Contraction of intestinal effector T cells by retinoic acid-induced purinergic receptor P2X7

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The intestinal environment harbors a large number of activated T cells, which are potentially inflammatory. To prevent inflammatory responses, intestinal T cells are controlled by various tolerogenic mechanisms, including T-cell apoptosis. We investigated the expression mechanism and function of the purinergic receptor P2X7 in contraction of intestinal CD4⁺ effector T cells. We found that P2X7 upregulation on CD4⁺ effector T cells is induced by retinoic acid through retinoic acid receptor α binding to an intragenic enhancer region of the P2rx7 gene. P2X7 is highly expressed by most intestinal αβ and γδ T cells, including T-helper type 1 (Th1) and Th17 cells. The intestinal effector T cells are effectively deleted by P2X7 activation-dependent apoptosis. Moreover, P2X7 activation suppressed T-cell-induced colitis in Rag1⁻/⁻ mice. The data from vitamin A-deficient and P2rx7⁻/⁻ mice indicate that the retinoic acid-P2X7 pathway is important in preventing aberrant buildup of activated T cells. We conclude that retinoic acid controls intestinal effector T-cell populations by inducing P2X7 expression. These findings have important ramifications in preventing inflammatory diseases in the intestine.

INTRODUCTION

The intestinal immune system is in a state of constant activation because of the ample presence of foreign antigens and microbial products in the gut lumen. The majority of intestinal CD4⁺ T cells are activated cells, and many of these T cells recognize antigens generated by commensal bacteria.¹ Although intestinal effector T cells have important roles in regulating the gut microbiota and mounting immune responses to pathogens, activated T cells can mediate adverse inflammatory responses, leading to colitis, allergic responses, or other chronic inflammatory diseases. To prevent inflammatory responses, the intestinal immune system must have effective tolerance mechanisms. Major mechanisms of immune tolerance in the intestine include generation and maintenance of FoxP3⁺ and/or IL-10⁺ (interleukin-10-positive) regulatory T cells (Tregs), induction of anergic lymphocytes, and disposal of excessive effector cells, the latter of which is called effector cell contraction.²,³ T-cell contraction is mediated by apoptotic signaling induced by activation-induced cell death pathways mediated by FAS/FAS ligand, perforin, T-cell receptor proximal kinases, reactive oxygen species, and P2X7 signaling.⁴-¹⁰ P2X7 is a purinergic receptor and adenosine triphosphate (ATP)-gated cation channel.¹¹ P2X7 is activated by nicotinamide adenine dinucleotide (NAD)-induced adenosine diphosphate (ADP) ribosylation, which is catalyzed by the enzyme ADP-ribosyltransferase 2 (ART2) in rodents.¹² P2X7 is also activated by high levels of extracellular ATP.¹³ Activation of P2X7 induces rapid influx of cations, intracellular signaling, and pore formation on the cell membrane for apoptosis.¹⁴ P2X7 is expressed by T cells, macrophages, mast cells, epithelial cells, and neuronal cells.¹⁵-¹⁷ P2X7 activation can lead to inflammasome activation and cytokine expression in monocyteic lineage cells.¹⁸-²⁰ T-cell subsets such as FoxP3⁺ T cells, cytotoxic T cells, and follicular helper T cells express P2X7 and are sensitive to P2X7-mediated cell death,⁴,⁵,²¹,²² which can either suppress or enhance immunity depending on the function of each cell type. In humans, loss-of-function polymorphisms in the P2RX7 gene are linked to chronic lymphocytic leukemia and increased susceptibility to pathogens such as Mycobacterium tuberculosis.²³,²⁴ It has been reported that naive/resting CD4⁺ T cells and retinoic acid (RA)-treated CD8⁺ T cells express P2X7,⁴,²⁵ but the expression...
mechanism and the function of P2X7 in intestinal T-cell homeostasis remain poorly elucidated.

We studied the regulation mechanism of P2rx7 expression and the function of P2X7 in regulating effector T cells, particularly T-helper type 1 (Th1) and Th17 cells, in the intestine. Here, we report that RA induces P2X7 expression in Th1 and Th17 cells in the intestine by activating an RA-responsive enhancer region in the mouse P2rx7 gene. P2X7 deficiency leads to aberrant expansion of Th1 and Th17 cells in the small intestine (SI). NAD-dependent ADP ribosylation of P2X7 induces the contraction of intestinal Th1 and Th17 cell populations in the steady state and during active immune responses to bacterial pathogens. NAD treatment also depleted inflammatory effector T cells and suppressed tissue inflammation in the intestine. Our results provide a regulatory mechanism for P2X7 expression in effector T cells and identify a role for the RA-induced P2X7 in the control of inflammatory T cells in the intestine.

RESULTS
RA induces the expression of P2rx7 and Art2b in intestinal CD4^+ T cells
Transcriptome analysis of cultured mouse CD4^+ T cells revealed that P2rx7 expression is induced by RA but suppressed by an RA receptor α (RARα) antagonist, Ro41-5253 (Figure 1a). A follow-up real-time reverse transcription-PCR examination confirmed that RA greatly induced P2rx7 expression, whereas the RARα antagonist Ro41-5253 suppressed its expression in cultured CD4^+ T cells (Figure 1b). Along with P2rx7, the expression of the gene for the ADP-ribsosyltransferase (ART2.2), Art2b, was also induced by RA (Figure 1b). Using a highly sensitive three-step antibody staining method, we also found that RA induces P2X7 protein expression on the surface of activated CD4^+ T cells (Figure 1c). To further study the impact of RA on P2X7 expression in vivo, we used vitamin A-deficient (VAD) mice and examined P2X7 expression by CD4^+ T cells in the SI, colon, mesenteric lymph node (MLN), and spleen in vivo. We found that SI and large intestinal (LI) CD4^+ T cells highly expressed P2X7, whereas those in lymphoid tissues expressed P2X7 only at basal levels (Figure 1d). Importantly, the P2X7 expression on intestinal CD4^+ T cells was greatly decreased in VAD mice (Figure 1d), indicating a positive role of vitamin A in P2X7 expression by CD4^+ T cells. Immunofluorescence staining revealed that most CD4^+ T cells in the lamina propria (LP), along with intraepithelial T cells, expressed P2X7 in intestinal villi (Figure 1e).

The sensitivity of the P2rx7 gene to RA is regulated by an intragenic enhancer region
RA induces gene expression by activating RAR-RXR receptors that bind RA-responsive elements on many genes. Analysis of published chromatin immunoprecipitation (ChIP)-sequencing data indicates the presence of two major intragenic RARα binding regions (I and II) in the mouse P2rx7 gene (Figure 2a). However, the putative P2rx7 promoter region did not have any significant RARα-binding activity. The RARα-binding regions had epigenetic modifications such as H3K4me and H3K27Ac, which are consistent with high transcriptional activity.27 T-cell activation in the presence of RA-induced RARα binding and histone 3 (H3) acetylation on region II (Figure 2b). The enhancer activity of region II, which is located between exons 2 and 3, was tested in primary CD4^+ T cells by a luciferase reporter assay. RA-dependent transcriptional reporter activity was detected when region II was ligated downstream of the P2rx7 promoter in the luciferase reporter plasmid (Figure 2c). Therefore, this region has an RA-dependent enhancer activity and is referred to as “the RA-responsive P2rx7 enhancer.”

RA makes CD4^+ T cells susceptible to NAD-induced apoptosis in a P2X7-dependent manner
P2X7 activation on T cells induces phosphatidylserine exposure and apoptosis.14 Because of the differential expression of P2X7 by RA- and Ro41-5253-treated T cells, we compared their sensitivity with NAD-induced apoptosis. RA-treated CD4^+ T cells were highly sensitive to NAD-induced apoptosis, whereas Ro41-5253-treated T cells were insensitive to NAD (Figure 3a). This dependence on RA was observed on CD4^+ T cells, activated with different T-cell activators such as concanavalin A (Figure 3a) and OVA 323-339 (not shown). αATP (oxidized ATP; an irreversible antagonist for P2X7) effectively blocked the NAD-induced apoptosis. Moreover, the CD4^+ T cells, isolated from the MLN of vitamin A-normal (VAN) mice, were significantly more susceptible to NAD-induced apoptosis than their counterparts from VAD mice (Figure 3b). Next, we examined the sensitivity of intestinal T cells to NAD-induced apoptosis in vivo following intravenous injection of NAD into mice. The CD4^+ T cells of the SI of VAN mice were effectively depleted following NAD injection, whereas those in VAD mice were resistant to NAD-induced cell death (Figure 3c). While wild-type (WT) T cells, induced for P2X7 expression with RA in vitro, were sensitive to NAD-induced apoptosis, P2rx7−/− CD4^+ T cells were resistant (Figure 3d). These results indicate that RA induces the expression of functional P2X7 on intestinal CD4^+ T cells and P2X7 activation depletes intestinal CD4^+ T cells.

Polymorphisms in the P2rx7 gene in C57BL/6 and BALB/c mice have been reported, leading to functional differences in P2X7 activity.28 Because of this polymorphism, we also examined P2X7 expression and function in regulating T cells in BALB/c mice. We found that the T cells from BALB/c mice expressed P2X7 in response to RA and were equally sensitive to NAD-induced apoptosis (Supplementary Figure 1a-d online).

There are many αβ and γδ T-cell subsets in the LP and intraepithelial compartments. We studied P2X7 expression by CD4- and/or CD8-expressing αβ and γδ T-cell subsets in the SI LP and intraepithelial compartments. We found that all of the αβ (CD4^+CD8α^+), CD4^+CD8α^−, CD8α^+CDβ^+), and CD8α^+^+ T-cell subsets highly expressed P2X7 and were susceptible to NAD-induced cell death (Figure 4a,b).
Antigenically stimulated memory/effector, but not naïve, CD4⁺ T cells preferentially express P2X7

It has been proposed that naïve or resting CD4⁺ T cells preferentially express P2X7 and are the main target of NAD-induced cell death. However, our data indicate that P2X7 expression is induced by RA on intestinal T cells, the majority of which are memory or effector T cells. Therefore, we closely examined the P2X7 expression by resting vs. activated cells based on CD69 expression and naïve vs. memory/effector-like T cells based on CD62L/CD44 expression. P2X7 expression on SI CD4⁺ T cells was concentrated on CD69⁺ activated T cells compared with CD69⁻ CD4⁺ cells (Figure 5a). Similarly, CD69⁻ CD4⁺ cells, generated in culture in the presence of RA,
expressed higher levels of P2X7 than did CD69− CD4+ cells (Figure 5b). Moreover, P2X7 expression highly correlated with the expression of the memory/effector T cell marker CD44 and was negligible on CD62L+ CD44− naïve-like CD4+ T cells (Figure 5c). In line with the pattern of P2X7 expression, NAD injection effectively depleted memory/effector CD62L− CD44+ CD4+ T cells but not naïve-type CD62L+ CD44− CD4+ T cells (Figure 5d). Because it was previously reported that FoxP3+ T cells highly express P2X7,22 we also examined P2X7 expression by FoxP3+ T cells. For CD62L+ CD44− naïve types, P2X7 expression was higher on FoxP3+ T cells, whereas it was higher on CD44+ FoxP3− memory T cells compared with CD44+ FoxP3− memory T cells (Supplementary Figure 2a online). In culture, RA induced P2X7 expression both in FoxP3− and FoxP3+ CD4+ T cells.

Figure 2  An enhancer region in the P2X7 gene has binding sites for retinoic acid (RARα) and makes the P2X7 gene responsive to RA. (a) The structure of P2rx7 promoter and enhancer regions along with RARα binding, H3K4 methylation, and H3K27 acetylation. (b) RARα binding and histone 3 (H3) acetylation at putative P2rx7 enhancer regions. A chromatin immunoprecipitation (ChIP) assay was performed using anti-RARα and anti-acetylated H3 on CD4+ naïve T cells activated with anti-CD3/CD28 for 3 days in the presence of RA or Ro41-5253. (c) The transcriptional activity of the P2rx7 enhancer region was determined with a luciferase reporter assay. Reporter plasmids were transfected into activated CD4+ T cells, cultured for 6 h in the presence or absence of RA, and assayed for luciferase activity. Relative luciferase units (RLUs) normalized by PGL4-P2rx7 control levels are shown. Combined data from three to six independent experiments are shown. *Significant differences between indicated groups. IgG, control immunoglobulin G.
In support of the findings, reanalysis of publicly available transcriptome data (GSE15907) for P2rx7 revealed that CD4\textsuperscript{+} CD44\textsuperscript{+} CD122\textsuperscript{+} memory T cells, but not CD4\textsuperscript{+} CD62L\textsuperscript{+} naive T cells, highly expressed this gene (Supplementary Figure 3).

**Figure 3** Retinoic acid (RA) increases CD4\textsuperscript{+} T-cell sensitivity to nicotinamide adenine dinucleotide (NAD)-induced cell death in vitro and in vivo. (a) RA-treated CD4\textsuperscript{+} T cells are more susceptible than Ro41-treated CD4\textsuperscript{+} T cells to NAD-induced cell death. Naive CD4\textsuperscript{+} T cells were cultured with concanavalin A in the presence of interleukin-2 (IL-2) and RA (or Ro41-5253) for 4–5 days. The cells were treated with NAD (100 μM) or control media for 2 h, and the frequency of apoptotic CD4\textsuperscript{+} cells was determined by annexin V and propidium iodide (PI) staining. (b) Mesenteric lymph node (MLN) CD4\textsuperscript{+} T cells isolated from vitamin A-deficient (VAD) mice were relatively more resistant than those from vitamin A-normal (VAN) mice to NAD-induced cell death. (c) P2X7\textsuperscript{-} small intestinal (SI) CD4\textsuperscript{+} T cells were preferentially depleted by NAD-induced cell death in vivo. Mice were injected intravenously with NAD (10 mg per mouse), and numbers of CD4\textsuperscript{+} T cells were determined by flow cytometry 20 h after the NAD injection. (d) P2rx7\textsuperscript{−/−} cells are resistant to NAD-induced cell death. Cultured CD4\textsuperscript{+} T cells from wild-type (WT) and P2rx7\textsuperscript{−/−} mice were treated with NAD as in (a). Representative and combined data (n=3 for a, b, and d; n=5 for c) are shown. *Significant differences between indicated groups. oATP, oxidized ATP; PBS, phosphate-buffered saline.

(Supplementary Figure 2b). In support of the findings, reanalysis of publicly available transcriptome data (GSE15907) for P2rx7 revealed that CD4\textsuperscript{+} CD44\textsuperscript{+} CD122\textsuperscript{+} memory T cells, but not CD4\textsuperscript{+} CD62L\textsuperscript{+} naive T cells, highly expressed this gene (Supplementary Figure 3).
We next investigated if antigen stimulation drives P2X7 expression on intestinal CD4+ T cells in vivo. We adoptively transferred ovalbumin-specific OT-II T cells into CD45.1 congenic mice and intragastrically immunized these mice with ovalbumin. The P2X7 expression on CD4+ T cells was induced by ovalbumin, and the OT-II P2X7+ cells were detected in the SI and LI but rarely in the spleen and MLN (Figure 5e), indicating that antigen stimulation in the intestinal environment is required for robust induction of P2X7 expression by T cells.

P2X7 controls the size of Th1 and Th17 cell populations in the intestine

Th1 and Th17 cells are major CD4+ memory/effector T-cell subsets in the intestine. Because of the preferential expression of P2X7 by memory/effector T cells in the intestine, we examined P2X7 expression by Th1 and Th17 cells. SI Th1 and Th17 cells highly expressed P2X7 (Figure 6a), which suggests that these effector T cells are likely to be the major targets of P2X7-mediated apoptosis. In support of this, high positive correlations were found between the expression of P2X7 and T-bet or RORγt (retinoid-related orphan receptor-γt), the master transcription factors for Th1 or Th17 cells (Figure 6b). The P2X7 expression by intestinal Th1 and Th17 cells was decreased in the VAD compared with the VAN condition (Figure 6c). Moreover, RA induced P2X7 expression on in vitro-generated Th1, Th17, and Th2 cells (Supplementary Figure 6a online). These results identify intestinal Th1 and Th17 cells as potential targets of P2X7-mediated apoptosis.

A potential function of P2X7 in the intestine is to induce apoptosis of excessive Th1 and Th17 cells. If this is the case, P2X7 deficiency would increase the number of Th1 and Th17 cells in the intestine. To test this hypothesis, we examined the size of Th1 and Th17 cell populations in WT vs. P2rx7−/− mice during the steady state and infection by Citrobacter rodentium. The absolute number of Th1 cells in the SI were significantly increased in P2X7-deficient mice in the steady state and this change was even more pronounced during infection by C. rodentium (Figure 7a). The number of Th17 cells was also increased in the SI and LI of P2X7-deficient mice. Interestingly, the abnormal increase of effector T cells was also observed in the MLN and spleen during infection (Supplementary Figure 5a online). It was also noted that P2X7 expression by CD4+ memory/effector cells was increased at variable levels in secondary lymphoid tissues and LI during infection (Supplementary Figure 5b). We next administered NAD into WT and P2rx7−/− mice to determine if Th1 and Th17 cells are effectively depleted in the intestine. NAD injection depleted intestinal Th1 and Th17 cells in WT, but hardly in P2rx7−/−, mice following NAD administration or upon NAD treatment ex vivo (Figure 7b and Supplementary Figure 6a online). In line with the data, Th1, Th17, and Th2 cells, generated in vitro in the presence of RA, were effectively depleted by NAD (Supplementary Figure 6b–d).

We further performed a competitive repopulation study for WT and P2rx7−/− bone marrow (BM) cells to create mice that have both WT and P2rx7−/− hematopoietic cells in the same hosts. The SI had abnormal expansion of P2rx7−/− Th1 and Th17 cells compared with their WT counterparts in the same host (Figure 7c). Similar differences were observed in the MLN but the differences were relatively smaller. These results
Figure 5  T-cell activation in the presence of retinoic acid (RA) \textit{in vitro} or in the gut-tissue environment \textit{in vivo} induces P2X7 expression and increases sensitivity to nicotinamide adenine dinucleotide (NAD)-induced cell death. (a) Expression of P2X7 by CD69\textsuperscript{+} vs. CD69\textsuperscript{−} CD4\textsuperscript{+} T cells in small intestine (SI) lamina propria (LP) and mesenteric lymph node (MLN). (b) Expression of P2X7 by CD69\textsuperscript{+} vs. CD69\textsuperscript{−} CD4\textsuperscript{+} T cells activated \textit{in vitro} with RA or Ro41-5253 and concanavalin A. Naive OT-II CD4\textsuperscript{+} T cells were activated for 4–5 days in the presence of CD11c\textsuperscript{+} splenocytes and OVA\textsubscript{323–339}. (c) Expression of P2X7 by naı̈ve and memory/effector-like CD4\textsuperscript{+} T-cell subsets distinguished by CD44 and CD62L expression. (d) Comparison of gut-naı̈ve and memory/effector CD4\textsuperscript{+} T-cell subsets for their sensitivity to NAD-induced cell death. (e) Antigenic stimulation induces P2X7 on CD4\textsuperscript{+} T cells preferentially in the small and large intestines. CD45.1\textsuperscript{+} mice were transferred intravenously with naı̈ve CD45.2\textsuperscript{+} OT-II T cells and immunized intragastrically with ovalbumin. Representative and combined data (n = 5 for a, n = 3 for b, n = 6 for c, n = 7 for d, n = 7 for e) are shown. *Significant differences from the first groups or between indicated groups. MFI, mean fluorescence intensity; WT, wild type. A full color version of this figure is available at the MucosalImmunology journal online.
indicate that P2X7 is required to prevent the excessive buildup of Th1 and Th17 cell in the intestine.

NAD administration restrained inflammatory intestinal Th1 and Th17 cells and suppressed colitis

To gain insights into the role of P2X7 in regulating inflammatory T cells in the intestine, we next examined P2X7 expression by CD4+ T cells in the T-cell-induced colitis in Rag1−/− mice. P2X7 expression was upregulated in both the SI and LI of T-cell-transferred Rag1−/− mice (Figure 8a). The P2X7 expression levels on inflammatory intestinal T cells in Rag1−/− mice were significantly higher compared with that of normal intestinal T cells in control C57BL/6 mice. Particularly, the expression of P2X7 by CD4+ T cells in the LI of Rag1−/− mice was particularly high. This indicates a potentially important role for the P2X7 pathway in reining in inflammatory T cells. To test this hypothesis, we injected NAD into T-cell-transferred Rag1−/− mice. Repeated NAD treatment depleted >~90% of Th1 in the colitis model (Figure 8b). Moreover, minor populations such as Th17 and FoxP3+ cells were also depleted. NAD injection suppressed weight loss, inflammation-related colon contraction, and tissue inflammation (Figure 8c–e).

DISCUSSION

The results of this study reveal an important role of vitamin A metabolites in regulating intestinal effector T cells through P2X7 upregulation. We identified an enhancer region that makes the murine P2rx7 gene sensitive to RA. RA-induced P2X7 is required to restrain the size of effector T-cell populations in the intestine. This function of P2X7 is important in suppressing the overpopulation of effector T cells in intestinal tissues and, therefore, has the potential to prevent T-cell-induced inflammatory diseases in the intestine.

It has been unclear when and how P2X7 is upregulated on CD4+ T cells during T-cell differentiation. Early studies suggested that CD4+ T cells in peripheral lymph nodes are sensitive to P2X7-induced cell death. A key question is if there is any specificity in P2X7 expression in terms of cell type, differentiation stage, and tissue location. We systematically compared P2X7 expression by naïve and memory/effector CD4+ T-cell populations in the intestine and lymphoid tissues. Naïve CD4+ T cells hardly expressed P2X7 but memory and effector CD4+ T cells highly expressed P2X7. Moreover, there were differences among organs (i.e., intestine vs. lymphoid tissues) in P2X7 expression by T cells. In the steady state, CD4+ T cells in the SI most highly express P2X7, which is followed by those of the LI. In contrast, relatively fewer CD4+ T cells in lymphoid tissues expressed P2X7. Moreover, it was Th1 and Th17 cells that expressed P2X7 most highly in the intestine. Thus, P2X7 expression is developmentally and tissue specifically regulated in CD4+ T cells.

A key finding of this study is the identification of an enhancer region in the mouse P2rx7 gene, which induces the gene in response to RA. RA is highly produced by dendritic cells and epithelial cells in the intestine, particularly in the SI. RA has pleiotropic functions in regulating the immune system. RA affects the differentiation and function of T cells and other cell types and induces gut-homing receptors for T-cell migration into the intestines. By inducing gut-homing receptors, RA populates the intestine with antigen-experienced effector T cells. While this promotes intestinal immunity, the same function can cause overpopulation of effector T cells and tissue inflammation. Our transcriptome analysis of RA-treated T cells indicates that P2X7 is coexpressed by T cells that express...
gut-homing receptors such as CCR9 and Itg-a4. This indicates that gut-homing CD4 T cells are a target of P2X7-mediated apoptosis. It has been reported that RA induces P2X7 expression in intraepithelial CD8 T cells. Our data indicate that most ab and gd T-cell subsets in intestinal LP and intraepithelial compartments express P2X7 and are susceptible to NAD-induced cell death. Therefore, the RA-P2X7 pathway is likely to affect most T-cell subsets in the intestine.

The intestinal tissues harbor many effector T cells reactive to commensal bacteria and dietary antigens. ATP and NAD, the two P2X7-activating metabolites, are released from stressed or activated cells, which are generated following tissue damage and inflammatory responses. ATP can be also produced by the gut microbiota or host cells upon stimulation with commensal bacteria and pathogens. Our results indicate that intestinal effector T cells are restrained by the P2X7-mediated apoptosis in the steady state and during inflammatory responses. This is supported by the abnormally expanded Th1 and Th17 cell populations in P2X7-deficient mice, particularly during active immune responses such as infection or inflammatory responses.

Figure 7  P2rx7−/− mice have aberrantly expanded populations of T-helper type 1 (Th1) and Th17 cells in the steady state and during active immune responses. (a) Frequencies and absolute numbers of Th1 and Th17 cells in the intestine of wild-type (WT) and P2rx7−/− mice in the steady state and during Citrobacter rodentium infection. Mice were examined 2 weeks after infection with C. rodentium. (b) Sensitivity of small intestine (SI) WT vs. P2rx7−/− Th1 and Th17 cells to nicotinamide adenine dinucleotide (NAD)-induced cell death. (c) Th1 and Th17 populations following competitive bone marrow repopulation. A 1:1 mixture of Thy1.1+CD45.2+ WT and Thy1.2+CD45.2+ P2rx7−/− bone marrow (BM) was transplanted into lethally irradiated CD45.1+ congenic mice. The mice were killed 9 weeks post-BM transfer. The boxes and bars, respectively, represent the 25–75th and 0–100th percentile data ranges. Representative and combined data (n = 11–14 for a; n = 12 for b) are shown. *Significant differences between two groups. IFN, interferon, IL, interleukin; LI, large intestine; MLN, mesenteric lymph node; PBS, phosphate-buffered saline.
In support of our findings, it has been reported that P2X7-deficient mice suffer from more severe inflammatory responses compared with WT mice following infection with *Toxoplasma gondii* or during an experimental inflammatory response in the central nervous system. This is further supported by our data that NAD administration effectively depleted inflammatory Th1 and Th17 cells and suppressed the T-cell-induced colitis in *Rag1*<sup>−/−</sup> mice. Thus, P2X7 controls the size of activated T-cell populations in the intestine.

P2X7 is also expressed by mouse and human FoxP3<sup>+</sup> Tregs. For example, Aswad *et al.* reported that spleen Tregs express P2X7 and are more susceptible to NAD or ATP-induced apoptosis compared to non-Tregs. It is true that Tregs in secondary lymphoid tissues such as spleen and lymph nodes express P2X7 at levels higher compared with that by non-Tregs. This is owing to the fact that even naïve-like Tregs express P2X7 at medium levels, whereas conventional non-Treg naïve CD4<sup>+</sup> T cells hardly express P2X7. In the SI, however, non-Treg effector T cells express P2X7 at levels higher than Tregs. RA induces P2X7 also in FoxP3<sup>+</sup> T cells. Therefore, RA-induced P2X7 expression probably affects both effector T cells and Tregs in the intestine. Because Tregs express P2X7 even at the naïve stage, Tregs may depend less on RA in P2X7 expression than non-Tregs. We also observed that antigen stimulation is required to induce P2X7 expression in naïve CD4<sup>+</sup> T cells. Thus, the results of our study expand the role of P2X7 to memory/effector T cells, including Th1 and Th17 effector T cells. A recent study revealed that P2X7 controls the function of follicular helper T cells in Peyer’s patches. In this regard, follicular helper T cells are also effector CD4<sup>+</sup> T cells, specialized in helping B cells.

In summary, effector T cells, including Th1 and Th17 cells, are effectively restrained by P2X7-dependent apoptosis in the intestine. RA induces the expression of P2X7 and ART2.2 in activated CD4<sup>+</sup> T cells producing effector cytokines. RA induces P2X7 expression through RAR<sub>α</sub> binding to an enhancer region between exons 2 and 3 on the *P2rx7* gene for epigenetic modification and enhanced gene expression. We conclude that RA, the key factor that promotes T-cell migration to the intestine for immunity, also has a significant role in containing the activity of various effector T cells in the intestine.
We suggest that this pathway provides a negative feedback loop in the intestinal immune network to suppress aberrant T-cell activity and inflammatory diseases.

METHODS

Animals and diet. All experiments with animals in this study were approved by the Purdue Animal Care and Use Committee (PACUC). P2rx7−/− mice (stock number 005576) and Rag1−/− C57BL/6 mice (stock number 002216) were purchased from the Jackson Laboratory (Bar Harbor, ME). VAD and VAN mice were generated by feeding late-term (15–16 days postcoitus) pregnant females with AIN-93G custom diet containing retinyl acetate at 2,500 IU kg−1 of diet for VAN or 0 IU kg−1 of diet for VAD, respectively (TD. 07267 and TD. 00158; Harlan Teklad, Indianapolis, IN). The weaned mice were kept on the same diet for at least 10 weeks before the experiment.

Infection and induction of colitis. WT and P2rx7−/− C57BL/6 mice (6 weeks old, sex-matched males or females) were infected intragastrically with C. rodentium (10⁹ colony-forming unit per mouse). Rag1−/− C57BL/6 mice were injected intraperitoneally with naïve CD4+ T cells (5 × 10⁶ cells per mouse) to induce colitis. Mice were monitored for weight change and killed 2 or 4 weeks later. The colon was histologically examined after hematoxylin and eosin staining. Colitis scores were assessed based on levels of leukocyte infiltration, loss of normal crypt structure, and mucosal hyperplasia on a scale of 4 by two different researchers. When indicated, NAD (20 mg; Sigma-Aldrich, St Louis, MO) was injected intraperitoneally every 12 h.

BM or T-cell transfer.Recipient mice were subjected to lethal γ-irradiation (1,050 rads) and reconstituted intravenously with BM cells from WT and P2rx7−/− mice (4 × 10⁶ cells each). Mice were examined 9 weeks after BM transplantation. CD4+ naïve T cells (2 × 10⁶ cells per mouse), isolated from CD45.2+ OT-II mice, were transferred intravenously into CD45.1+ mice and then immunized intragastrically with OVA323–339 (10 μg; Sigma-Aldrich) and cholera toxin (10 μg; Sigma Aldrich) on days 1 and 4. Mice were killed 5 days after the immunization.

Cell isolation and culture. Naïve CD4+ T cells, isolated as described previously, were cultured in complete RPMI-1640 medium supplemented with either regular or charcoal-treated fetal bovine serum. The cells were activated with concanavalin A (1.5 μg ml−1; Sigma-Aldrich), OVA323–339, or plate-bound anti-CD3 antibody (0.2–2 μg ml−1) and soluble anti-CD28 antibody (2 μg ml−1) (BioXcell, Lebanon, NH) with human IL-2 (1.5 μg ml−1) (BioXcell, Lebanon, NH) for 3–5 days. Tfi, Th17, and Tregs were cultured as described previously.35,37 Cytokines were purchased from either BioLegend (San Diego, CA) or PeproTech (Rocky Hill, NJ). T cells were cultured with RA (all-trans R, 1–10 nm) or Ro41-5253 (100 or 1,000 nM; Sigma-Aldrich) for 2 h in complete RPMI-1640. When indicated, cells were pretreated with periodate-oxidized ATP (a P2X7 receptor antagonist, 1 mM; Sigma-Aldrich) for 2 h before the NAD treatment. Cells were then washed twice with ice-cold phosphate-buffered saline, and then stained with antibodies to CD4, CD44, IFNγ, and IL-17. When indicated, Annexin V and propidium iodide staining was performed in Annexin V binding buffer (BioLegend). To assess the short-term depletion effect of NAD on CD4+ T cells in vivo, mice were injected intravenously with NAD (10 mg) and killed 2 or 20 h later. The results obtained at 2- and 20-h time points were similar and all data shown in this report were obtained at the 20-h time point.

ChIP assay. RARx binding and H3 acetylation sites on the P2rx7 gene were identified by analyzing publicly available Chip-seq data (GSE60356) with Integrated Genome Browser (IGB Bioinformatics Application Note). To determine RA-dependent regulation of RARx binding and H3 acetylation sites on the P2rx7 gene, a ChIP assay was performed with the SimpleChIP Kit (Cell Signaling Technologies, Danvers, MA) using the primers described in Supplementary Table 1 online.

Quantitative real-time PCR and microarray data analyses. Total RNA was extracted with TRizol (Invitrogen, Grand Island, NY), and cDNA synthesis was performed with the High-Capacity cDNA Reverse Transcription Kit (Invitrogen). Quantitative real-time PCR was performed with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Grand Island, NY) using the primers described in Supplementary Table 1 online. β-Actin expression was also measured for data normalization. Microarray data (GSE20500 and GSE15907) were described previously.43,44 All plots were made with the multiplot module of GenePattern.

Statistical significance of differences between indicated groups was obtained by Student’s t-test and two-way analysis of variance. P values <0.05 were considered significant. Error bars in all figures indicate s.e.m.

SUPPLEMENTAL MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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AUTHOR CONTRIBUTIONS
S.H. performed experiments, analyzed data, and prepared most of the figures with experimental contributions from L.F. and M.K. C.K. conceived the project and obtained funding; analyzed data and drafted the manuscript with S.H.

DISCLOSURE
The authors declared no conflict of interest.

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