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Inhibition of Pim1 kinase prevents peanut allergy by enhancing Runx3 expression and suppressing T_H2 and T_H17 T-cell differentiation

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Abstract

Background—The provirus integration site for Moloney murine leukemia virus (Pim) 1 kinase is an oncogenic serine/threonine kinase implicated in cytokine-induced cell signaling, whereas Runt-related transcription factor *(Runx)* has been implicated in the regulation of T-cell differentiation. The interaction of Pim1 kinase and Runx3 in the pathogenesis of peanut allergy has not been defined.

Objectives—We sought to determine the effects of Pim1 kinase modulation on Runx3 expression and T_H2 and T_H17 cell function in an experimental model of peanut allergy. Methods: A Pim1 kinase inhibitor was administered to peanut-sensitized and challenged wild-type and *Runx3*$^{+/-}$ mice. Symptoms, intestinal inflammation, and Pim1 kinase and Runx3 mRNA expression and protein levels were assessed. The effects of Pim1 kinase inhibition on T_H1, T_H2, and T_H17 differentiation *in vivo* and *in vitro* were also determined.

Results—Peanut sensitization and challenge resulted in accumulation of inflammatory cells and goblet cell metaplasia and increased levels of Pim1 kinase and T_H2 and T_H17 cytokine production but decreased levels of Runx3 mRNA and protein in the small intestines of wild-type mice. All of these findings were normalized with Pim1 kinase inhibition. In sensitized and challenged *Runx3*$^{+/-}$ mice, inhibition of Pim1 kinase had less effect on the development of the full spectrum of intestinal allergic responses. *In vitro* inhibition of Pim1 kinase attenuated T_H2 and T_H17 cell differentiation and expansion while maintaining *Runx3* expression in T-cell cultures from wild-type mice; these effects were reduced in T-cell cultures from *Runx3*$^{+/-}$ mice.

Conclusion—These data support a novel regulatory axis involving Pim1 kinase and *Runx3* in the control of food-induced allergic reactions through the regulation of T_H2 and T_H17 differentiation.

Keywords

Pim1 kinase; Runx3; peanut; intestinal allergy; T_H2; T_H17

In hosts with peanut allergy, several cell types are recruited to the intestine and activated to release cytokines and chemokines, contributing to intestinal inflammation.[1-4] In addition to IL-4 and IL-13, increased levels of IL-17A have been found in the small intestine and mesenteric lymph nodes (MLNs) in a mouse model of food allergy.[2] The data suggest that

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$CD4^+$ T cells, which produce both T_H2 and T_H17 cytokines, play an important role in food-associated allergic disease.

The provirus integration site for Moloney murine leukemia virus (Pim), a proto-oncogene encoding a family of serine/threonine protein kinases, has multiple cellular functions.[5] Pim1 kinase has been implicated in cytokine-dependent signaling in hematopoietic cells and T lymphocytes,[6,7] and kinase expression was enhanced during T-cell activation.[8] Pim1 kinase increases T-cell proliferation by enhancing the activity of nuclear factor of activated T cells (NFAT) c1, increasing IL-2 production in T cells.[7] Pim1 kinase expression was upregulated in the lungs of mice after sensitization and challenge with allergen.[9]

Pim1 kinase regulates Runt-related transcription factor (Runx) expression *in vitro*.[6] In this family of transcription factors, Runx3 is required for epigenetic silencing in cytotoxic lineage thymocytes.[10] Runx3 cooperates with T-box transcription factor (T-bet) to repress the production of IL-4 by binding to the IL-4 silencer in the T_H2 cytokine locus and promotes the production of IFN-γ in T_H1 cells.[11-13] Loss of Runx3 results in the spontaneous development of inflammatory bowel disease, as well as allergic asthma.[14,15]

We investigated the role of Pim1 kinase and its relationship to Runx3 expression in an experimental model of peanut-induced intestinal allergy. Pim1 kinase was essential to the development of peanut-induced intestinal allergy. Moreover, inhibition of this kinase prevented the intestinal inflammation and attenuated T_H2 and T_H17 differentiation and cytokine production by regulating *Runx3*.

Methods

For further description of the methods used in this study, see the Methods section in this article's Online Repository at www.jacionline.org.

Mice

Five- to 6-week-old female wild-type (WT) C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, Me). Runx3 heterozygous (*Runx3*$^{+/-}$) mice were provided by Dr James Hagman (National Jewish Health, Denver, Colo). All studies were conducted under a protocol approved by the Institutional Animal Care and Use Committee of National Jewish Health.

Preparation of peanut protein

Crude peanut extract (PE) was prepared as described in the Methods section in this article's Online Repository.

Sensitization and intragastric challenge

The experimental protocol for sensitization and challenge to peanut was previously described.[2] Because C57BL/6 mice did not have peanut-induced intestinal allergy to the same extent as BALB/c mice because of their limited development of peanut-specific IgE antibody, *Runx3*$^{+/-}$ and C57BL/6 mice were passively sensitized with serum containing peanut-specific IgE.[2] All systemically and passively sensitized and challenged *Runx3*$^{+-}$ and C57BL/6 mice had diarrhea by the seventh day of challenge.

Pim1 kinase inhibitor and treatment in vivo

The small-molecule Pim1 kinase inhibitor (AR460770; Array Biopharma, Boulder, Colo) cellular inhibitory concentration of 50% was 93, 9200, and 340 nmol/L for Pim1, Pim2, and Pim3, respectively.[9] PE-sensitized and challenged mice received different doses (0-100 mg/

kg) of the inhibitor by means of gavage and based on earlier experiments.[9] For more information, see the Methods section in this article's Online Repository.

Assessment of hypersensitivity reactions

Symptoms were evaluated as previously reported[16] and described in the Methods section in this article's Online Repository.

Histology

The jejunum was processed and stained with periodic acid–Schiff, chloroacetate esterase, and anti-mouse major basic protein antibody (kindly provided by Dr J. J. Lee, Mayo Clinic, Scottsdale, Ariz) for detection of mucosal mucus-containing cells, mast cells, and eosinophils, respectively, as previously described.[2,17,18] Numbers of CD4, CD8, Pim1, Pim3, and Runx3 mucosal cells were identified by means of immunohistochemical staining with anti-mouse CD4, CD8, Pim1, Pim3, and Runx3 antibodies (Abcam, Cambridge, Mass), respectively.

Cytokine levels in cell culture

Levels of the cell-culture supernatant IL-4, IL-13, IL-17A, and IFN-γ were measured by means of ELISA (eBioscience, San Diego, Calif), as described by the manufacturer.

Measurement of peanut-specific antibody levels

Serum peanut-specific IgE, IgG_1, and IgG_{2a} levels were measured by using ELISA, as described previously.[16]

Histamine levels in plasma

Histamine levels in plasma were measured as described in the Methods section in this article's Online Repository.

T-cell differentiation and treatment with the Pim1 kinase inhibitor *in vitro*

Differentiation of T_H1, T_H2, or T_H17 cells was performed as previously described[19,20] and in the Methods section in this article's Online Repository.

Western blot analysis

Cell lysates were prepared from jejunal tissue and cultured cells as previously described[2,21] and in the Methods section in this article's Online Repository.

Quantitative real-time PCR

RNA was extracted from jejunal tissue homogenates or from CD4 T cells cultured *in vitro* with Trizol (Invitrogen, Carlsbad, Calif). cDNA was generated with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, Calif). Quantitative real-time PCR was performed on the ABI Prism 7300 sequence detection system (Applied Biosystems, Foster City, Calif). All primers and probes used were purchased as TagMan Gene Expression Assays from Applied Biosystems. Fold change was calculated by using the ΔΔ cycle threshold method.

Anti-Runx3 antibody

Rabbit anti-human Runx3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) was biotinylated with the EZ-link sulfo-NHS-LC-biotin kit (Pierce, Rockford, Ill). Allophycocyanin-conjugated streptavidin (eBioscience) was used to detect biotinylated primary Runx3 antibodies.

Intracellular cytokine staining and flow cytometry

Cells from MLNs or differentiated CD4 T cells were labeled with anti-CD3 or anti-CD4 antibody (eBioscience) and stained for intracytoplasmic IL-4, IL-13, IL-17A, IFN-γ, and Runx3 using antibodies from BD Biosciences (San Jose, Calif) or as described above (Runx3 antibody).[22] Cells were analyzed on a FACSCalibur (BD Biosciences) by using CellQuest software (BD Biosciences).

Cell proliferation

T_H1-, T_H2-, or T_H17-polarized CD4 T cells were incubated with anti-CD3 and anti-CD28 (eBioscience) at 37°C for 24 hours. Tritiated thymidine (PerkinElmer, Boston, Mass) was added to the cultures for another 6 hours, and incorporation was measured in a liquid scintillation counter (Packard Bioscience Company, Meriden, Conn).

Cell viability and apoptosis

Cell viability was determined using a trypan blue dye exclusion assay. Cell apoptosis was detected by means of flow cytometry with surface staining with 7AAD and Annexin V (BD Biosciences).

Statistical analysis

ANOVA was used to determine the levels of difference among all groups. Comparisons for all pairs used the Tukey-Kramer highest significance difference test. *P* values for significance were set at .05. All results were expressed as means ± SEMs.

Results

Pim1 kinase levels are upregulated in the small intestines of peanut-sensitized and challenged mice

After PE sensitization and challenge (Fig 1, A), Pim1 kinase protein expression was increased in the jejunums of WT and *Runx3*$^{+/-}$ mice (Fig 1, B). Increases in Pim1 kinase protein levels were greater in the jejunums of sensitized and challenged *Runx3*$^{+/-}$ mice compared with those seen in WT mice. *Pim1* kinase mRNA levels were 2- and 3-fold higher in the jejunums of PE-sensitized and challenged WT and *Runx3*$^{+/-}$ mice, respectively (Fig 1, C); *Pim2* and *Pim3* mRNA levels were not altered after sensitization and challenge of WT or *Runx3*$^{+/-}$ mice. Pim1 kinase was expressed predominantly in the lamina propria of the jejunum, and positive cell numbers were increased by approximately 5- and 7-fold in WT and *Runx3*$^{+/-}$ mice, respectively (Fig 1, D and E). The numbers of Pim3-positive cells were lower, with little alteration after PE sensitization and challenge.

Runx3 is downregulated in the small intestines of peanut-sensitized and challenged mice

Levels of *Runx3* mRNA were approximately 20% to 30% lower in sham-sensitized *Runx3*$^{+/-}$ mice than in WT mice (Fig 2, A). *Runx3* and *Runx/core binding factor β* (*Cbfβ*) mRNA levels were decreased in the small intestines of PE-sensitized and challenged WT and *Runx3*$^{+/-}$ mice. Levels of *Runx3* and *Cbfβ* mRNA, but not *Runx1* mRNA, were approximately 2-fold lower in the jejunums of PE-sensitized and challenged WT and *Runx3*$^{+/-}$ mice compared with those seen in control animals. Levels of *Runx3* mRNA were significantly lower in the jejunums of sensitized and challenged *Runx3*$^{+/-}$ mice than in WT mice. In parallel, Runx3 protein expression was also lower in the jejunums of sensitized and challenged mice and lower in *Runx3*$^{+/-}$ mice than in WT mice (Fig 2, B). Immunohistochemical analysis revealed that Runx3 protein was mainly expressed in the lamina propria, and levels of expression were decreased by 2- to 3-fold in sensitized and challenged WT and *Runx3*$^{+/-}$ mice (Fig 2, C). Numbers of Runx3$^+$ cells were also

significantly lower in PE-sensitized and challenged *Runx3*$^{+/-}$ mice compared with those seen in WT mice.

Inhibition of Pim1 kinase attenuates PE-induced intestinal responses *in vivo*

These data suggested that Pim1 kinase and Runx3 play essential roles in the control of intestinal allergy. To test this hypothesis, we investigated whether inhibition of Pim1 kinase alters the severity of PE-induced intestinal allergy using the small-molecule inhibitor AR460770. The specificity of AR460770 for Pim1 kinase was previously demonstrated.[9] Administration of the inhibitor to sensitized WT mice resulted in a dose-dependent inhibitory effect on intestinal allergy induction; 30 to 100 mg/kg of the inhibitor fully prevented the development of diarrhea and symptoms in PE-sensitized and challenged WT mice. In contrast, inhibitor treatment of *Runx3*$^{+/-}$ mice resulted in reduced inhibitory effects; 30 to 100 mg/kg of the inhibitor partially inhibited diarrhea and symptoms in *Runx3*$^{+/-}$ mice. These effects were significantly lower in *Runx3*$^{+/-}$ than in WT mice (Fig 3, A and B).

Mast cells are involved in the response to PE sensitization and challenge.[2] We monitored mast cell degranulation by quantitating plasma levels of histamine within 30 minutes of the last challenge. Levels of histamine were increased after sensitization and challenge, and in *Runx3*$^{+/-}$ mice levels were significantly increased over levels seen in WT mice. Levels of histamine in inhibitor (30 and 100 mg/kg)–treated WT mice were significantly decreased, almost to baseline levels, after sensitization and challenge. In *Runx3*$^{+/-}$ mice after inhibitor treatment, levels were significantly decreased, although to a smaller extent than in WT mice (Fig 3, C).

When administered after sensitization and during challenge, the inhibitor had no effect on peanut-specific IgE, IgG_1, and IgG_{2a} serum levels in WT or *Runx3*$^{+/-}$ mice (see Fig E1 in this article's Online Repository at www.jacionline.org).

PE-sensitized and challenged mice demonstrated increased numbers of mast cells, eosinophils, and periodic acid–Schiff–positive goblet cells in the mucosa of the small intestine (Fig 3, D-F, and see Figs E2-E4 in this article's Online Repository at www.jacionline.org). WT mice treated with the inhibitor at a dose of 30 to 100 mg/kg demonstrated markedly reduced numbers of these cells. In contrast, the decreases in numbers of these cells were significantly lower in inhibitor (100 mg/kg)–treated *Runx3*$^{+/-}$ mice. The lamina propria of the sham-sensitized group contained few CD4 and CD8 T cells in WT or *Runx3*$^{+/-}$ mice. These numbers were significantly increased in the untreated PE-sensitized and challenged WT group and reduced to baseline levels in the treated WT group (Fig 3, G). In contrast, CD4 T-cell numbers were significantly increased in the untreated PE-sensitized and challenged *Runx3*$^{+/-}$ mice, and after treatment, there were only modest decreases in the numbers of CD4 T cells; the effect on numbers of CD8 T cells was similar in *Runx3*$^{+/-}$ and WT mice (Fig 3, G).

Collectively, these results indicated that Pim1 kinase activation played an essential role in enhancing allergic diarrhea, intestinal inflammation, and goblet cell metaplasia and was inversely associated with Runx3 levels. In *Runx3*$^{+/-}$ mice the inhibitor exhibited reduced effects on all of these parameters, supporting the notion of an interaction (negative) between Pim1 kinase and Runx3.

Pim1 kinase regulates IL-13 and IL-17 production

In addition to T_H2 cells, T_H17 cells have been implicated in allergic disease.[23-25] After 7 days of PE challenges, intestinal tissue from sensitized WT and *Runx3*$^{+/-}$ mice demonstrated significant increases in *IL4, IL6, IL13*, and *IL17A* but not *IFNG* mRNA

expression. Furthermore, *IL4, IL6*, and *IL13* mRNA expression were significantly higher in *Runx3*$^{+/-}$ mice compared with that seen in WT mice (Fig 4, A). After treatment with AR460770, mRNA expression levels for these cytokines returned to control levels in WT but not in *Runx3*$^{+/-}$ mice. The expression levels of *GATA3, NFATc1*, and retinoic acid–related orphan receptor γt (*RORγt*) mRNA were also significantly increased in sensitized and challenged WT and *Runx3*$^{+/-}$ mice, whereas *T-bet* and repressor of GATA mRNA levels were not altered (Fig 4, B). After treatment with the inhibitor, these increased levels also returned to control levels in WT but not in *Runx3*$^{+/-}$ mice.

To assess the effect of Pim1 kinase inhibition on T-lymphocyte cytokine production, we isolated MLN CD4 T cells from WT and *Runx3*$^{+/-}$ mice treated with the inhibitor or vehicle and stimulated them with anti-CD3/anti-CD28. PE sensitization and challenge resulted in significant increases in the numbers of IL-4–, IL-13–, and IL-17A–producing CD4 T cells from WT and *Runx3*$^{+/-}$ mice (Fig 4, C, and see Fig E5 in this article's Online Repository at www.jacionline.org). Numbers of IL-13–producing, but not IL-4– or IL-17A–producing, CD4 T cells were significantly increased in PE-sensitized and challenged *Runx3*$^{+/-}$ compared with WT mice. Mice treated with the inhibitor exhibited 2-to 4-fold and 1.5- to 2-fold decreases in the numbers of these CD4 cytokine–producing cells in WT and *Runx3*$^{+/-}$ mice, respectively. The percentages of CD4^{+}IFN-γ^{+} cells were not altered by sensitization and challenge or treatment with the inhibitor.

Pim1 regulates Runx3 expression

We next determined whether inhibition of Pim1 kinase affects Runx3 expression and whether Runx3 plays an important role in peanut allergy. Protein was extracted from jejunal tissue of WT and *Runx3*$^{+/-}$ mice, and Western blot analysis showed a decrease in levels of Runx3 in PE-sensitized and challenged WT and *Runx3*$^{+/-}$ mice; levels of Runx3 protein were decreased to a greater degree in *Runx3*$^{+/-}$ mice (Fig 5, A). In mice treated with AR460770, Runx3 levels were restored to baseline levels in WT but not in *Runx3*$^{+/-}$ mice. In parallel, levels of *Runx3* and *Cbfβ* mRNA were also decreased in sensitized and challenged WT and *Runx3*$^{+/-}$ mouse tissue, with levels of *Runx3* mRNA lower in the PE-sensitized and challenged *Runx3*$^{+/-}$ mice than in WT mice (Fig 5, B). *Runx3* mRNA levels were restored to control values after treatment with the inhibitor in WT but not in *Runx3*$^{+/-}$ mice. Runx3 protein expression was decreased in the jejunums of PE-sensitized and challenged WT and *Runx3*$^{+/-}$ mice and was similarly restored to almost control values after treatment with the inhibitor in WT but not *Runx3*$^{+/-}$ mice (Fig 5, C, and see Fig E6 in this article's Online Repository at www.jacionline.org). Together, these data indicated that PE sensitization and challenge resulted in Pim1 activation and reductions in levels of Runx3 protein and mRNA. In WT mice treatment with the Pim1 kinase inhibitor normalized these levels to baseline, although not in *Runx3*$^{+/-}$ mice.

Effects of Pim1 kinase inhibition on T_H1, T_H2, and T_H17 cell differentiation and Runx3 expression *in vitro*

Considering the effects of Pim1 kinase inhibition on Runx3 and T_H2/T_H17 cytokine production *in vivo*, we investigated the effect of Pim1 kinase inhibition on *Runx3* expression and T-cell differentiation and function *in vitro*. Isolated naive CD4^{+}CD45RB^{+} T cells from WT and *Runx3*$^{+/-}$ mice were cultured under T_H1-, T_H2-, and T_H17-polarizing conditions in the presence or absence of the inhibitor for 6 days and then stimulated with the combination of anti-CD3/anti-CD28. Overall, T_H2 cells expressed higher levels of Pim1 mRNA than T_H1 cells in WT and *Runx3*$^{+/-}$ mice but expressed lower levels of Runx3 mRNA than T_H1 cells in WT and *Runx3*$^{+/-}$ mice (see Fig E7 in this article's Online Repository at www.jacionline.org). The inhibitor suppressed T_H2 and T_H17 cell expansion in cells from WT mice in a dose-dependent manner; 0.1 to 1 μmol/L inhibited cell number

increases (Fig 6, A), and 1 μmol/L inhibited T_H2 and T_H17 cell proliferation, as assessed using tritiated thymidine incorporation (Fig 6, B). T_H1 cell expansion was not significantly affected. In contrast, the inhibitor (1 μmol/L) suppressed T_H2 and T_H17 cell expansion to a lesser extent in cells from *Runx3*$^{+/-}$ mice. The numbers of untreated *Runx3*$^{+/-}$ T_H2 and T_H17 cells were significantly higher than in WT cells (Fig 6, A and B). As with WT cells, *Runx3*$^{+/-}$ T_H1 cell expansion was not significantly affected. In parallel, the decreases in T_H2 and T_H17 cytokine levels in the polarized T-cell cultures (IL-4, IL-13, and IL-17A, respectively) from *Runx3*$^{+/-}$ mice were lower than in WT cell cultures (Fig 6, C); IFN-γ levels were not affected by the inhibitor. These effects were not due to altered cell viability (see Figs E8 and E9 in this article's Online Repository at www.jacionline.org).

In the polarized T-cell cultures we examined the effects of AR460770 on the expression of *Runx3* and lineage-specific transcription factors using quantitative PCR. In polarized WT but not *Runx3*$^{+/-}$ T_H2 cultures treated with the inhibitor, *Runx3* mRNA expression was upregulated (Fig 6, D). Similar results were seen inT_H17-polarized cells from WT and *Runx3*$^{+/-}$ mice. In parallel, levels of *IL13* and *GATA3* and *IL17A* and *RORγt* mRNA expression were decreased in T_H2 and T_H17 cells from WT but not from *Runx3*$^{+/-}$ mice (Fig 6, D). No effects were detected in T_H1 cells.

We examined the effects of Pim1 kinase inhibition on Runx3 protein and cytokine levels in the polarized T-cell cultures from WT and *Runx3*$^{+/-}$mice using intracellular staining. The inhibitor increased the percentages of $CD4^+Runx3^+$ cells in the polarized T_H2 and T_H17 cell cultures from WT but not in the polarized cultures from *Runx3*$^{+/-}$ mice (Fig 6, E, and Fig E10 in this article's Online Repository at www.jacionline.org). In parallel, the percentages of $CD4^+IL\text{-}13^+$ and $CD4^+IL\text{-}17A^+$ cells were significantly decreased in inhibitor-treated T_H2 and T_H17 cell cultures from WT but not *Runx3*$^{+/-}$ mice (Fig 6, F). Of note, the percentage of $CD4^+IL\text{-}13^+$ cells was significantly higher in untreated T_H2 cell cultures from *Runx3*$^{+/-}$ than from WT mice, whereas the percentage of $CD4^+IFN\text{-}\gamma^+$ cells was not affected in untreated and treated T_H1 cell cultures from WT or *Runx3*$^{+/-}$mice. These data demonstrated that inhibition of Pim1 kinase affected T_H2 and T_H17 but not T_H1 differentiation and promoted expression of *Runx3*. Thus Pim1 kinase functions as a positive regulator for T_H2 and T_H17 differentiation and expansion and as a negative regulator of *Runx3* expression.

Discussion

The family of Pim protein kinases includes 3 members; Pim1 and Pim2 are primarily expressed in hematopoietic cells, whereas Pim3 is expressed in brain, kidney, and mammary tissue. Pim1 is involved in cytokine signaling and has been implicated in many signal transduction pathways.[5,26] Pim2 and Pim3 have largely overlapping functions.[5,27] Several studies have implicated Pim kinases in hematopoiesis, T-cell development, and differentiation.[28,29] *Pim* gene expression is induced by several factors, particularly by cytokines, including IL-2, IL-3, IL-12, IL-15, and IFN-γ.[30,31] Pim1 is expressed in cell types linked to allergic disease, such as T cells and eosinophils[28,32]; is upregulated in peripheral T cells after antigen activation[33]; and influences both mouse and human CD4 T-cell activation and differentiation.[29,33]

In this study we investigated the roles of Pim1 kinase and Runx3 in peanut-induced allergic intestinal responses. Because homozygous *Runx3* deficiency is embryonically lethal, we used *Runx3*$^{+/-}$ mice, which expressed roughly 30% lower Runx3 mRNA and protein levels than WT mice. We first demonstrated that Pim1 kinase expression was upregulated in the jejunums of sensitized and challenged WT mice, with little change in either Pim2 or Pim3 expression. Next, we determined the role of Pim1 kinase using a selective small-molecule inhibitor. *In vivo* treatment with this inhibitor, which was administered after sensitization

but during the oral challenge phase, reduced the incidence and severity of diarrhea and intestinal inflammation (mast cell, eosinophil, and CD4 and CD8 T-cell accumulation and goblet cell metaplasia) in WT mice accompanied by decreases in IL-13 and IL-17A levels in the intestines and MLNs. The inhibitor did not alter the development of specific antibodies, including PE-specific IgE, likely because sensitization was completed before treatment. These data identified for the first time that Pim1 kinase contributed in important ways to the development of peanut-induced allergic responses.

There are several possibilities whereby Pim1 kinase regulates the initiation of an allergic response. IL-13 is a pivotal T_H2 effector cytokine in several allergic diseases.[34,35] We previously demonstrated[2] and confirmed here increased IL-13 levels in the intestines of sensitized and challenged mice. Furthermore, targeting IL-13 alone with a soluble IL-13 receptor 2 protein, which neutralized IL-13,[36] resulted in almost complete elimination of allergic diarrhea and suppressed intestinal inflammation and goblet cell metaplasia *in vivo*.[2] The major sources of IL-13 are activated T cells and mast cells.[2,37] Inhibition of Pim1 kinase resulted in the reduction of T-cell and mast cell recruitment and activation, resulting in lower levels of IL-13 and IL-17A and an improved outcome after challenge of sensitized mice. In addition to IL-13, IL-17A might contribute to severe allergic responses in mice by enhancing IL-13 function.[25] CD4 T_H2 memory/effector cells contribute to IL-17 cytokine production and promote the exacerbation of allergic disease.[38]

Differentiation of T_H1, T_H2, and T_H17 cells is mainly regulated by cytokines and transcription factors.[29,39-42] *In vivo* our data showed that inhibition of Pim1 kinase significantly reduced not only T_H2 expansion and cytokine production but also expression of the T_H2 lineage-specific transcription factor *GATA3* in WT mice. In addition, expression levels of *IL17A* and *RORγt* were inhibited, indicating that inhibition of Pim1 kinase activity suppressed T_H2 and T_H17 differentiation and cytokine production in WT mice and was mediated through inhibition of GATA3 and RORγt transcription factor induction, respectively. On the other hand, T_H1 differentiation was unaffected.

To understand the consequences of Pim1 kinase inhibition, we focused on a potential downstream transcriptional regulator, Runx3. Runx, a novel family of transcription factors, is associated with the development of allergic responses.[43] There are 3 mammalian *Runx* genes: *Runx1, Runx2*, and *Runx3. Runx1* is required for hematopoiesis,[44] and *Runx2* is a regulator of osteogenesis.[45] *Runx3* resides on human chromosome 1p36.1,[46] which maps to a region containing susceptibility genes for asthma,[47] and on mouse chromosome 4,[48] which contains a susceptibility gene for atopic dermatitis.[49] Loss of *Runx3* results in spontaneous development of inflammatory bowel disease,[14] as well as constitutive airway hyperreactivity and eosinophilic inflammation.[43] We demonstrated that *Runx3* (but not *Runx1* or *Runx2*) expression was downregulated in the jejunums of sensitized and challenged WT and $Runx3^{+/-}$ mice and that the increases in Pim1 kinase mRNA and protein levels were greater in the jejunums of peanut-sensitized and challenged $Runx3^{+/-}$ mice than in WT mice. The decreases in *Runx3* expression were prevented in WT but not $Runx3^{+/-}$ mice after inhibition of Pim1 kinase, suggesting an important inverse relationship between Pim1 kinase and Runx3 expression. By all accounts, the effects of Pim1 kinase inhibition *in vivo* were lower in $Runx3^{+/-}$ than in WT mice. T_H2 cytokine (*IL4, IL6*, and *IL13*) mRNA expression was significantly higher in the intestines of sensitized and challenged $Runx3^{+/-}$mice compared with that seen in WT mice. In $Runx3^{+/-}$ mice inhibition of Pim1 kinase resulted in reduced inhibitory effects on T_H2 and T_H17 expansion and cytokine production, as well as expression of *GATA3* and *RORγt*. As in WT mice, T_H1 differentiation was unaffected.

Naive CD4 T cells from WT and $Runx3^{+/-}$ mice were treated with the Pim1 kinase inhibitor under T_H1-, T_H2-, or T_H17-polarizing conditions to extend the link between Pim1 kinase

and Runx3. We observed that the inhibitor suppressed T_H2 and T_H17 cell differentiation in cells from WT but not *Runx3*$^{+/-}$ mice in vitro, which was detected as lower expression levels of *GATA3/IL13* and *RORγt/IL17A*. The expression levels of *Runx3* mRNA and the percentages of $CD4^+Runx3^+$ cells were upregulated in CD4 T cells from WT but not *Runx3*$^{+/-}$ mice induced to differentiate into T_H2 or T_H17 cells in the presence of the Pim1 kinase inhibitor. In contrast, inhibition of Pim1 kinase activity had little to no effect on levels of expression of *IFNG* and *IFNG* mRNA and protein or the percentages of $CD4^+$IFN-γ^+ cells from WT and *Runx3*$^{+/-}$ mice under T_H1 differentiating conditions. In addition to blocking T_H2 and T_H17 differentiation, the inhibitor prevented anti-CD3/anti-CD28–triggered expansion of these cells from WT mice but not the proliferation of T_H1 cells. The inhibitor suppressed T_H2 and T_H17 cell expansion to a lesser extent in cells from *Runx3*$^{+/-}$ mice.

Although the direct interactions between Pim1 kinase and Runx3 require further definition, collectively, these results demonstrate that Pim1 kinase inhibition attenuates T_H2 and T_H17 differentiation in cells from WT mice by suppressing expression of T_H2 and T_H17 lineage-specific transcription factors, limiting expansion of T_H2 and T_H17 cells, and reducing T_H2 and T_H17 cytokine production. At the same time, Runx3 expression was maintained. In cells from *Runx3*$^{+/-}$ mice, Pim1 kinase inhibition had less to no effect on T_H2 and T_H17 differentiation, nor could Runx3 expression be maintained. Thus it appears that in the generation of an allergic response, as shown here for PE-induced intestinal allergy, Pim1 kinase is upregulated, Runx3 is downregulated, and T_H2 and T_H17 differentiation is facilitated. As a corollary, targeting Pim1 kinase results in maintenance of Runx3 expression and prevention of T_H2 and T_H17 differentiation, resulting in a markedly reduced allergic response. Targeting this novel regulatory axis involving Pim1 kinase and *Runx3* offers new therapeutic opportunities for the control of food-induced allergic reactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

Cbfβ	Core binding factor β
MLN	Mesenteric lymph node
NFAT	Nuclear factor of activated T cells
PE	Peanut extract
Pim	Provirus integration site for Moloney murine leukemia virus
RORγt	Retinoic acid–related orphan receptor γt
Runx	Runt-related transcription factor
T-bet	T-box transcription factor
WT	Wild-type

Clinical implications

Pim1 kinase and Runx3 are potential new targets for the treatment of peanut-induced anaphylaxis and intestinal inflammation.

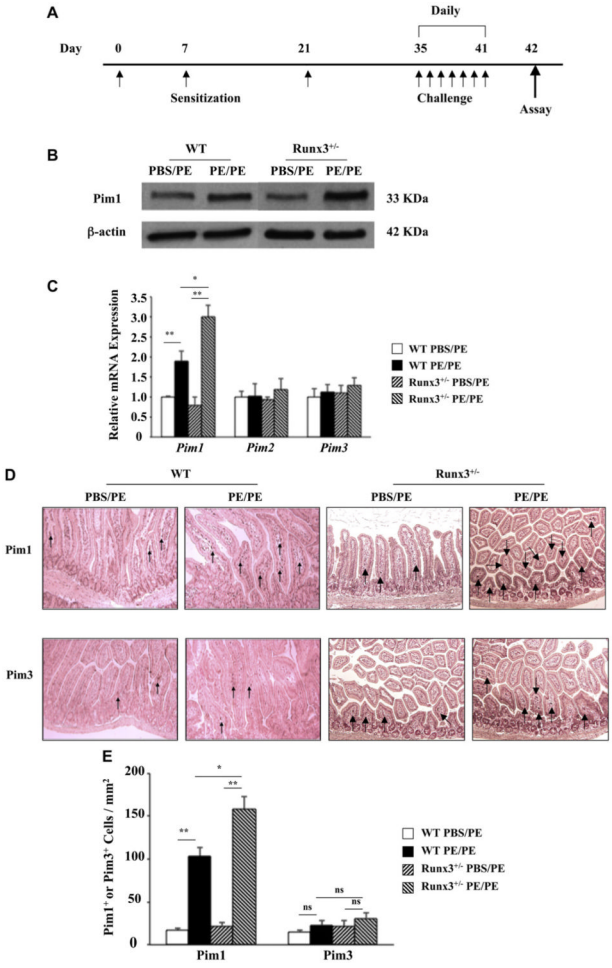


Fig 1.

Pim1 kinase is expressed in WT and *Runx3*$^{+/-}$ mouse jejunum. **A**, Protocol for induction of peanut allergy. **B**, Western blot analysis of Pim1 kinase expression. The results are from the same experiment and the same membranes, but intervening lanes have been cut out for the photograph. **C**, Relative mRNA expression of Pim family members. *D*, Representative immunohistochemical staining for Pim1 and Pim3 kinases. **E**, Quantitation of mucosal Pim1 and Pim3 kinase–expressing cells. Results were from 3 independent experiments; each experiment included 4 mice per group (n = 12). **P* < .05 and ***P* < .01. *PBS/PE*, Sham sensitized but PE challenged; *PE/PE*, PE sensitized and challenged.

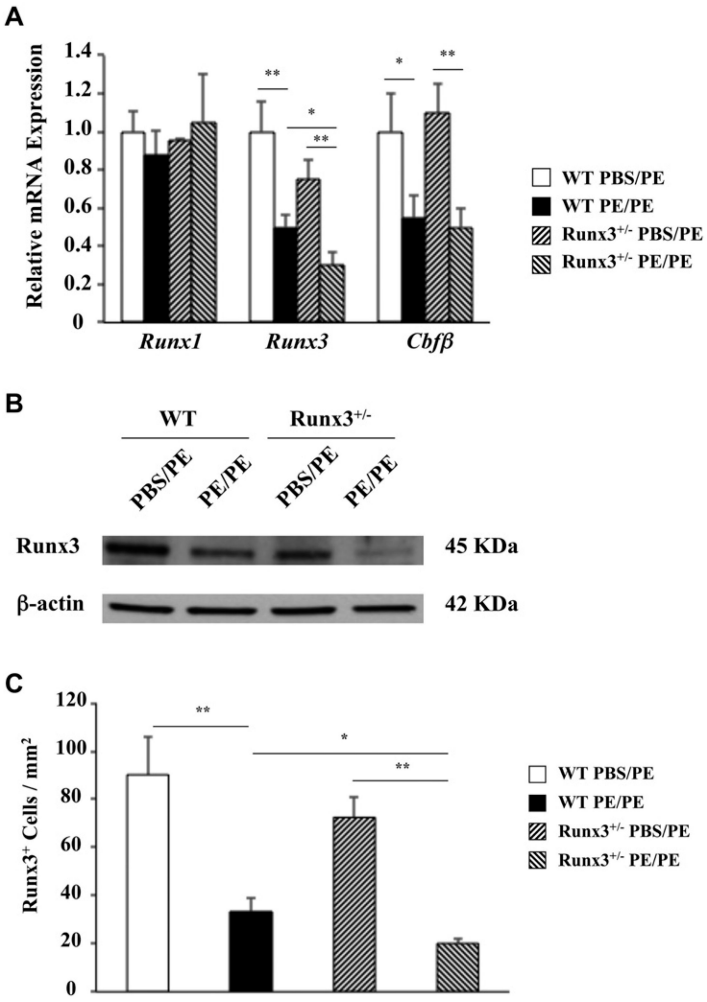
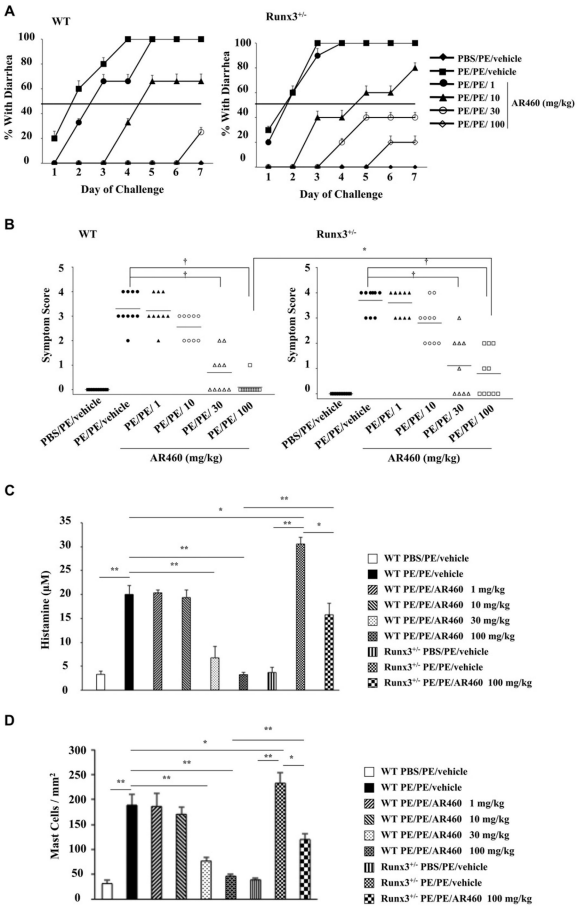


Fig 2.

Expression of Runx3 in the mouse jejunum. **A**, Relative expression levels of *Runx3, Runx1*, and *Cbfβ* mRNA in jejunums of WT and *Runx3*$^{+/-}$ mice. **B**, Runx3 protein levels in jejunums of WT and *Runx3*$^{+/-}$ mice. **C**, Quantitation of mucosal Runx3-expressing cell numbers in jejunums of WT and *Runx3*$^{+/-}$ mice. Results are from 2 independent experiments; each experiment included 4 mice per group. *$P < .05$ and **$P < .01$. *PBS/PE*, Sham sensitized but PE challenged; *PE/PE*, PE sensitized and challenged.



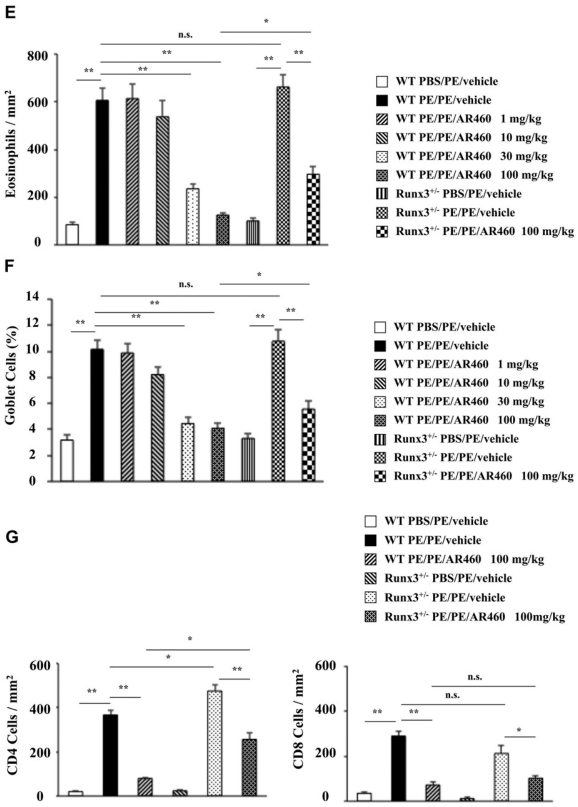


Fig 3.

Inhibition of Pim1 kinase reduces intestinal responses. **A**, Kinetics of development of diarrhea. The incidence of diarrhea was significantly lower in inhibitor (100 mg/kg)-treated WT mice than in *Runx3*$^{+/-}$ mice ($P < .05$). **B**, Symptom scores were assessed 30 minutes after oral challenge. **C**, Plasma histamine levels were assessed within 30 minutes of the last oral challenge. **D-G**, Quantitation of mucosal mast cells, eosinophils, goblet cells, and CD4 and CD8 T cell numbers in the jejunum. Results were from 3 independent experiments; each experiment included 4 mice per group. *$P < .05$, **$P < .01$, and †$P < .001$. *PBS/PE*, Sham sensitized but PE challenged; *PE/PE*, PE sensitized and challenged.

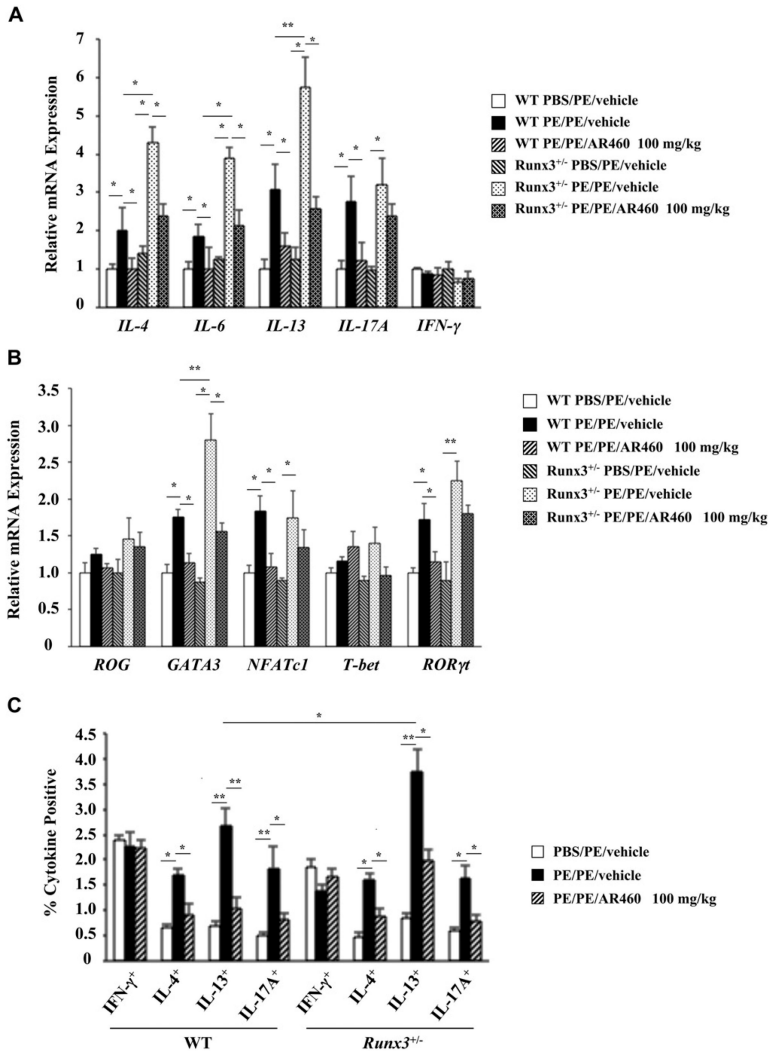


Fig 4.

Effect of Pim1 kinase inhibition on cytokine and transcription factor expression. **A**, T_H1, T_H2, and T_H17 mRNA expression in jejunums of WT and *Runx3*$^{+/-}$ mice treated with AR460770 or vehicle. **B**, T_H1, T_H2, and T_H17 transcription factor expression in jejunums of WT and *Runx3*$^{+/-}$ mice treated with AR460770 or vehicle. *ROG*, Repressor of GATA. **C**, Percentages of IFN-γ^+–, IL-4$^+$–, IL-13$^+$–, and IL-17A$^+$–producing MLN CD3$^+$CD4$^+$ cells of WT and *Runx3*$^{+/-}$ mice treated with the inhibitor or vehicle. Data are from 3 independent experiments (n = 12). *$P < .05$ and **$P < .01$. *PBS/PE*, Sham sensitized but PE challenged; *PE/PE*, PE sensitized and challenged.

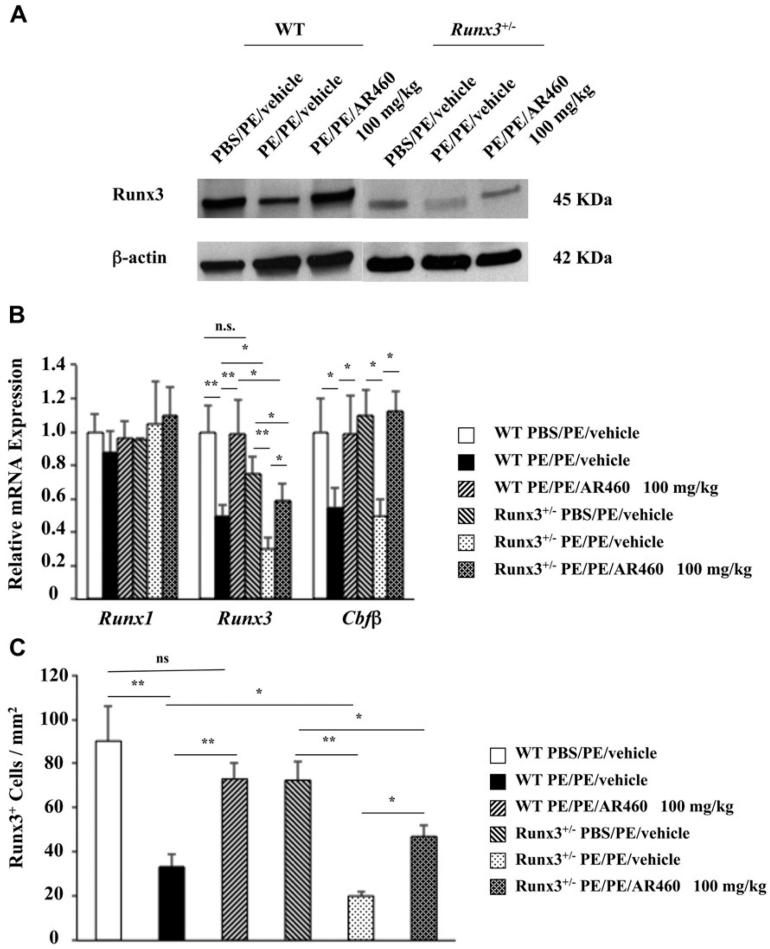
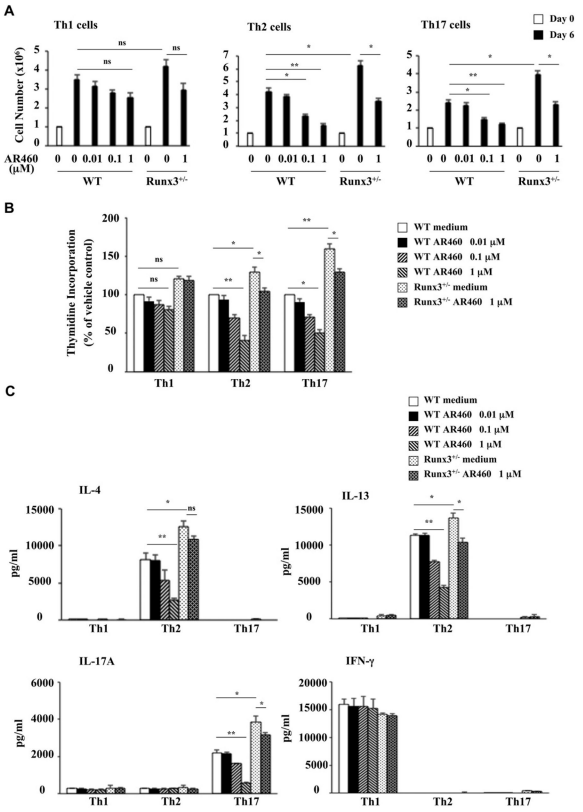


Fig 5.

Pim1 kinase regulates *Runx3* transcription factor expression in the intestine. **A**, Runx3 protein levels in jejunums of Pim1 kinase inhibitor–treated PE/PE WT and *Runx3*$^{+/-}$ mice. The results are from the same experiment and the same membranes, but intervening lanes have been cut out for the photograph. **B**, *Runx* mRNA expression in the jejunums of WT and *Runx3*$^{+/-}$ mice treated with or without AR460770. **C**, Quantitation of mucosal *Runx3*$^{+/-}$ cell numbers in the jejunums of WT and *Runx3*$^{+/-}$ mice. Results are from 3 independent experiments with 4 mice per group. **P* < .05 and ***P* < .01. *PBS/PE*, Sham sensitized but PE challenged; *PE/PE*, PE sensitized and challenged.



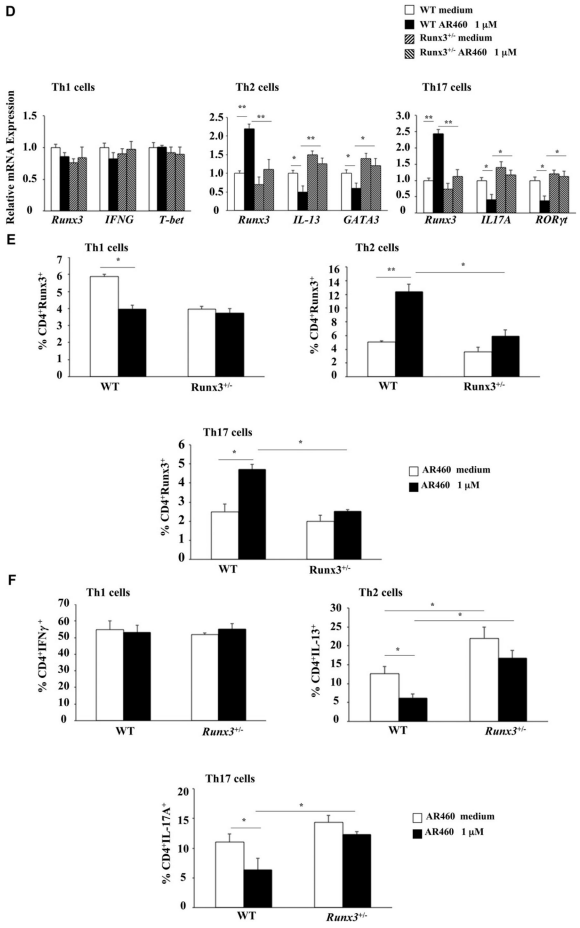


Fig 6.

Pim1 kinase inhibitor modulates *Runx3* expression and suppresses the differentiation of naive CD4 T cells into T_H2 and T_H17 lineages *in vitro*. **A**, Cell proliferation reported as number of cells. **B**, Cell proliferation measured using tritiated thymidine incorporation. **C**, Cytokine levels in supernatants of cultured CD4 T cells under T_H1-, T_H2-, and T_H17-polarizing conditions. **D**, *Runx3* and cell-specific transcription factor mRNA expression in naive CD4 T cells differentiated in vitro into T_H2 or T_H17 cells. **E** and **F**, Intracellular staining of *Runx3* and cytokines in polarized T_H1, T_H2, and T_H17 cells. Results are from 3 independent experiments. $*P < .05$ and $**P < .01$.