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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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. (J Allergy Clin Immunol 2012;130:932-44.)

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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 $CD4^+$ 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

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

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