those infused CD4 T cells was determined. Fig. 3D shows that, even with regular serum ADA levels, ADA−/− T cells mounted little response to allogenic targets. Overall, Fig. 3 shows that T cell-associated ADA is important for T cell activation in vivo.](http://www.jimmunol.org/2017/6801/6801-Fig2)
thymic apoptosis with a relatively normal peripheral lymphocyte distribution. To characterize any potential immunological defects with the maintenance of PEG-ADA, 4-wk-old thymic and splenic populations were analyzed for T and B cell markers. D2.0 NOD mice show largely normal single and double CD4 and CD8 markers in the thymus (Fig. 4B), suggesting that the mice have near-normal thymic development with PEG-ADA rescue. The splenic CD8⁺, CD4⁺, and B220⁺ populations also are not grossly affected (Fig. 4B). CD11c⁺ DCs were similar in number in NOD and D2.0 NOD mice. However, similar to ADA inhibition in DC activation, D2.0 NOD BMDCs produced less TNF-α in response to several PAMPs (Fig. 4C).

8.3 CD8⁺ T cell transfer

Serum ADA levels fluctuated over time with the weekly injection of PEG-ADA. The level immediately after the injection was substantially higher than in the WT control (data not shown). Therefore, NOD KO mice are not an ideal host in which to study immune functions with respect to ADA, and they also develop diabetes at time points comparable to NOD mice. To study ADA’s role in DC activation in an in vivo setting under routine environmental ADA levels, D2.0 NOD DCs were cultured from bone marrow, pulsed with NRP-V7 peptide, and transferred into 8.3 mice. 8.3 CD8⁺ T cells stimulated by NRP-V7 presented by DCs become activated, and the host is expected to become diabetic rapidly. This was indeed the case. Six of seven NOD WT DC recipients became diabetic. In comparison, only one recipient of D2.0 NOD DCs showed high urine glucose (Fig. 5A). This result indicated that NOD DCs without intrinsic ADA are far less capable of stimulating 8.3 CD8⁺ T cells, in line with our hypothesis.

To study 8.3 CD8⁺ T cell activation in this setting closely, we measured T cell-activation markers. Following DC/T cell transfer, 8.3 CD8⁺ T cells in NOD recipients showed high levels of CD69 and CD44, pointing to an activated phenotype (Fig. 5B). In comparison, 8.3 CD8⁺ T cells in D2.0 NOD recipients showed lower expression of CD69 and CD44 (Fig. 5B). This observation is in line with the diabetes readout. To measure T cell proliferation, 8.3 CD8⁺ T cells were labeled with CFSE and injected into NOD mice, as well as NOD or D2.0 NOD mice. Proliferation of the injected cells was measured by CFSE dilution, and 8.3 CD8⁺ T cells in D2.0 NOD mice exhibited lower levels of proliferation (Fig. 5C). These results suggested that intrinsic ADA deficiency substantially reduces DCs’ ability to trigger autoimmune T cell activation.

Adenosine suppression: what is the target?

We previously reported that DC-associated ADA plays a crucial role in supporting DC activation. Fig. 5 further establishes that ADA is also important for DC activation in NOD mice. However, it is not known whether T cell-associated ADA is also crucial in T1D development. This knowledge is important because it points to the potential target of therapeutic intervention. Therefore, we performed an experiment reciprocal to Fig. 5A. We purified CD8 and CD4 T cells separately from both NOD and D2.0 NOD mice, and 10 million of each were injected into NOD.scid hosts. The separate purification procedure, although tedious, avoids the use of anti-CD3 that could activate T cells. Furthermore, the resulting mixture (CD4+CD8) does not contain any donor APCs, which allows Ag presentation by the host, ADA-proficient DCs (Fig. 6). Although D2.0 T cells seemed less likely to trigger T1D in NOD.
scid mice, the difference between the two donor strains was less profound than in the DC transfer. Combining Figs. 5 and 6, it is clear that even in an ADA normal environment, cell-associated ADA plays an important role in DC cell activation in autoimmune diabetes and, to some extent, in T cell Ag-specific activation.

**Discussion**

Adenosine regulation of immune activation has long been a topic of interest. The earliest work by Ohta and Sitkovsky (28) indicated that A2AR KO mice had a diminished ability to handle sepsis. This seminal discovery established the role of adenosine signaling in inflammation suppression and laid the foundation for our present study. The adenosine regulation on the immune system has been mainly attributed to its regulation on T cell activation. Linden’s group (7) and Sitkovsky’s group (6) showed that adenosine suppressed CD4 T cell activation, reducing CD25 expression in response to anti-CD3 stimulation. More recently, two reports suggested that the involvement of adenosine in immune activation may involve more than direct downregulation of T cells. First, it was found that NKT cells mediate severe liver damage in A2AR KO mice.
Labeled with CFSE. A total of 107 cells was injected into NOD or D2.0 recipients; thin lines, D2.0 NOD recipients. A total of 106 NOD or D2.0 NOD BMDCs was purified with CD11c MACS for NOD DCs.

Glucose levels on two consecutive days (FIG. 5A, except that 10 million each of CD4 and CD8 cells from NOD or D2.0 NOD mice were purified and mixed before infusion into NOD.scid mice. The rate of diabetes was determined as in Fig. 5 (n = 6 for each group).

FIGURE 5. Adoptive transfer of ADA−/− DCs into NOD mice. A, A total of 10^6 NOD or D2.0 NOD BMDCs was purified with CD11c MACS beads and pulsed with 1 μg/ml NRP-V7 peptide for 2 h. The cells were washed and injected i.v. into 8.3 NOD mice. The procedure was repeated for the next 2 wk. The onset of T1D was determined by positive urine glucose levels on two consecutive days (n = 3 for D2.0 NOD DCs; n = 7 for NOD DCs). B and C, Purified CD8 T cells from 8.3 NOD mice were labeled with CFSE. A total of 10^7 cells was injected into NOD or D2.0 NOD mice. Six days later, CFSE+ cells were stained for CD69 and CD44 expression (B) or measured for their proliferation (C). Thick lines, NOD recipients; thin lines, D2.0 NOD recipients.

KO strains (8). Other reports indicated that CD39, the ectoenzyme mediating the conversion from ATP to adenosine on the cell surface, may be used by Tregs to regulate adenosine levels and, thus, exert their suppressive effects on other T cells (4, 5). With respect to T1D, Hasko’s group (29) reported several years ago that in drug-induced T1D (in CD-1 mice), A2BR signaling inhibited diabetogenesis. That report established a role for adenosine signaling in T1D and set the groundwork for our work. It largely remains an open question as to how adenosine-mediated suppression works on APCs and how it affects cross-presentation of host Ags, a crucial step in autoimmunity.

Three years ago, our group suggested the nearly complete loss of ADA expression in NOD will be relevant, one needs to ascertain the most susceptible cellular target of adenosine suppression. In this report, we address both issues: adenosine regulation is a basal regulator of DC activity and subsequent DC-induced T cell activation in vivo, and DCs’ ADA deficiency (or inhibition) seems to impact overall immune activation.

Prior to this report, strong evidence existed that DCs are a main player in T1D. One line of investigation pointed out that a wave of pancreatic cell death precedes the onset of diabetes (20). We reported that cell death releases endogenous adjuvants, such as uric acid, that strongly activate DCs (31, 32). However, if such a factor is removed, autoreactive T cells show substantially reduced response to an islet-specific transgenic neo Ag (OVA, in RIP-OVA diabetes model) (33). Removal of pancreatic LNs leads to decreased diabetes incidence, suggesting that DC migration to secondary lymphoid organs is critical (34). Furthermore, DCs are known to be first activated in the islets when other immune cells (T cells) are resting (35). Ag-specific T cells are activated in draining LNs. This pattern of Ag activation is consistent with the route of DC migration after activation. In our previous work, we provided substantial evidence that modulation of adenosine influences DC activation, implicating its potential roles in vivo.

We believe that this is the first study to produce an animal model in which to dissect the role of ADA as a regulator for T1D autoimmunity. The main finding is that ADA acts as a promoter of DC activation and may contribute to the onset of autoimmune T cell dysregulation in NOD mice. The high ADA expression on NOD DCs and, hence, their hyperactivity, is likely intrinsic to this strain, because BMDCs generated with GM-CSF/IL-4 display this phenotype. The regulation of ADA protein expression in NOD will be

FIGURE 6. T cells from D2.0 NOD mice in driving T1D. Similar to Fig. 5A, except that 10 million each of CD4 and CD8 cells from NOD or D2.0 NOD mice were purified and mixed before infusion into NOD.scid mice. The rate of diabetes was determined as in Fig. 5 (n = 6 for each group).
a topic of interest in the future. We suggest that high ADA on NOD DCs triggers their activation in response to stimulations and may serve as a clinical target for drug intervention. Certainly, the next important step is to determine whether high ADA levels are also a feature of DCs from diabetic patients.

Our mouse model is not a true functional deficiency of ADA. To rescue the lethality, ADA is provided as a PEG conjugate. The combined approach, using ADA rescue minigene in the placenta and weekly injection of PEG-ADA, restores normal birth and development and, to a large extent, avoids pathologies associated with ADA deficiency, such as pulmonary stress. Their life span can be as long as the WT, but it is far from ideal. The blood ADA levels fluctuate. In our estimation, upon PEG-ADA injection, blood ADA levels are significantly higher than in WT controls (data not shown). This may explain why ADA KO NOD mice develop diabetes, because the DCs and T cells are under less adenosine suppression than in WT mice. Because mice without ADA do not survive, a compromised measurement is to study how ADA-deficient DCs behave in a WT environment. With such an assay, we established that ADA expression by DCs is a prerequisite for DCs’ induction of autoimmune diabetes. This provides strong support for our in vitro experiments in which ADA+− NOD DCs were suppressed with regard to their cytokine production and ability to induce T cell activation. Interestingly, despite the large number of previous reports of adenosine’s suppressive effect on T cells, ADA KO 8.3 CD8+ T cells seemed to maintain a certain capacity to trigger T1D in scid mice. Although it is difficult to quantify which population is more severely affected by the lack of ADA, it is prudent to state that in T1D, both DC and T cell activations are influenced by their ADA levels, in line with our (9, 10, 15) and Čsoóka et al. (36) previous work. In fact, a detailed understanding of ADA’s effect on various immune cells is important for a practical reason: potential intervention of T1D. To assess the effect of ADA inhibition on T1D, we administered a clinical ADA inhibitor (deoxycoformycin) to NOD mice for several months. Some treated mice still developed diabetes in addition to an array of other anomalies (data not shown). This is not surprising because adenosine signaling is involved in many physiological processes. In particular, it interferes with CAMP regulation in glucose-induced insulin secretion and reduces insulin sensitivity (37, 38). In addition, long-term presence of high adenosine desensitizes P1Rs and renders DCs and T cells insensitive to adenosine suppression (10, 15). Therefore, the complete inhibition of ADA is not a desired method. Our results emphasize the need for targeted and temporally controlled ADA inhibition in T1D treatment.

It is important to note that although ADA plays an important role in DC activation, high ADA does not cause T1D by itself, because other Swiss strains are diabetes resistant. Regarding the role of ADA in sustaining immune activation, our work does not support the costimulatory activation of surface ADA. In humans, work by Morimoto and colleagues (39) and Franco and colleagues (40) suggested that ADA binds to surface CD26. The bound ADA serendipitously interacts with adenosine receptor A1 or A2B, forming a costimulation-like bridge. This is certainly not the case for our study; mouse ADA did not bind CD26, and intracellular deficiency of ADA led to suppressed DC activation. Therefore, it is very likely that our early proposal that DC-associated ADA deaminates adenosine and, thus, sustains DC activation still holds (9). Overall, this work indicated that the role of hyperactivated ADA on NOD DCs is likely a major reason why T1D develops rapidly in NOD mice. It reveals a new line of investigation, and potential pharmacological means may be developed as a result of this work to provide a new treatment for this autoimmune disease.

Acknowledgments

We thank Dr. Kenneth Rock (University of Massachusetts Medical School) for CD4-depleting Ab.

Disclosures

The authors have no financial conflicts of interest.

References

Corrections


There was a mistake in processing the FACS plots that resulted in a duplication of two panels. In Fig 4B, top row of the FACS plots, the right plot of the middle two plots (KO of Spleen) was incorrect. The revised Fig. 4 is published below. The legend for Fig. 4 was correct as published and is shown below for reference.

FIGURE 4. Characterization of ADA-deficient NOD mice. A, D2.0 ADA KO alleles and the rescue minigene were bred into NOD mice for 10 generations. These mice were genotyped by PCR for the presence of the minigene and KO allele and the absence of the WT allele (1/8 probability). B, Cells from indicated sources from D2.0 NOD and NOD mice were stained for various immune markers. Overall, D2.0 NOD immune development was not drastically different from that of NOD. Pooled data from three or four independent experiments are presented in bar graphs to show the range. The lower right panel shows the small numbers of DCs. C, Similar to Fig. 2A, NOD or D2.0 NOD BMDCs were not treated or were stimulated with 5 μg/ml CpG, 5 ng/ml LPS, or 0.0025% v/v killed E. coli. TNF-α production was measured after 24 h. LB, left bottom; LT, left top; RB, right bottom; RT, right top.