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(54) Title: METHODS AND COMPOSITIONS FOR THE TREATMENT OF IMMUNE DISORDERS

(57) Abstract: The invention features methods of treating an immune disorder characterized by elevated Pim1 marker levels in a subject by administering a retinoic acid compound. Additionally, the invention features methods of treating immune disorders (e.g., immune disorders characterized by elevated Pim1 marker levels) by administering a retinoic acid compound in combination with an anti-inflammatory, anti-viral, or anti-microbial compound.
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METHODS AND COMPOSITIONS FOR THE TREATMENT OF IMMUNE DISORDERS

Cross Reference to Related Application

This application claims benefit of U.S. Provisional Application No. 61/490,338, filed May 26, 2011, which is incorporated by reference in its entirety.

Field of the Invention

In general, the invention relates to the treatment of immune disorders (e.g., immune disorders characterized by elevated Pin1 marker levels) with retinoic acid compounds.

Background of the Invention

Immune disorders are characterized by the inappropriate activation of the body's immune defenses. Rather than targeting infectious invaders, the immune response targets and damages the body's own tissues or transplanted tissues. The tissue targeted by the immune system varies with the disorder.

For example, in multiple sclerosis, the immune response is directed against the neuronal tissue, while in Crohn's disease the digestive tract is targeted.

Immune disorders affect millions of individuals and include conditions such as asthma, allergic intraocular inflammatory diseases, arthritis, atopic dermatitis, atopic eczema, diabetes, hemolytic anaemia, inflammatory dermatoses, inflammatory bowel or gastrointestinal disorders (e.g., Crohn's disease and ulcerative colitis), multiple sclerosis, myasthenia gravis, pruritus/inflammation, psoriasis, rheumatoid arthritis, cirrhosis, and systemic lupus erythematosus.

A major cellular pathway in the pathogenesis of autoimmunity is the TLR/IRAK1/IRF/IFN pathway. For example, levels of IFNα (type I interferon) are elevated in patients with autoimmune diseases, including systemic lupus erythematosus (SLE), and are central to disease pathogenesis, correlating with autoantibodies and disease development. Recent genetic studies in SLE patients and lupus-prone mice have identified variants in the genes critical for the TLR/IRAK1/IRF/IFN pathways, including TLR7, IRAKI and IRF5. In addition, several TLR inhibitors are in development for treatment of SLE. Notably, IRAKI genetic variants have recently been identified in human SLE. IRAKI, a well-established pivotal player in TLRs and inflammation, is located on the X chromosome, which may help account for the fact that SLE is more common in women. Importantly, studies using mouse models, where the IRAKI gene is removed, have demonstrated a key role for this kinase in the TLR7/9/IRF pathway that produces large quantities of IFNα in response to viral infection. Immune cells responsible for producing large quantities of IFNα are called pDCs. IRAKI gene deletion prevents TLR dependent activation of IRF5/7 in pDCs and IFNα production. Significantly, autoantibody complexes obtained from SLE patients contain DNA and RNA and are taken up by pDCs to activate TLR7 and TLR9 leading
to secretion of cytokines and IFNoc. Moreover, TLR activation is known to inhibit activity of glucocorticoids, a frontline drug used to treat SLE. Although IRAKI activity is regulated by phosphorylation upon TLR activation, little is known about whether it is subject to further control after phosphorylation and whether such regulation has any role in SLE.

The prevalence of asthma is increasing in the developed world, but the underlying mechanisms are not fully understood, and therapeutic modalities remain limited. Asthma is a chronic inflammatory disease of the airways that is induced by overexpression of multiple proinflammatory genes regulated by various signal pathways in response to exposure to any of numerous allergens, including Toll-like receptor/interleukin-1 receptor (TLR/IL-IR) signaling activated by house dust mite (HDM) allergens and IL-33, respectively. A major regulatory mechanism in these signal pathways and gene activation is Pro-directed phosphorylation (pS/T-P), but until recently little was known about whether and how they are regulated following phosphorylation.

Current treatment regimens for immune disorders typically rely on immunosuppressive agents. The effectiveness of these agents can vary and their use is often accompanied by adverse side effects.

Thus, improved therapeutic agents and methods for the treatment of autoimmune disorders are needed.

**Summary of the Invention**

In one aspect, the invention provides a method of treating an immune disorder in a subject by administering a retinoic acid compound to the subject in an amount sufficient to treat the subject, wherein the subject is determined to have elevated levels of a Pinl marker (e.g., Ser71 phosphorylation) prior to the administration.

In another aspect, the invention features a method of treating an immune disorder in a subject by determining Pinl marker levels (e.g., reduced Ser71 phosphorylation) in a sample (e.g., tumor samples, blood, urine, biopsies, lymph, saliva, phlegm, and pus) from the subject and administering a retinoic acid compound to the subject if the sample is determined to have elevated Pinl marker levels.

In any of the foregoing aspects, the method can also include the administration of a second therapeutic compound (e.g., at a low dosage). The second compound can be administered separately, or in a single formulation with the retinoic acid compound. The second compound can be, e.g., an anti-inflammatory, anti-microbial, or anti-viral compound. Additionally, or alternatively, any one of the foregoing methods can include determining Pinl marker levels in the sample after the administration of a retinoic acid compound.

In any of the foregoing aspects, the retinoic acid compound may be selected from 13-cis-retinoic acid, all-trans-retinoic acid, retinol, retinyl acetate, retinal, AC-55649, or any of the compounds listed in Fig. 9B.

The elevated Pinl marker level of any of the foregoing methods can be due to, e.g., an inherited trait or a somatic mutation.
The immune disorder of any of the foregoing methods can, e.g., result from disregulation of Toll-like receptor signaling or type I interferon-mediated immunity, including acne vulgaris; acute respiratory distress syndrome; Addison's disease; adrenocortical insufficiency; adrenogenital syndrome; allergic conjunctivitis; allergic rhinitis; allergic intraocular inflammatory diseases, ANCA-associated small-vessel vasculitis; angioedema; ankylosing spondylitis; aphthous stomatitis; arthritis, asthma; atherosclerosis; atopic dermatitis; autoimmune disease; autoimmune hemolytic anemia; autoimmune hepatitis; Behcet's disease; Bell's palsy; berylliosis; bronchial asthma; bullous herpetiformis dermatitis; bullous pemphigoid; carditis; celiac disease; cerebral ischaemia; chronic obstructive pulmonary disease; cirrhosis; Cogan's syndrome; contact dermatitis; COPD; Crohn's disease; Cushing's syndrome; dermatomyositis; diabetes mellitus; discoid lupus erythematosus; eosinophilic fasciitis; epicondylitis; erythema nodosum; exfoliative dermatitis; fibromyalgia; focal glomerulosclerosis; giant cell arteritis; gout; gouty arthritis; graft-versus-host disease; hand eczema; Henoch-Schonlein purpura; herpes gestationis; hirsutism; hypersensitivity drug reactions; idiopathic cerato-scleritis; idiopathic pulmonary fibrosis; idiopathic thrombocytopenic purpura; inflammatory bowel or gastrointestinal disorders, inflammatory dermatoses; juvenile rheumatoid arthritis; laryngeal edema; lichen planus; Loeffler's syndrome; lupus nephritis; lupus vulgaris; lymphomatous tracheobronchitis; macular edema; multiple sclerosis; musculoskeletal and connective tissue disorder; myasthenia gravis; myositis; obstructive pulmonary disease; ocular inflammation; organ transplant rejection; osteoarthritis; pancreatitis; pemphigoid gestationis; pemphigus vulgaris; polyarteritis nodosa; polymyalgia rheumatica; primary adrenocortical insufficiency; primary biliary cirrhosis; pruritus scroti; pruritus/inflammation, psoriasis; psoriatic arthritis; Reiter's disease; relapsing polychondritis; rheumatic carditis; rheumatic fever; rheumatoid arthritis; rosacea caused by sarcoidosis; rosacea caused by scleroderma; rosacea caused by Sweet's syndrome; rosacea caused by systemic lupus erythematosus; rosacea caused by urticaria; rosacea caused by zoster-associated pain; sarcoidosis; scleroderma; segmental glomerulosclerosis; septic shock syndrome; serum sickness; shoulder tendinitis or bursitis; Sjogren's syndrome; Still's disease; stroke-induced brain cell death; Sweet's disease; systemic dermatomyositis; systemic lupus erythematosus; systemic sclerosis; Takayasu's arteritis; temporal arteritis; thyroiditis; toxic epidermal necrolysis; tuberculosis; type-1 diabetes; ulcerative colitis; uveitis; vasculitis; and Wegener's granulomatosis.

By the term "immune disorder" is meant a disorder characterized by deregulation of Toll like receptor and/or type 1 interferon.

As used herein, the term "Pinl marker" refers to a marker which is capable of being indicative of Pinl activity levels in an organism or a sample of the invention. Pinl markers include nucleic acid molecules (e.g., mRNA, DNA) which correspond to some or all of a Pinl gene, peptide sequences (e.g., amino acid sequences) which correspond to some or all of a Pinl protein, nucleic acid sequences which are homologous to Pinl gene sequences, peptide sequences which are homologous to Pinl peptide sequences, antibodies to Pinl protein, substrates of Pinl protein, binding partners of Pinl protein, and activity of Pinl.
By "elevated levels of a Pinl marker" is meant a level of Pinl marker that is altered thereby indicating elevated Pinl activity. "Elevated levels of a Pinl marker" include levels at least 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 500%, 1000%, or greater than, or 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% less than the marker levels measured in a normal, disease free subject or tissue.

By the term "retinoic acid compound" is meant a compound that is either (a) the diterpene retinoic acid, or a derivative thereof, or (b) a compound having the structure \( R^1 \cdot \text{Ar}^1-L^1 \cdot \text{Ar}^2-L^2-C(=0)R^3 \) (Formula (I)). Exemplary retinoic acid compounds described herein include the compounds identified in Figs. 9A-9C. The term "diterpene retinoic acid" encompasses any stereoisomer of retinoic acid (e.g., the retinoic acid may be in the all-trans configuration (ATRA) or one or more of the double bonds may be in the cis configuration (e.g., 13-cis retinoic acid (13cRA)). Derivatives of the diterpene retinoic acid include reduced forms such as retinal, retinol, and retinyl acetate. In Formula (I), each of \( \text{Ar}^1 \) and \( \text{Ar}^2 \) is, independently, optionally substituted aryl or an optionally substituted heteroaryl; \( \text{R}^1 \) is \( \text{H} \), an optionally substituted alkyl group, an optionally substituted alkenyl group, or an optionally substituted alkynyl group; each of \( \text{L}^1 \) and \( \text{L}^2 \) is selected, independently from a covalent bond, an optionally substituted \( \text{C}_{1-10} \) alkylene, an optionally substituted \( \text{C}_{2-10} \) alkenylene (e.g., \(-\text{CH}=\text{CH}_2\), \(-\text{COCH}=\text{CH}_2\), \(-\text{CH}=\text{CHCO}_2\), a dienyl group, or a trienyl group), optionally substituted \( \text{C}_{2-10} \) alkyylene (e.g., \(-\text{C}3\text{C}3\)), or \(-\text{R}^4\text{C}3\text{CO}_2\)), where \( n \) is 0 or 1, \( \text{R}^4 \) is \( \text{H} \) or \( \text{OH} \), and \( \text{R}^5 \) is \( \text{H} \) or optionally substituted alkyl; and \( \text{R}^3 \) is \( \text{H} \), \( \text{OR}^3 \) or \( \text{N}(\text{R}^3)^2 \), where each \( \text{R}^4 \) is selected, independently, from \( \text{H} \), optionally substituted alkyl, or optionally substituted heteroalkyl.

The term "aryl," as used herein, represents a mono- or bicyclic \( C_6-C_{14} \) group with \( 4n+2 \) \pi electrons in conjugation and where \( n \) is 1, 2, or 3. Aryl groups also include ring systems where the ring system having \( 6n+2 \) \pi electrons is fused to a non-aromatic cycloalkyl or a non-aromatic heterocyclyl. Phenyl is an aryl group where \( n = 1 \). Aryl groups may be unsubstituted or substituted with, e.g., 1, 2, 3, or 4 substituent groups as defined herein. Still other exemplary aryl groups include, but are not limited to, naphthyl, 1,2-dihydropaphthyl, 1,2,3,4-tetrahydropaphthyl, fluorenyl, indanyl, and indenyl.

The term "cycloalkyl," as used herein, represents a monovalent saturated or unsaturated non-aromatic cyclic hydrocarbon group from three to ten carbons, unless otherwise specified, and is exemplified by cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, bicyclo[2.2.1]heptyl, and the like. In some embodiments, the cycloalkyl is a polycyclic (e.g., adamantyl). Cycloalkyl groups may be unsubstituted or substituted with, e.g., 1, 2, 3, or 4 substituent groups as defined herein.

The term "heteroaryl," as used herein, represents an aromatic (i.e., containing \( 4n+2 \) pi electrons within the ring system) 5- or 6-membered ring containing one, two, three, or four heteroatoms independently selected from the group consisting of nitrogen, oxygen, and sulfur, as well as bicyclic, tricyclic, and tetracyclic groups in which any of the aromatic ring is fused to one, two, or three heterocyclic or carbocyclic rings (e.g., an aryl ring). Exemplary heteroaryls include, but are not limited to, furan, thiophene, pyrrole, thiazole (e.g., 1,2,3-thiadiazole or 1,2,4-thiadiazole), oxadiazole (e.g.,
1.2.3-oxadiazole or 1,2,5-oxadiazole), oxazole, isoxazole, isothiazole, pyrazole, thiazole, triazole (e.g.,
1.2.4-triazole or 1,2,3-triazole), pyridine, pyrimidine, pyrazine, pyrimidine, triazine (e.g., 1,2,3-triazine
1,2,4-triazine, or 1,3,5-triazine), 1,2,4,5-tetrazine, indolyl, quinolinyl, isoquinolinyl, benzimidazolyl,
benzothiazolyl, and benzoazoxazolyl. Heteroaryls may be unsubstituted or substituted with, e.g., 1, 2, 3, or 4
substituents groups as defined herein.

The term "heterocyclyl," as used herein represents a non-aromatic 5-, 6- or 7-membered ring,
unless otherwise specified, containing one, two, three, or four heteroatoms independently selected from
the group consisting of nitrogen, oxygen, and sulfur. Heterocyclyl groups may be unsubstituted or
substituted with, e.g., 1, 2, 3, or 4 substituent groups as defined herein.

Where a group is substituted, the group may be substituted with 1, 2, 3, 4, 5, or 6 substituents
groups. Optional substituent groups include, but are not limited to: Cl-6 alkyl, C2-4 alkenyl, C2-4 alkynyl,
aryl, heteroaryl, cycloalkyl, heterocyclyl, halogen (-F, -Cl, -Br, or -I), azido(-N3), nitro (-NO2), cyano
(-CN), acyloxy(-OC(=0)R'), acyl (-C(=0)R'), alkoxy (-OR'), amino (-NH2), amido (-NR'C(=0)R''),
acyl (-OC(=0)R'), amido (-NH-C(=0)R'), carbamoyl (-OC(=0)NRR'' or -NRC(=0)OR'), hydroxy
(-OH), oxo (=O), isocyano (-NC), sulfonate (-S=0 2OR), sulfonamide
(-S(=0) 2NRR' or -NRS(=0) 2R'), or sulfonyl (-S(=0) 2R), where each R or R' is selected, independently,
from H, C1-6 alkyl, C1-5 alkenyl, C1-5 alkynyl, aryl, or heteroaryl. In some embodiments, the substituent
groups themselves may be further substituted with, for example, 1, 2, 3, 4, 5, or 6 substituents as defined
herein. For example, a Cl-6 alkyl, aryl, or heteroaryl group may be further substituted with 1, 2, 3, 4, 5, or
6 substituents as described herein.

The retinoic acid compounds of the invention inhibit Pin1 activity (e.g., as determined by the
fluorescence polarization-based displacement assay or PPIase assay as described herein). This inhibition
can be, e.g., greater than 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or greater.

By "corticosteroid" is meant any naturally occurring or synthetic steroid hormone which can be
derived from cholesterol and is characterized by a hydrogenated cyclopentanoperhydrophenanthrene ring
system. Naturally occurring corticosteroids are generally produced by the adrenal cortex. Synthetic
corticosteroids may be halogenated. Functional groups required for activity include a double bond at Δ4,
a C3 ketone, and a C20 ketone. Corticosteroids may have glucocorticoid and/or mineralocorticoid
activity. In preferred embodiments, the corticosteroid is either fludrocortisone or prednisolone.

Exemplary corticosteroids include algestone, 6-alpha-fluoroprednisolone, 6-alpha-
methylprednisolone, 6-alpha-methylprednisolone 21-acetate, 6-alpha-methylprednisolone 21-
hemisuccinate sodium salt, 6-alpha,9-alpha-difluoroprednisolone 21-acetate 17-butyrate, amcinafal,
beclomethasone, beclomethasone dipropionate, beclomethasone dipropionate monohydrate, 6-beta-
hydroxycortisol, betamethasone, betamethasone- 17-valerate, budesonide, clobetasol, clobetasol
propionate, clobetasone, clofcortolone, clofcortolone pivalate, cortisone, cortisone acetate, cortodoxone,
deflazacort, 21-deoxycortisol, depodone, descinolone, desonide, desoximethasone, dexamethasone,
dexamethasone -21-acetate, dichlorisone, diflorasone, diflorasone diacetate, diflucortolone, doxibetasol,
fludrocortisone, flumethasone, flumethasone pivalate, flumoxonide, flunisolide, fluocinonide, fluocinolone acetonide, 9-fluorocortisone, fluorohydroxyandrostenedione, fluorometholone, fluorometholone acetate, fluoxymesterone, fluprednidene, fluprednisolone, flurandrenolide, formocortimal, halcinonide, halometasone, halopredone, hycaneside, hydrocortisone, hydrocortisone acetate, hydrocortisone butyrate, hydrocortisone cypionate, hydrocortisone sodium phosphate, hydrocortisone sodium succinate, hydrocortisone probutate, hydrocortisone valerate, 6-hydroxydexamethasone, isoflupredone, isoflupredone acetate, isoprednidene, meclorisonsone, methylprednisolone, methylprednisolone acetate, methylprednisolone sodium succinate, paramethasone, paramethasone acetate, prednisolone, prednisolone acetate, prednisolone metasulphobenzoate, prednisolone sodium phosphate, prednisolone tebutate, prednisolone-21-hemisuccinate free acid, prednisolone-21-acetate, prednisolone-21(beta-D-glucuronide), prednisone, prednylidene, proclonide, tralione, triamcinolone, triamcinolone acetonide, triamcinolone acetonide 21-palmitate, triamcinolone diacetate, triamcinolone hexacetonide, and wortmannin. Desirably, the corticosteroid is fludrocortisone or prednisolone.

"Treatment," as used herein, refers to the application or administration of a therapeutic agent (e.g., a retinoic acid compound) to a patient, or application or administration of a therapeutic agent to an isolated tissue or cell line from a patient, who has a disease, a symptom of disease or a predisposition toward a disease, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect the disease, the symptoms of disease or the predisposition toward disease, or to slow the progression of the disease.

As used herein, the terms "sample" and "biological sample" include samples obtained from a mammal or a subject containing Ptnl which can be used within the methods described herein, e.g., tissues, cells and biological fluids isolated from a subject, as well as tissues, cells and fluids present within a subject. Typical samples from a subject include tissue samples, tumor samples, blood, urine, biopsies, lymph, saliva, phlegm, pus, and the like.

By a "low dosage" or "low concentration" is meant at least 5% less (e.g., at least 10%, 20%, 50%, 80%, 90%, or even 95%) than the lowest standard recommended dosage or lowest standard recommended concentration of a particular compound formulated for a given route of administration for treatment of any human disease or condition. For example, a low dosage of an anti-inflammatory, antimicrobial, or anti-viral compound formulated for oral administration will differ from a low dosage of an anti-inflammatory, anti-microbial, or anti-viral compound formulated for intravenous administration.

**Brief Description of the Drawings**

**Figure 1: Ptnl is activated and required for cytokine and especially type I IFN secretion following TLR stimulation.**

(a-c) Impaired TLR7/9-induced cytokine production from Ptnl KO mDCs. Bone-marrow-derived mDCs were stimulated with 100 ng/mL LPS, 1 µg/mL Pam3CSK4, 0.1 µg/mL R848 or 0.1 µM
CpG-B. Levels of IL-6 (a), IL-12p40 (b), TNFa (c) measured in cell-culture supernatants after 12 h are shown.

(d, e) IFN-α levels in supernatants after R848 and CpG-A treatment of purified splenic pDCs (B220+CD11cint) (d) and Flt3L-induced bone-marrow-derived pDCs for 24 h (e).

(f, g) IFN-α levels in supernatants following stimulation of splenic (f) and Flt3L-induced bone-marrow-derived pDCs (g) for 24 h with Influenza A (H1N1) virus or MCMV. IFN-α concentrations were measured by ELISA. Bars indicate means ±s.d. of triplicate determinations.

(h) Splenic pDCs were stimulated with PBS, R848 or CpG DNA for 6 h. Expression of IFN-α or β mRNAs was measured by quantitative real-time RT-PCR analysis. Data were normalized to the levels of GAPDH expression in means ±s.d. of triplicates.

(i) Pinl catalytic activity, but not protein level, is increased upon TLR7/9 stimulation. Purified human PBMC were treated for 30 min either with PBS (blue), R848 (black) or CpG DNA (red) and lysed, followed by protease-coupled isomerase activity assay for Pinl activity. Results are representative of 3 independent experiments. Following the Pinl protease coupled isomerase activity assay, fractions of lysates were subjected to immunoblotting analysis using Pinl antibody with tubulin as a control (inset).

Figure 2: Proteomic approach identifies IRAKI as a major Pinl substrate upon TLR stimulation.

(a) Proteomic identification of IRAKI as a TLR-induced Pinl binding protein. THP1 cells stimulated with R848 for 45 min were lysed and subjected to GST-Pinl pulldown followed by SDS-PAGE and colloidal CBB staining. Specific GST-Pinl interacting bands were excised and 7 peptides were identified to IRAKI by LC-MS (Fig. 12A).

(b) TLR-dependent interaction between Pinl and IRAKI, assayed by GST-Pinl pulldown. RAW264.7 cells stimulated with PBS or either R848 or CpG for 30 min were subjected to immunoblotting analysis using IRAKI antibodies after pull down with GST or GST-Pinl.

(c) TLR-dependent interaction between endogenous Pinl and IRAKI, assayed by Co-IP. THP1 cells were stimulated with poly(I:C), R848 or CpG and subjected to immunoprecipitation with anti-Pinl antibodies or control IgG, followed by immunoblotting with IRAKI antibodies.

(d) The IRAKI-Pinl interaction is sensitive to phosphatase treatment. TLR7-HEK293T cells were transfected with FLAG-IRAK1 and stimulated with R848 and lysates were untreated or treated with CIP phosphatase for 60 min at 30°C, followed by GST-Pinl pulldown experiments.

(e) The Pinl-IRAK1 interaction is dependent on the intrinsic kinase activity of IRAKI. FLAG-KD-IRAK1, either alone or in combination with IRAKI were expressed in IRAKI-deficient 293T cells, followed by GST pulldown experiments.

(f) Pinl binds directly to phosphorylated WT IRAKI, but not KD IRAKI. FLAG-IRAK1 and FLAG-KD IRAKI were expressed in IRAKI-deficient 293T cells and purified using FLAG-agarose, followed by Far-Western analysis using GST-Pinl WW domain to detect Pinl binding using anti-GST antibody. Membranes were re-probed with FLAG antibody as a control.
(g) Pinl binds to activated WT IRAKI, but not KD IRAKI in MEFs. FLAG-IRAKI and its KD mutant were expressed in MEFs using retroviral infection and then treated with R848 or control buffer, followed by GST pulldown experiments.

(h) Multiple pSer-Pro motifs in the undetermined domain (UD) of IRAKI are required for Pinl binding. FLAG-IRAKI and its mutants were expressed in MEFs using retroviral infection, and then treated with R848 or control buffer, followed by GST pull down experiments.

(i) S173 phosphorylation of IRAKI is induced upon TLR7/9 stimulation.

Figure 3: Phosphorylated S131/S144/S173-Pro sites in the IRAK-1 UD bind and are isomerized by Pinl

(a) Representative chemical shift perturbations in $^1$H-$^1$H HSQC spectra resulting from titration with IRAK-1 peptides phosphorylated at Ser131, Ser144, and Ser173. Apo peaks are shown in red, and sequential colors represent increasing concentrations of peptides, purple being highest.

(b) Representative binding curves for WW domain residues, showing weighted chemical shift changes ($\Delta \delta = \sqrt{\Delta \delta_{\text{HH}1}^2 + (0.154 \Delta \delta_{\text{HH}2})^2}$) as a function of total concentration of peptide. Residues plotted are Ser16 (●), Ser18 (x), Gln33 (●), the sidechain of Trp34 (●), and Glu35 (A). Lines represent global fits.

(c) 2D $^1$H-$^1$H ROESY spectra (mixing time of 100 ms) of IRAK-1 phosphopeptides in the presence (top panels) or absence (bottom panels) of a catalytic amount of Pinl. The appearance of exchange crosspeaks (arrows) between peaks corresponding to the cis and trans isomers confirms that Pinl acts catalytically on these sequences.

Figure 4 Pinl is essential for IRAKI activation upon TLR ligation.

(a) Pinl KO completely blocks IRAKI activation in mouse cells following TLR7 stimulation. Pinl WT and KO Flt3-derived pDCs (bottom) or TLR7-expressing MEF cells (top) were simulated with R848 for the indicated times and analyzed for the characteristic IRAKI shift by immunoblotting with IRAKI antibodies, with IRAK4 and Pinl levels as controls.

(b) Pinl KO completely blocks activation of IRAKI, but not IRAK4 following TLR7 stimulation. Peritoneal macrophage from Pinl WT and KO mice were stimulated with R848 for the indicated times and kinase activity of IRAKI and IRAK4 was assessed by IP kinase autophosphorylation assay. Protein levels of IRAKI, IRAK4 and Pinl were assayed as controls.

(c) Pinl knockdown blocks IRAKI activation in human cells following TLR7 and TLR9, but not TLR3 stimulation. Human THPI monocytes were infected with viral control shRNA or shRNA targeting Pinl and simulated with poly (I:C) (TLR3), R848 or CpG ligands for the indicated times, followed by analyzing the characteristic IRAKI shift using immunoblotting.
(d) In vivo kinase assay demonstrates IRAKI kinase activity in Pinl WT, but not Pinl KO cells. Retroviral FLAG-IRAKI, and KO-IRAK1 or vector (VCT) control were coexpressed with a HA-N-terminal 220 aa fragment of IRAKI as a substrate in Pinl WT and KO MEFs (schematic diagram). IRAKI kinase activity was determined by immunoblotting with HA antibodies to assess the characteristic mobility shift in IRAKI N-terminal 220aa due to trans-phosphorylation by co-expressed IRAKI proteins.

(e) Pinl KO abolishes TLR dependent activation of exogenous IRAKI in vivo. FLAG-IRAKI and its KO mutant were co-expressed with TLR7 in Pinl WT and KO MEF cells using retroviral vectors and stimulated with R848 for the indicated times, followed by analyzing the characteristic IRAKI mobility shift using immunoblotting.

(f) Pinl, but not its WW domain-binding mutant (W34A) or catalytically inactive PPIase domain mutant (K63A), fully rescues IRAKI activation in Pinl KO cells. Pinl KO cells stably expressing FLAG-IRAKI were transfected with either WT-Pinl, K63A-Pinl, W34A-Pinl or PPIase domain of Pinl and TLR7 and stimulated for the indicated times, followed by analyzing the characteristic IRAKI mobility shift using immunoblotting.
Figure 5 Pinl facilitates IRAKI release from the receptor complex to activate IRF7 following TLR ligation.

(a) Activated and phosphorylated IRAKI is released from MyD88 in Pinl WT cells, but inactive IRAKI is not in Pinl KO cells. HA-MyD88 and FLAG-IRAKI were co-expressed in Pinl WT and KO MEF using retroviral expression vectors, followed by immunoprecipitation with anti-HA antibody and then immunoblotting with anti-FLAG antibody.

(b) Pinl knockdown inhibits the interaction of IRF7 with TRAF6. THPI cells expressing Pinl-RNAi or control RNAi were stimulated with CpG for the indicated times and the interaction of IRF7 and TRAF6 was examined by Co-IP.

(c, d) Pinl knockdown prevents IRF7 nuclear translocation in human THPI cells. Following TLR7 (c) or TLR9 (d) ligation for the indicated times, nuclear and cytoplasmic fractions of THPI cells were prepared, followed by immunoblotting with IRF7 antibody. The purity of nuclear and cytosolic fractions was evaluated by immunoblotting with tubulin or lamin A/C antibodies, respectively.

(e) Pinl KO prevents IRF7 nuclear translocation after TLR7 or TLR9 ligation in pDCs. After R484 or CpG stimulation, Pinl WT and KO pDCs were immunostained with IRF7 antibodies and counter-stained with DAPI, followed by confocal microscopy.

Figure 6 Pinl is required for IRF7 activation and IFN-α production upon TLR ligation in vitro.

(a, b) Pinl is required for IRF7 activation in response to TLR7/9 activation. Pinl WT and KO cells transiently co-expressing a UAS_{(GAL)}-reporter plasmid, Gal4-IRF7 and TLR7 (a) or TLR9 (b) were stimulated with R848 or CpG, respectively, followed by luciferase assay 12 h later using renilla luciferase to normalize for transfection efficiency.

(c, d) Re-expression of Pinl, but not its mutants, fully rescues impaired IRF7 activation and IFN-α production in Pinl KO cells. Pinl WT and KO MEFs stably expressing IRAKI were transiently co-transfected with UAS_{(GAL)} and Gal4-IRF7 and empty vector (EV). Pinl, WW domain mutant (W34A) or PPIase domain mutant (K63A), followed by luciferase assay (c) and IFN-α ELISA (d), with Pinl WT MEFs stably expressing IRAKI transfected with EV as a control. Expression levels of WT, W34A and K63A Pinl proteins are shown below graphs in (c) and (d). ND, not detectable.

(e) Overexpression of KD IRAKI inhibits IRF7 activity in Pinl WT, but does not affect basal IRF7 activity in Pinl KO MEFs. Pinl WT and KO MEFs were transiently transfected with Gal4-IRF7, UAS(Gai), MyD88 (20 ng) and various amounts of KD IRAKI or control vector, as indicated, followed by assaying IRF7 activity using Renilla as a control for normalization.

(f, g) Pinl KO or IRAKI mutations that prevent IRAKI from being a Pinl substrate abolish IRF7 activation and IFN-α production. Pinl WT and KO cells stably expressing empty vector (EV), IRAKI or IRAKI mutants S110A, S131, S144, S173A, 3A (S131+S144+S173A) or KD were co-transfected with UAS_{(GAL)} and Gal4-IRF7 to assess IRF7 reporter activity (f) or with IRF7 to measure IFN-α production (g). Expression levels of IRAKI and its various mutants are shown below the graph (t).
Pinl KO or IRAKI mutations that prevent IRAKI from being a Pinl substrate abolish antiviral activity. VSV production in plaque-forming units (PFU) per mL 24 h after infection of monolayer L cells (0.1 PFU/cell) previously treated with supernatants from Pinl WT and KO cells stably expressing EV, IRAKI or IRAKI mutants S110A, S131, S144, S173A, 3A or KD (h), with representative pictures of VSV plaques shown in (i). All samples were measured in triplicates. Vertical bars represent S.D.

Figure 7 Pinl is required for TLR-mediated, type I interferon-dependent innate and adaptive immunity in vivo.

(a-c) Pinl KO mice completely fail to mount robust IFN-a response upon TLR7/9 activation. Pinl WT and KO mice were injected with 50 nmol of R-848 (i.v.) (a), 5 μg CpG-A complexed to DOTAP (i.v.) (b), or MCMV 5x 10^4 PFU (i.p.) (c), followed by assaying serum IFN-a levels at different time points. (n=3)

(d, e) Pinl KO mice are highly vulnerable to viral infection. Pinl WT and KO mice were injected with 2.5x10^4 PFU MCMV, followed by monitoring changes in body weights over time (d) or with 10^5 PFU MCMV, followed by monitoring morbidity daily for 14 days (n=6) (e).

(f) Pinl KO mice are severely defective in triggering the TLR-mediated, IFN-dependent adaptive immunity. Pinl WT and KO mice were immunized with ovalbumin, anti-CD40 and CpG-A complexed to DOTAP and six days later, splenocytes were isolated and subjected to FACS analysis using antibodies against CD8a and CD44 antibody and a MHC tetramer. The data shown were gated on CD8a-positive events and are representative of three independent experiments. The numbers indicate the percentage of tetramer-positive cells relative to the total number of CD8a+ T cells.

Figure 8 Essential Role for Pinl in TLR-IRAK1-IRF-IFN signaling events in innate and adaptive immunity.

(a) Upon activation of TLRs, especially TLR7/9, Pinl activity is upregulated and IRAKI is autophosphorylated in the UD, which allows Pinl to bind to and isomerize phosphorylated IRAK. Such Pinl -catalyzed conformational change facilitates the dissociation of IRAKI from the receptor complex and recruitment of TRAF6, which combines with IRAKI to activate IRF7 by promoting nuclear translocation for the induction of type I interferon to mediate innate and adaptive immunity.

(b) Although Pinl KO neither affects the recruitment of IRAKI to the TLR complex, nor the activation of other TLR activated kinases such as IRAK4 and MAP kinases, it specifically prevents IRAKI activation and release from the receptor complex so that TRAF6 is not recruited and IRF7 activated, leading to loss of type I interferon production and its mediated innate and adaptive immunity.

Figure 9A is a schematic of the chemical structure of 13-cis-retinoic acid and all-trans retinoic acid.

Figure 9B is a schematic showing additional retinoic acid compounds.
Figure 9C is a series of schematics showing the indicated retinoic acid compounds and β-carotene.

Figure 10 shows Pinl deficiency moderately inhibits inflammatory cytokine production in macrophages but does not affect proinflammatory cytokines in the pDCs and IFN-β in mDCs.

(a, b) Bone marrow derived macrophages were stimulated with various THL ligands over night. IL-6 (a) and IL-12p40 (b) levels in supernatants was measured by ELISA. Splenic pDCs were isolated by negative selection sing MACS beads according to the manufacturer's guidelines and stimulated with CpG or R848 for 24 h.

(c, d) IL-6 and IL-12p40 concentrations were measured in supernatants by ELISA. Purity of isolated pDCs was >95% as assessed by flow cytometry using antibodies against PDCA1, B220 and CD11c.

(e) Bone marrow derived mDC were stimulated with CpG for 24 h and IFN-β levels were measured in the supernatants by ELISA. Results present mean values of three independent experiments.

Figure 11 shows Pinl KO has no effects on overall population of immune cells and their TLR expression.

(a) CD4+ T cells, CD8+ T cells, B cells, mDCs and pDCs isolated form spleens and lymph nodes of Pinl WT and KO mice were determined by FACS analysis using various cell markers.

(b) TLR7 and TLR9 expression in T cells, B cells, macrophages, mDCs and pDCs isolated from Pinl WT and KO mice were analyzed using FACS analysis.

(c) TLR2 and TLR4 expression in Pinl WT and KO splenic B cells and mDCs isolated from Pinl WT and KO mice were analyzed using FACS analysis.

Figure 12 shows that the Pinl-IRAK1 interaction is mediated by IRAKI phosphorylation and Pinl WW domain.

(a) Peptides matching IRAKI form LC-MS identification of TLR7 dependent Pinl binding partners.

(b) Schematic diagram of Pinl illustrating the modular domains (WW domain and PPIase domain), their respective function and inactivating point mutations in key functional residues.

(c) The Pinl-IRAK1 interaction is sensitive to phosphatase treatment. Raw264.7 cells were stimulated with R848 and extracts were untreated or treated with CIP phosphatase, followed by GST-Pinl pulldown (PD).

(d) The WW domain of IRAKI mediates the Pinl-IRAK1 interaction. FLAG-IRAK1 was expressed in IRAKI-null HEK293T cells, followed by GST pulldown using GST, GST-Pinl, GST-WW or GST-PPIase domain.
(e) The Pin-IRAK1 interaction is blocked by inactivating the Pinl WW domain function using the W34A mutation. FLAG-IRAK1 was expressed in IRAK1-null HEK293T cells, followed by GST pulldown using GST, GST-Pinl or W34A-Pinl protein.

(f) Pinl binds specifically to IRAKI but not IRAK2 or IRAK4. HA tagged human IRAKI, IRAK2, and IRAK4 were transfected into RAW 264.5 cells followed by treatment with PBS, R848 (TLR7) or CpG ODN (TLR9). Interaction with Pinl was determined by GST-Pinl PD was immunoblotting with anti-HA antibodies.

Figure 13 shows that the Pinl-IRAK1 interaction is mediated by ProS/T-rich undetermined domain (UD).

(a) Schematic diagram of IRAKI illustrating the functional domains including death domain, ProS/T-rich UD, and the kinase domain. Amino acid sequence of the ProS/T-rich UD highlighting the potential Pinl binding sites (dark gray).

(b) Mapping IRAKI domains for binding to Pinl shows that deletion of the hUD or kinase-dead K239S mutation abolished Pinl binding. FLAG-IRAK1 deletion mutants were expressed in IRAKI deficient 293T cells, followed by GST-Pinl pulldown.

Figure 14 shows the identification of IRAKI phosphorylation sites by LC-MS.

(a,b) LC-MS chromatograms demonstrating phosphorylation of S131 and S144 in IRAKI.

FLAG IRAKI was purified using anti-FLAG agarose and eluted followed by purification using GST-Pinl. Following separation by SDS-PAGE and CBB staining bands were digested with trypsin and analyzed by LC-MS.

(c) Schematic diagram of the peptides detected by LC-MS from tryptic digests and highlighting potential Pinl binding sites in the UD of IRAKI. Squares on peptides indicate phospho-serine/threonine residues. Note: no peptides were detected for the region spanning S173.
Figure 15 shows the detection of phosphorylation of endogenous and exogenous IRAKI on S173.

(a) Anti-pSer173 antibodies specifically recognize the WT IRAKI but not the S173A mutant. FLAG WT and S173A IRAKI were highly overexpressed in IRAK null 293 cells and purified using FLAG beads. Immunoblotting was performed using anti-FLAG or anti-phospho-Ser173 antibodies.

(b) THP1 cells were stimulated with PBS, R848 (TLR7) and GST-Pinl PD was performed followed by immunoblotting with anti-pSer173 IRAKI antibody. Total cell lysates were immunoblotted with total IRAKI antibodies.

Figure 16 shows Pinl knockdown completely blocks IRAKI activation in response to activation in human monocytes.

(a) Human THP1 monocytes were stably infected with control shRNA or shRNA targeting Pinl and then stimulated with Pam3CSK4 (TLR2 ligand) for the indicated times, followed by analyzing the characteristic IRAKI mobility shift associated with IRAKI full activation by immunoblotting with IRAKI antibody. IRAK4 levels were used as a loading control.

(b) Human THP1 monocytes were stably infected with control shRNA or shRNA targeting Pinl and then stimulated with LPS (TLR4 ligand) for the indicated times, followed by analyzing the characteristic IRAKI mobility shift associated with IRAKI full activation by immunoblotting with IRAKI antibody. IRAK4 levels were used as a loading control.

Figure 17 shows Pinl knockout does not affect activation of MAPKs in response to activation of TLR7/9 in pDCs.

(a) Following stimulation with R848 for the indicated times, activation of ERKs, JNKs and p38 MAPK in Pinl +/- and Pinl -/- Flt3 ligand derived pDCs was determined by immunoblotting with phospho-specific antibodies, with total ERKs, JNK and p38 MAPL antibodies as loading controls.

(b) Following stimulation with CpG for the indicated times, activation of ERKs, JNKs and p38 MAPK in Pinl +/- and Pinl -/- Flt3 ligand derived pDCs was determined by immunoblotting with phospho-specific antibodies, with total ERKs, JNK and p38 MAPL antibodies as loading controls.

Figure 18 shows that genetic deletion of Pinl does not affect activation of NF-κB or MAPK in TLR4 stimulating macrophage or activation of NF-κB in TLR7 or TLR9 stimulated pDC.

(a, b) Bone marrow derived macrophages form Pinl WT and Pinl KO mice were stimulated with LPS for the indicated times. The levels of pERK or pJNK (a) or IκBα (b) were determined by immunoblotting with phospho-specific MAPK antibodies or anti-IκBα antibodies, with tubulin levels being used as a control and Pinl levels were confirmed by immunoblotting with anti-Pinl antibodies.

(c, d) Bone marrow derived Flt3-pDC from Pinl WT and Pinl KO mice were stimulated with R848 or CpG-ODN for the indicated times, followed by immunoblotting with anti-IκBα antibodies.
Figure 19 shows that Pinl deficiency inhibits TLR4 and TLR7 induction of proinflammatory cytokine production in vivo.

(a,b) IL-6 and IL-12p40 serum levels following i.p. injection of Pinl WT and Pinl KO mice with LPS were measured by ELISA at indicated times.

(c,d) IL-6 and IL-12p40 serum levels following i.p. injection of Pinl WT and Pinl KO mice with R848 were measured by ELISA at indicated times.

Figure 20 shows Pinl bound to IRAKI and NF-κB upon IL-33 stimulation and Pinl KO abolished IL-33 induced IRAKI activation, calcium influx and NF-κB activation.

(A) GST-Pinl pulldown showed Pinl binding to activated form of IRAKI in THP monocytes after stimulation with IL-33 (100ng/ml).

(B) IL-33 activated IRAKI in Pinl WT, but not KO MEFs in a time-dependent manner.

(C) IL-33 induced calcium influx in eosinophils derived from Pinl WT, but not KO bone marrows, as measured by Fura 2-AM.

(D, E) GST-Pinl pulldown (D) and Co-IP (E) showed Pinl binding to p65 NF-κB in Pinl WT, but not KO MEFs after IL-33 stimulation.

(F) IL-33 activated NF-κB in Pinl WT, but not KO MEFs, as shown by p65 gel-shift assay (EMSA).

(G) Preliminary NMR results showing region of IRAKI-UD sequence containing the three pSP sites implicated in Pinl activation.

(H) NMR measurement (inset, Nzz spectrum of [15N-Ala175] UD157-180 peptide) and data fitting to yield Pinl-catalysis rate at pS173P.

(I) 15N-HfHSQC spectrum of recombinant 15N-IRAK1-UD (residues 85-212) indicates disorder and the presence of various cis isomers (minor populations).

Figure 21 shows Pinl might be hyperphosphorylated and activated in SLE.

(A) PBMCs were isolated from normal and SLE patients and immunoblotted with Pinl antibodies specific to pS16 (top) or S16 (bottom) of the WW domain.

(B) PBMCs were isolated from normal and SLE patients and were subjected to Pinl PPIase assays.
Figure 22 shows phosphorylation of Pinl by PKA inhibits its function.  
(A, B) S16 phosphorylation of Pinl in the WW domain abolishes its substrate binding activity.  
(C-E) S71 phosphorylation of Pinl on PPlase domain abolishes its catalytic activity.

Figure 23 shows phosphorylation of Pinl by PKA on S16 and S71 prevents Pinl from binding to and isomerizing its substrates.  
(A) Phosphorylation of Pinl by PKA on S16 and S71, as detected by our antibodies that recognized Ser16-Pinl, pSer16-Pinl, or pS71-Pinl.  
(B) S16 phosphorylation formed H-bonds with S18 & S19, preventing substrates from entering the binding site.  
(C) S71 phosphorylation formed H-bonds with R69, preventing substrates from entering the catalytic active site.

Figure 24 shows activation of Pinl in B cells by TLR9.  
(A) Detection of Pinl in Pinl WT but not Pinl KO MEFs by icFACS using APC-labeled Pinl mAb, which recognized a non-phosphorylated active Pinl.  
(B) Splenocytes were stimulated for 48 hours with 1 umol CpG, followed by detecting activation of Pinland B cells using APC-Pinl and PE-CD69 in B220 positive cells.

Figure 25 shows activation of Pinl by PP2A in vitro and in vivo.  
(A) PP2A dephosphorylated Pinl that was phosphorylated by PKA, as detected by 32P-labeling.  
(B) PP2A dephosphorylated Pinl that was phosphorylated by PKA, as detected by phospho-specific antibodies.  
(C) PP2A fully restored the PPlase activity of Pinl that was inhibited by PKA phosphorylation.  
(D) Pinl became dephosphorylated in human PBMCs after TLR9 stimulation with CpG, which was reversed by a pretreatment with 5nM PP2A inhibitor okadaic acid (OA).  
(E) Pinl became dephosphorylated in T cells isolated from PP2A transgenic mice, but not wild-type controls.

Figure 26 shows generation of Pinl conditional knockout in immune cells.  
(A) Pinl CO targeting mice were crossed with CMV-Flp mice to delete the Neo cassette and produce Pinl CO allele (Pinlfl).  
Pinlfl/fl mice will be crossed with various lupus prone mice and then with CD11c-Cre, CD4-Cre, or CD19-Cre mice to generate Pinl CO specifically in DCs, T, or B cells.  
(B) Pinl CO in neurons confirmed by immunoblot.

Figure 27 shows HTS identified novel Pinl inhibitors that blocked cytokine production induced by TLR9.
(A) Using a FP-based HTS, we identified ATRA and Cpd4 ad new Pinl inhibitors.
(B) Both ATRA and Cpd4 are confirmed to compete with a peptide inhibitor from binding Pinl.
(C) Both ATRA and Cpd4 are confirmed to compete with a peptide inhibitor to inhibit Pinl catalytic activity.
(D) Both ATRA and Cpd4 are confirmed to compete with a peptide inhibitor from binding Pinl to inhibit Pinl -dependent cancer cell growth.
(E) ATRA induced the degradation of Pinl and its substrate D1 in cancer cells.
(F-H) ATRA inhibited cytokine production (pg/ml) of pDCs in response to TLR activation.
(I) ATRA bound to Pinl active site, as shown by the co-crystal structure.

Figure 28 shows Pinl is activated by IL-33 and its KO fully suppressed Th2 response and asthma induced by IL-33 in mice and in vitro.

(A) Pinl isomerase activity in THP monocytes was increased by 5 ng/ml of IL-33.
(B) Pinl KO completely suppressed IL-6 secretion induced by IL-33 in MEFs.
(C) Pinl KO fully blocked Th2 response in mouse BAL fluid after intranasal IL-33.
(D-F) Pinl KO effectively inhibited intranasal IL-33 induced asthma, as shown by histological examination (D), and number of total cells (E) or eosinophils (F). (n=4).

Figure 29 shows Pinl KO reduced Th2 response and asthma after OVA allergen challenge in mice.

(A) Pinl KO reduced OVA-induced asthma in mice, as shown by histological examination.
(B) Pinl KO reduced OVA-induced asthma in mice, as shown by Th2 response in mouse BAL fluid.
(C) Pinl KO reduced OVA-induced asthma in mice, as shown by number of total cells.
(D) Pinl KO reduced OVA-induced asthma in mice, as shown by eosinophils, (n = 4).

Figure 30 shows Pinl KO suppressed proinflammatory cytokine secretion induced by LPS or HDM.

(A) Pinl KO suppressed IL-6 secretion from MEFs after overnight treatment with 0.1 ug/ml LPS.
(B) Pinl KO suppressed IL-6 secretion from MEFs after 90 min after injection of mice with 4 mg/ml LPS (B).
(C) Pinl KO blocked IL-6 secretion induced by various concentrations of HDM in MEFs.
(D) Pinl KO blocked IL-6 secretion induced by various concentrations of HDM in mast cells.

Figure 31 shows identification of retinoic acids as potential Pinl inhibitors.

(A) trans-RA bound to Pinl more efficiently that cis-RA.
(B) Dose-dependant Pinl inhibition by cis-RA (left) or trans-RA (right).
(C) SKBR3 and T47D cancer cells were more sensitive to RAs than normal MCF-IOA cells.
(D) RAs degraded Pinl in drug-responsive SKBR3 cells (right), but not in drug-irresponsive MCF 10A (left).

(E) Pinl KO MEFs were resistant to trans-RA anti-proliferative effects (left), but expression of WT Pinl, but not its mutant, restored sensitivity of Pinl KO MEFs to trans-RA.

Figure 32 shows essential moiety of trans-RA for Pinl inhibition.

(A) Retinoids only with carboxylic acid, but not other groups potently inhibit Pinl.

(B) Crystal structure confirms trans-RA in the Pinl active site, with the carboxylic acid forming H-bonds with key R68 and K63.

Figure 33 shows trans-RA inhibited Pinl activation and IL-6 production in eosinophils induced by IL-33.

(A) BM-derived eosinophils were stimulated without or with 100ng/ml IL-33 and different levels of trans-RA, followed by Pinl western.

(B) BM-derived eosinophils were stimulated without or with 100ng/ml IL-33 and different levels of trans-RA, followed by PPIase assay.

(C) BM-derived eosinophils were stimulated without or with 100ng/ml IL-33 and different levels of trans-RA, followed by IL-6 ELISA.

Figure 34 shows Proteinuria in NZBWFl mice is significantly reduced by Pinl inhibitor ATRA. Female lupus-prone NZBWFl mice were treated with placebo or ATRA for 3.5 months and proteinuria was evaluated by Bio-Rad assay.

Figure 35 shows cutaneous inflammation is reduced in Pinl KO mice. Female Pinl WT and KO mice were shaved and left untreated or treated dermally with TLR ligands to induce skin inflammation followed by analysis of skin sections by H&E staining. Arrows indicate thickening of the keratinocyte layer (hyperkeratosis).

Detailed Description of the Invention

In general, the invention features methods of treating an immune disorder characterized by elevated Pinl marker levels in a subject by administering a retinoic acid compound. Additionally, the invention features methods of treating an immune disorder (e.g., an immune disorder characterized by elevated Pinl marker levels), by administering a retinoic acid compound in combination with one or more additional anti-inflammatory, anti-microbial, or anti-viral compounds.

Inhibitors of Pinl (e.g., retinoic acid compounds) are useful for treating immune disorders (e.g., disorders characterized by increased Pinl activity or resulting from disregulation of Toll-like receptor signaling or type I interferon-mediated immunity). Furthermore, because Pinl associated aberrant
IRAKI activation and type I IFN overproduction occurs in various immune diseases, Pinl inhibition would be expected to behave synergistically with many anti-inflammatory compounds.

I. Pinl

Phosphorylation on serine/threonine-proline motifs restrains cis/trans prolyl isomerization, and also creates a binding site for the essential protein Pinl. Pinl binds and regulates the activity of a defined subset of phosphoproteins, as well as participating in the timing of mitotic progression. Both structural and functional analyses have indicated that Pinl contains a phosphoserine/threonine-binding module that binds phosphoproteins, and a catalytic activity that specifically isomerizes the phosphorylated phosphoserine/threonine-proline. Both of these Pinl activities are essential for Pinl to carry out its function in vivo.

Pinl has previously been shown to act on IRF3 to affect IFN-β production upon TLR3 or RIG-I activation. However, recent results have shown that unlike IRF3- or TLR3-deficient mice, IRF7 or IRAKI-deficient mice completely fail to mount a type I IFN antiviral responses due to loss of type I IFN secretion from pDCs. Our results have uncovered an essential role for Pinl as a novel regulator of IRAKI activation in TLR signaling and type I IFN-mediated innate and adaptive immunity and revealed that Pinl inhibitors, which are under active development, may represent a novel therapeutic approach that would allow selective inhibition of the type I IFN response while leaving proinflammatory cytokine production unaffected.

Pinl is highly conserved and contains a protein-interacting module, called WW domain, and a catalytically active peptidyl-prolyl isomerase (PPIase). Pinl is structurally and functionally distinct from members of two other well-characterized families of PPIases, the cyclophilins and the FKBP. PPIases are ubiquitous enzymes that catalyze the typically slow prolyl isomerization of proteins, allowing relaxation of local energetically unfavorable conformational states. Phosphorylation on Ser/Thr residues immediately preceding Pro not only alters the prolyl isomerization rate, but also creates a binding site for the WW domain of Pinl. The WW domain acts a novel phosphoserine-binding module targeting Pinl to a highly conserved subset of phosphoproteins. Furthermore, Pinl displays a unique phosphorylation-dependent PPIase that specifically isomerizes phosphorylated Ser/Thr-Pro bonds and regulates the function of phosphoproteins.

II. Measurement of PIN1 marker levels

The present invention pertains to the treatment of immune disorders identified as coinciding with elevated Pinl marker levels with retinoic acid compounds. In some aspects, the invention features the determination of Pinl marker levels in a subject; where retinoic acid is administered in subjects where Pinl marker levels are determined to be elevated. In other aspects, the invention can also feature the measurement of Pinl marker levels subsequent to the administration of retinoic acid compounds in order to evaluate the progress of therapy in treating the immune disorder.
Accordingly, one aspect of the present invention relates to diagnostic assays for measuring levels of Pinl marker, as well as Pinl activity, in the context of a biological sample (e.g., blood, urine, biopsies, lymph, saliva, phlegm, and pus) to thereby determine whether an individual is a candidate for treatment with a retinoic acid compound. The invention features both treatment of subjects exhibiting symptoms of an immune disorder and individuals at risk for developing an immune disorder.

**Diagnostic Assays**

An exemplary method for detecting the presence or absence of Pinl protein or nucleic acid in a biological sample involves obtaining a biological sample from a test subject and contacting the biological sample with a compound or an agent capable of detecting Pinl protein or a nucleic acid (e.g., mRNA, genomic DNA) that encodes Pinl protein such that the presence of Pinl protein or nucleic acid is detected in the biological sample. A preferred agent for detecting Pinl mRNA or genomic DNA is a labeled nucleic acid probe capable of hybridizing to Pinl mRNA or DNA. The nucleic acid probe can be, for example, a Pinl nucleic acid or a corresponding nucleic acid such as an oligonucleotide of at least 15, 30, 50, 100, 250 or 500 nucleotides in length which is capable of specifically hybridizing under stringent conditions to Pinl mRNA or genomic DNA. Other suitable probes for use in the diagnostic assays of the invention are described herein.

A preferred agent for detecting Pinl marker is an antibody capable of binding to Pinl protein, preferably an antibody with a detectable label. Antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (e.g., Fab or F(ab')2) can be used. The term "labeled," with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (i.e., physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin.

With respect to antibody-based detection techniques, one of skill in the art can raise anti-Pinl antibodies against an appropriate immunogen, such as isolated and/or recombinant Pinl or a portion or fragment thereof (including synthetic molecules, such as synthetic peptides) using no more than routine experimentation. Synthetic peptides can be designed and used to immunize animals, such as rabbits and mice, for antibody production. The nucleic and amino acid sequence of Pinl is known (Hunter et al., WO 97/17986 (1997); Hunter et al., U.S. Pat. Nos. 5,952,467 and 5,972,697, the teachings of all of which are hereby incorporated by reference in their entirety) and can be used to design nucleic acid constructs for producing proteins for immunization or in nucleic acid detection methods or for the synthesis of peptides for immunization.

Conditions for incubating an antibody with a test sample can vary depending upon the tissue or cellular type. Incubation conditions can depend on the format employed in the assay, the detection
methods employed, and the type and nature of the antibody used in the assay. One skilled in the art will recognize that any one of the commonly available immunological assay formats (such as radioimmunoassays, enzyme-linked immunoa sorbent assays, diffusion based Ouchterlony, or rocket immunofluorescent assays) can readily be adapted to employ the antibodies of the present invention.


The detection method of the invention can be used to detect Pinl mRNA, protein, or genomic DNA in a biological sample in vitro as well as in vivo. For example, in vitro techniques for detection of Pinl mRNA include northern blot hybridizations and in situ hybridizations. In vitro techniques for detection of Pinl protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations, immunofluorescence, or quantitative sequencing reactions. In vitro techniques for detection of Pinl genomic DNA include Southern hybridizations. The detection of genomic mutations in Pinl (or other genes that effect Pinl marker levels) can be used to identify inherited or somatic mutations. Furthermore, in vivo techniques for detection of Pinl protein include introducing into a subject a labeled anti-Pinl antibody. For example, the antibody can be labeled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

In another embodiment, the biological sample contains protein molecules from the test subject. Alternatively, the biological sample can contain mRNA molecules from the test subject or genomic DNA molecules from the test subject. A preferred biological sample is a serum sample isolated by conventional means from a subject.

In another embodiment, the methods further involve obtaining a control biological sample from a control subject, contacting the control sample with a compound or agent capable of detecting Pinl marker such that the presence of Pinl marker is detected in the biological sample, and comparing the presence of Pinl marker in the control sample with the presence of Pinl marker in the test sample.

The immunological assay test samples of the present invention may include cells, protein or membrane extracts of cells, blood or biological fluids such as ascites fluid or cerebrospinal fluid. The test sample used in the above-described method is based on the assay format, nature of the detection method and the tissues, cells or extracts used as the sample to be assayed. Methods for preparing protein extracts or membrane extracts of cells are well known in the art and can be readily adapted in order to obtain a sample which is compatible with the system utilized. The invention also encompasses kits for detecting the presence of Pinl in a biological sample. For example, the kit can comprise a labeled compound or agent capable of detecting Pinl protein or mRNA in a biological sample; means for determining the amount of Pinl in the sample; and means for comparing the amount of Pinl in the sample with a known
standard. The compound or agent can be packaged in a suitable container. The kit can further comprise instructions for using the kit to detect Pinl protein or nucleic acid.

Pinl marker levels can also be measured in an assay designed to evaluate a panel of target genes, e.g., a microarray or multiplex sequencing reaction. In the embodiments of the invention described herein, well known biochemical methods such as northern blot analysis, RNase protection assays, southern blot analysis, western blot analysis, in situ hybridization, immunocytochemical procedures of tissue sections or cellular spreads, and nucleic acid amplification reactions (e.g., polymerase chain reactions) may be used interchangeably. One of skill in the art would be capable of performing these well-established protocols for the methods of the invention. (See, for example, Ausubel, et al., "Current Protocols in Molecular Biology," John Wiley & Sons, NY, N.Y. (1999)).

Diagnostic assays can be carried out in, e.g., subjects diagnosed or at risk of an immune disorder. Such disorders include, without limitation, acne vulgaris; acute respiratory distress syndrome; Addison's disease; adrenocortical insufficiency; adrenogenital syndrome; allergic conjunctivitis; allergic rhinitis; allergic intraocular inflammatory diseases, ANCA-associated small-vessel vasculitis; angioedema; ankylosing spondylitis; aphthous stomatitis; arthritis, asthma; atherosclerosis; atopic dermatitis; autoimmune disease; autoimmune hemolytic anemia; autoimmune hepatitis; Behcet's disease; Bell's palsy; berylliosis; bronchial asthma; bullous herpetiformis dermatitis; bullous pemphigoid; carditis; celiac disease; cerebral ischaemia; chronic obstructive pulmonary disease; cirrhosis; Cogan's syndrome; contact dermatitis; COPD; Crohn's disease; Cushing's syndrome; dermatomyositis; diabetes mellitus; discoid lupus erythematosus; eosinophilic fasciitis; epicondylitis; erythema nodosum; exfoliative dermatitis; fibromyalgia; focal glomerulosclerosis; giant cell arteritis; gout; gouty arthritis; graft-versus-host disease; hand eczema; Henoch-Schonlein purpura; herpes gestationis; hirsutism; hypersensitivity drug reactions; idiopathic cerato-scleritis; idiopathic pulmonary fibrosis; idiopathic thrombocytopenic purpura; inflammatory bowel or gastrointestinal disorders, inflammatory dermatoses; juvenile rheumatoid arthritis; laryngeal edema; lichen planus; Loeffler's syndrome; lupus nephritis; lupus vulgaris; lymphomatous tracheobronchitis; macular edema; multiple sclerosis; musculoskeletal and connective tissue disorder; myasthenia gravis; myositis; obstructive pulmonary disease; ocular inflammation; organ transplant rejection; osteoarthritis; pancreatitis; pemphigoid gestationis; pemphigus vulgaris; polyarteritis nodosa; polymyalgia rheumatica; primary adrenocortical insufficiency; primary biliary cirrhosis; pruritus scroti; pruritus/inflammation, psoriasis; psoriatic arthritis; Reiter's disease; relapsing polychondritis; rheumatic carditis; rheumatic fever; rheumatoid arthritis; rosacea caused by sarcoidosis; rosacea caused by scleroderma; rosacea caused by Sweet's syndrome; rosacea caused by systemic lupus erythematosus; rosacea caused by urticaria; rosacea caused by zoster-associated pain; sarcoidosis; scleroderma; segmental glomerulosclerosis; septic shock syndrome; serum sickness; shoulder tendinitis or bursitis; Sjogren's syndrome; Still's disease; stroke-induced brain cell death; Sweet's disease; systemic dermatomyositis; systemic lupus erythematosus; systemic sclerosis; Takayasu's arteritis; temporal arteritis; thyroiditis; toxic epidermal necrolysis; tuberculosis; type-1 diabetes; ulcerative colitis; uveitis;
vasculitis; and Wegener's granulomatosis. The invention also features the treatment of immune disorders that increase susceptibility to microbial or viral infection, including HIV.

**Prognostic Assays**

The diagnostic methods described herein can furthermore be utilized to identify subjects having or at risk of developing a disease or disorder associated with aberrant Pinl expression or activity. For example, the assays described herein, such as the preceding diagnostic assays or the following assays, can be utilized to identify a subject having or at risk of developing a disorder associated with Pinl marker (e.g., an immune disorder). Thus, the present invention provides a method for identifying a disease or disorder associated with aberrant Pinl expression or activity in which a test sample is obtained from a subject and Pinl protein or nucleic acid (e.g., mRNA, genomic DNA) is detected, wherein the presence of Pinl protein or nucleic acid is diagnostic for a subject having or at risk of developing a Pinl-associated disorder and are, therefore, susceptible to treatment with a retinoic acid compound.

Furthermore, the present invention provides methods for determining whether a subject can be effectively treated with a retinoic acid compound for a disorder associated with aberrant Pinl expression or activity in which a test sample is obtained and Pinl protein or nucleic acid expression or activity is detected (e.g., wherein the abundance of Pinl protein or nucleic acid expression or activity is diagnostic for a subject that can be administered the agent to treat a disorder Pinl-associated disorder).

In one embodiment, the present invention provides methods for determining Pinl post-translational modifications. More importantly, phosphorylation of Pinl on Ser71 in the catalytic active site also prevents retinoic acid compounds from binding to Pinl active site and induce Pinl degradation and to inhibit Pinl function. Therefore, by detecting reduced Ser71 phosphorylation using phospho-specific Pinl antibodies that we have generated can be a method to select patients for RA treatments and to explain some patients may not respond to RA.

The methods of the invention can also be used to detect genetic alterations in a Pinl gene, thereby determining if a subject with the altered gene is at risk for a disorder associated with the Pinl gene and, consequently, a candidate for retinoic acid therapy. In preferred embodiments, the methods include detecting, in a sample of cells from the subject, the presence or absence of a genetic alteration characterized by at least one of an alteration affecting the integrity of a gene encoding a Pinl-protein, or the mis-expression of the Pinl gene. For example, such genetic alterations can be detected by ascertaining the existence of at least one of 1) a deletion of one or more nucleotides from a Pinl gene; 2) an addition of one or more nucleotides to a Pinl gene; 3) a substitution of one or more nucleotides of a Pinl gene, 4) a chromosomal rearrangement of a Pinl gene; 5) an alteration in the level of a messenger RNA transcript of a Pinl gene, 6) aberrant modification of a Pinl gene, such as of the methylation pattern of the genomic DNA, 7) the presence of a non-wild type splicing pattern of a messenger RNA transcript of a Pinl gene, 8) a non-wild type level of a Pinl-protein, 9) allelic loss of a Pinl gene, and 10) inappropriate post-translational modification of a Pinl-protein. As described herein, there are a large
number of assay techniques known in the art which can be used for detecting alterations in a Pinl gene. A preferred biological sample is a tissue or serum sample isolated by conventional means from a subject.

In certain embodiments, detection of the alteration involves the use of a probe/primer in a polymerase chain reaction (PCR) (see, e.g., U.S. Pat. Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, e.g., Landegran et al. (1988) Science 241:1077-1080; and Nakazawa et al. (1994) Proc. Natl. Acad. Sci. USA 91:360-364), the latter of which can be particularly useful for detecting point mutations in the Pinl-gene (see Abravaya et al. (1995) Nucleic Acids Res 0.23:675-682). This method can include the steps of collecting a sample from a patient, isolating nucleic acid (e.g., genomic, mRNA or both) from the sample, contacting the nucleic acid sample with one or more primers which specifically hybridize to a Pinl gene under conditions such that hybridization and amplification of the Pinl-gene (if present) occurs, and detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. It is anticipated that PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.


In an alternative embodiment, mutations in a Pinl gene from a sample cell can be identified by alterations in restriction enzyme cleavage patterns. For example, sample and control DNA is isolated, amplified (optionally), digested with one or more restriction endonucleases, and fragment length sizes are determined by gel electrophoresis and compared. Differences in fragment length sizes between sample and control DNA indicates mutations in the sample DNA. Moreover, the use of sequence specific ribozymes (see, for example, U.S. Pat. No. 5,498,531; hereby incorporated by reference) can be used to score for the presence of specific mutations by development or loss of a ribozyme cleavage site.

In other embodiments, genetic mutations in Pinl can be identified by hybridizing a sample and control nucleic acids, e.g., DNA or RNA, to high density arrays containing hundreds or thousands of oligonucleotides probes (Cronin, M. T. et al. (1996) Human Mutation 7: 244-255; Kozal, M. J. et al. (1996) Nature Medicine 2: 753-759). For example, genetic mutations in Pinl can be identified in two dimensional arrays containing light-generated DNA probes as described in Cronin, M. T. et al. supra. Briefly, a first hybridization array of probes can be used to scan through long stretches of DNA in a sample and control to identify base changes between the sequences by making linear arrays of sequential overlapping probes. This step allows the identification of point mutations. This step is followed by a
second hybridization array that allows the characterization of specific mutations by using smaller, specialized probe arrays complementary to all variants or mutations detected. Each mutation array is composed of parallel probe sets, one complementary to the wild-type gene and the other complementary to the mutant gene.

In yet another embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence the Pinl gene and detect mutations by comparing the sequence of the sample Pinl with the corresponding wild-type (control) sequence. Examples of sequencing reactions include those based on techniques developed by Maxam and Gilbert ((1977) Proc. Natl. Acad. Sci. USA 74:560) or Sanger ((1977) Proc. Natl. Acad. Sci. USA 74:5463). It is also contemplated that any of a variety of automated sequencing procedures can be utilized when performing the diagnostic assays ((1995) Biotechniques 19:448), including sequencing by mass spectrometry (see, e.g., PCT International Publication No. WO 94/16101; Cohen et al. (1996) Adv. Chromatogr. 36:127-162; and Griffin et al. (1993) Appl. Biochem. Biotechnol. 38:147-159).

Other methods for detecting mutations in the Pinl gene include methods in which protection from cleavage agents is used to detect mismatched bases in RNA/RNA or RNA/DNA heteroduplexes (Myers et al. (1985) Science 230:1242). In general, the art technique of "mismatch cleavage" starts by providing heteroduplexes formed by hybridizing (labeled) RNA or DNA containing the wild-type Pinl sequence with potentially mutant RNA or DNA obtained from a tissue sample. The double-stranded duplexes are treated with an agent which cleaves single-stranded regions of the duplex such as which will exist due to basepair mismatches between the control and sample strands. For instance, RNA/DNA duplexes can be treated with RNase and DNA/DNA hybrids treated with SI nuclease to enzymatically digesting the mismatched regions. In other embodiments, either DNA/DNA or RNA/DNA duplexes can be treated with hydroxylamine or osmium tetroxide and with piperidine in order to digest mismatched regions. After digestion of the mismatched regions, the resulting material is then separated by size on denaturing polyacrylamide gels to determine the site of mutation. See, for example, Cotton et al. (1988) Proc. Nat Acad Sci USA 85:4397; Saleeba et al. (1992) Methods Enzymol. 217:286-295. In a preferred embodiment, the control DNA or RNA can be labeled for detection.

In still another embodiment, the mismatch cleavage reaction employs one or more proteins that recognize mismatched base pairs in double-stranded DNA (so called "DNA mismatch repair" enzymes) in defined systems for detecting and mapping point mutations in Pinl cDNAs obtained from samples of cells. For example, the mutY enzyme of E. coli cleaves A at G/A mismatches and the thymidine DNA glycosylase from HeLa cells cleaves T at G/T mismatches (Hsu et al. (1994) Carcinogenesis 15:1657-1662). According to an exemplary embodiment, a probe based on a Pinl sequence, e.g., a wild-type Pinl sequence, is hybridized to a cDNA or other DNA product from a test cell(s). The duplex is treated with a DNA mismatch repair enzyme, and the cleavage products, if any, can be detected from electrophoresis protocols or the like. See, for example, U.S. Pat. No. 5,459,039; hereby incorporated by reference.
In other embodiments, alterations in electrophoretic mobility will be used to identify mutations in Pinl genes. For example, single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita et al. (1989) Proc Natl. Acad. Sci. USA: 86:2766, see also Cotton (1993) Mutat Res 285:125-144; and Hayashi (1992) Genet Anal Tech Appl 9:73-79). Single-stranded DNA fragments of sample and control Pinl nucleic acids will be denatured and allowed to renature. The secondary structure of single-stranded nucleic acids varies according to sequence, the resulting alteration in electrophoretic mobility enables the detection of even a single base change. The DNA fragments may be labeled or detected with labeled probes. The sensitivity of the assay may be enhanced by using RNA (rather than DNA), in which the secondary structure is more sensitive to a change in sequence. In a preferred embodiment, the subject method utilizes heteroduplex analysis to separate double stranded heteroduplex molecules on the basis of changes in electrophoretic mobility (Keen et al. (1991) Trends Genet. 7:5).

In yet another embodiment the movement of mutant or wild-type fragments in polyacrylamide gels containing a gradient of denaturant is assayed using denaturing gradient gel electrophoresis (DGGE) (Myers et al. (1985) Nature 313:495). When DGGE is used as the method of analysis, DNA will be modified to insure that it does not completely denature, for example by adding a GC clamp of approximately 40 bp of high-melting GC-rich DNA by PCR. In a further embodiment, a temperature gradient is used in place of a denaturing gradient to identify differences in the mobility of control and sample DNA (Rosenbaum and Reissner (1987) Biophys Chem 265:12753).

Examples of other techniques for detecting point mutations include, but are not limited to, selective oligonucleotide hybridization, selective amplification, or selective primer extension. For example, oligonucleotide primers may be prepared in which the known mutation is placed centrally and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki et al. (1986) Nature 324:163); Saiki et al. (1989) Proc. Natl Acad. Sci USA 86:6230). Such allele specific oligonucleotides are hybridized to PCR amplified target DNA or a number of different mutations when the oligonucleotides are attached to the hybridizing membrane and hybridized with labeled target DNA.

Alternatively, allele specific amplification technology which depends on selective PCR amplification may be used in conjunction with the instant invention. Oligonucleotides used as primers for specific amplification may carry the mutation of interest in the center of the molecule (so that amplification depends on differential hybridization) (Gibbs et al. (1989) Nucleic Acids Res. 17:2437-2448) or at the extreme 3′ end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prossner et al. (1993) Tibtech 11:238). In addition it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based detection (Gasparini et al. (1992) Mol. Cell Probes 6:1). It is anticipated that in certain embodiments amplification may also be performed using Taq ligase for amplification (Barany (1991) Proc. Natl. Acad. Sci USA 88:189). In such cases, ligation will occur only if there is a perfect match at the 3′ end of the 5′ sequence
making it possible to detect the presence of a known mutation at a specific site by looking for the presence or absence of amplification.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one probe nucleic acid or antibody reagent described herein, which may be conveniently used, e.g., in clinical settings to diagnose patients exhibiting symptoms or family history of a disease or illness involving a Pin1 gene.

Furthermore, any cell type or tissue in which Pin1 is expressed may be utilized in the prognostic assays described herein.

As with the diagnostic assay described above, prognostic assays of Pin1 activity can be included as part of a panel of target genes.


Monitoring the effects of retinoic acid treatment

In one embodiment, the present invention features a method for monitoring the effectiveness of treatment of a subject with a retinoic acid compound comprising the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the compound; (ii) detecting the level of expression or activity of a Pin1 protein, Pin1 phosphorylation on Ser71, mRNA, or genomic DNA in the pre-administration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the Pin1 protein, mRNA, or genomic DNA in the post-administration samples; (v) comparing the level of expression or activity of the Pin1 protein, mRNA, or genomic DNA in the pre-administration sample with the Pin1 protein, mRNA, or genomic DNA in the post administration sample or samples; and (vi) altering the administration of the retinoic acid compound to the subject accordingly. According to such an embodiment, Pin1 expression, phosphorylation or activity may be used as an indicator of the effectiveness of the retinoic acid compound, even in the absence of an observable response.

III. Methods of Treatment

The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) or afflicted with an immune disorder (e.g., a disorder associated with increased Pin1 expression or activity) with a retinoic acid compound.

Prophylactic Methods

In one aspect, the invention provides a method for preventing an immune disorder in a subject by administering to the subject a retinoic acid compound. Subjects at risk for a disease which is caused or
contributed to by aberrant Pinl expression or activity can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a retinoic acid compound can occur prior to the manifestation of symptoms characteristic of the Pinl aberrancy, such that a disease or disorder is prevented and/or its progression delayed.

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**Combination Therapies**

Anti-inflammatory agents are useful for treating immune disorders in combination with the retinoic acid compounds of the invention. Anti-inflammatory agents that can be used in combination with a retinoic acid compound include, without limitation, corticosteroids, NSAIDs (e.g., naproxen sodium, diclofenac sodium, diclofenac potassium, aspirin, sulindac, diflunisal, piroxicam, indomethacin, ibuprofen, nabumetone, choline magnesium trisalicylate, sodium salicylate, salicylsalicylic acid (salsalate), fenoprofen, flurbiprofen, ketoprofen, meclofenamate sodium, meloxicam, oxaprozin, sulindac, and tolmetin), COX-2 inhibitors (e.g., rofecoxib, celecoxib, valdecoxib, and lumiracoxib), biologics (e.g., inflixamab, adalimumab, etanercept, CDP-870, rituximab, and atilizumab), small molecule immunomodulators (e.g., VX 702, SCIO 469, doramapimod, RO 30201 195, SCIO 323, DPC 333, pranalcasan, mycophenolate, and merimepodib), non-steroidal immunophilin-dependent immunosuppressants (e.g., cyclosporine, tacrolimus, pimecrolimus, and ISAtx247), 5-amino salicylic acid (e.g., mesalamine, sulfasalazine, balsalazide disodium, and olsalazine sodium), DMARDs (e.g., methotrexate, leflunomide, minocycline, auranofin, gold sodium thiomalate, aurothioglucose, and azathioprine), hydroxychloroquine sulfate, and penicillamine.

In cases where there is a viral or microbial infection, the retinoic acid compounds of the invention can be administered with an antimicrobial agent, e.g., the penicillins (e.g., penicillin G, ampicillin, methicillin, oxacillin, and amoxicillin), the cephalosporins (e.g., cefadroxil, ceforanid, cefotaxime, and ceftriaxone), the tetracyclines (e.g., doxycycline, minocycline, and tetracycline), the aminoglycosides (e.g., amikacin, gentamycin, kanamycin, neomycin, streptomycin, and tobramycin), the macrolides (e.g., azithromycin, clarithromycin, and erythromycin), the fluoroquinolones (e.g., ciprofloxacin, lomefloxacin, and norfloxacin), and other antibiotics including chloramphenicol, clindamycin, cycloserine, isoniazid, rifampin, and vancomycin. Particularly useful formulations contain aminoglycosides, including for example amikacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, streptomycin, and tobramycin, or an antiviral agent, e.g. 1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, 9->2-hydroxyethoxy methylguanine, adamanantanamine, 5-ido-2'-deoxyuridine, trifluorothy midine, interferon, adenine arabinoside, protease inhibitors, thymidine kinase inhibitors, sugar or glycoprotein synthesis inhibitors, structural protein synthesis inhibitors, attachment and adsorption inhibitors, and nucleoside analogues such as acyclovir, penciclovir, valacyclovir, and ganciclovir.

Such compounds can act synergistically with a retinoic acid compound. Additionally, co-administration with a retinoic acid compound may result in the efficacy of the anti-inflammatory
compound at lower (and thus safer) doses (e.g., at least 5% less (e.g., at least 10%, 20%, 50%, 80%, 90%, or even 95%) less than when the anti-inflammatory compound is administered alone.

Therapy according to the invention may be performed alone or in conjunction with another therapy and may be provided at home, the doctor's office, a clinic, a hospital's outpatient department, or a hospital. Treatment optionally begins at a hospital so that the doctor can observe the therapy's effects closely and make any adjustments that are needed, or it may begin on an outpatient basis. The duration of the therapy depends on the type of disease or disorder being treated, the age and condition of the patient, the stage and type of the patient's disease, and how the patient responds to the treatment. Additionally, a person having a greater risk of developing an immune disease may receive treatment to inhibit or delay the onset of symptoms.

Routes of administration for the various embodiments include, but are not limited to, topical, transdermal, nasal, and systemic administration (such as, intravenous, intramuscular, subcutaneous, inhalation, rectal, buccal, vaginal, intraperitoneal, intraarticular, ophthalmic, otic, or oral administration). As used herein, "systemic administration" refers to all nondermal routes of administration, and specifically excludes topical and transdermal routes of administration.

In combination therapy (e.g., a retinoic acid compound with a second therapeutic agent), the dosage and frequency of administration of each component of the combination can be controlled independently. For example, one compound may be administered three times per day, while the second compound may be administered once per day. Combination therapy may be given in on-and-off cycles that include rest periods so that the patient's body has a chance to recover from any as yet unforeseen side effects. The compounds may also be formulated together such that one administration delivers both compounds.

Each compound of the combination may be formulated in a variety of ways that are known in the art. For example, the first and second agents may be formulated together or separately. Desirably, the first and second agents are formulated together for the simultaneous or near simultaneous administration of the agents. Such co-formulated compositions can include the two drugs together in the same pill, ointment, cream, foam, capsule, liquid, etc. It is to be understood that, when referring to the formulation of combinations of the invention, the formulation technology employed is also useful for the formulation of the individual agents of the combination, as well as other combinations of the invention. By using different formulation strategies for different agents, the pharmacokinetic profiles for each agent can be suitably matched.

The individually or separately formulated agents can be packaged together as a kit. Non-limiting examples include kits that contain, e.g., two pills, a pill and a powder, a suppository and a liquid in a vial, two topical creams, ointments, foams etc. The kit can include optional components that aid in the administration of the unit dose to patients, such as vials for reconstituting powder forms, syringes for injection, customized IV delivery systems, inhalers, etc. Additionally, the unit dose kit can contain instructions for preparation and administration of the compositions. The kit may be manufactured as a
single use unit dose for one patient, multiple uses for a particular patient (at a constant dose or in which the individual compounds may vary in potency as therapy progresses); or the kit may contain multiple doses suitable for administration to multiple patients ("bulk packaging"). The kit components may be assembled in cartons, blister packs, bottles, tubes, and the like.

IV. Experimental Results

Pinl is activated and specifically required for type I IFN secretion following TLR ligation.

To examine the role of Pinl in TLR signaling, we first compared cytokine production in response to various TLR ligands using DC subsets derived from Pinl wild-type (WT, +/+) and Pinl knockout (KO, -/-) mice. When stimulated with LPS (TLR4 ligand), Pam3 CSK4 (TLR2), R848 (TLR7) or CpG DNA (TLR9), Pinl KO myeloid DCs (mDCs) produced moderately less proinflammatory cytokines than Pinl WT controls (Fig. a-c). Consistently, reduced proinflammatory cytokine secretion was also detected following stimulation of Pinl KO macrophages with each of the TLR ligands (Fig. 10a, b). Stimulation of splenic plasmacytoid DCs (pDC) or Flt3 ligand-induced bone marrow pDCs with purified TLR7 or TLR9 ligand or with the influenza A virus (H1N1) (TLR7) or MCMV (TLR9) showed robust IFN-a secretion in Pinl WT cells (Fig. 1d-g), as shown. However, Pinl KO cells almost completely failed to produce IFN-a or IFN-β, as assayed by ELISA (Fig. 1d-g) and qRT-PCR analyses (Fig. 1h). These effects of Pinl deficiency on IFN-a production were highly specific because Pinl KO neither affected the population of immune cells nor their TLR expression (Fig. 11). Moreover, Pinl enzymatic activity, although not its protein level, was significantly elevated in R848- or CpG-stimulated human peripheral blood mononuclear cells (Fig. 1i), which is consistent with the findings that Pinl is kept inactivated until cellular cues are engaged. Thus, Pinl plays a moderate role in proinflammatory cytokine production in mDCs in response to various TLR ligands, but unexpectedly, is essential and specific for the type I IFN response in pDCs following TLR 7/9 ligation.

Proteomic approach identifies IRAKI as a major Pinl substrate upon TLR stimulation. To elucidate the molecular mechanism underlying the impact of Pinl on type I IFN secretion, we used a proteomic approach to identify Pinl substrates using a GST-Pinl affinity purification procedure under high-salt and -detergent conditions, a procedure that has been used to identify almost all known Pinl substrates. We used R848-stimulated human THPI cells, a monocytic cell line that has a functional IFN response to TLR 7/9 ligands and can be cultured in sufficient volumes. Following SDS-PAGE and mass spectrometry, one prominent and reproducible Pinl-binding protein at 100 kDa was identified to be IRAKI (Fig. 2a and Fig. 12a). Notably, the Pinl KO phenotypes shown in Fig. 1 are strikingly similar to those observed in IRAKI KO cells and mice. Furthermore, similar to IRAKI KO, no obvious effect of Pinl deficiency on IL-6 and IL-12p40 levels could be detected following pDCs stimulated with R848 or CpG (Fig. 10c, d). Moreover, no difference was observed for IFN-β section from Pinl WT and KO mDCs stimulated with CpG (Fig. 10e), which is consistent with the previous results that CpG stimulation of mDCs induces IFN-β production in a MyD88- and IKKα-dependent but IRAKI-independent manner.
These results prompted us to examine the role of Pinl in regulating IRAKI function in TLR signaling. We confirmed the TLR7/9-dependent interaction between IRAKI and Pinl in THPI monocytes and Raw264.7 macrophages by GST-Pinl binding assay (Fig. 2b) or co-immunoprecipitation (Co-IP) (Fig. 2c). Pinl predominantly bound to the activated form of IRAKI, which displayed a characteristic mobility shift on SDS gels after TLR ligation (Fig. 2b, c, arrows), suggesting that Pinl might bind specifically to phosphorylated IRAKI. Indeed, this binding was mediated by the Pinl WW domain (Fig. 12b, d), a known pSer/Thr-Pro-binding module, but was abolished either by IRAKI dephosphorylation prior to Pinl binding assay (Fig. 2d, 12c) or mutating a key functional residue in the WW domain (Fig. 12b, e). In addition, Pinl did not bind to the related kinases IRAK2 and IRAK4 following TLR7 and TLR9 stimulation (Fig. 12f). Thus, following TLR 7/9 ligation, Pinl is activated and IRAKI is phosphorylated, which allows Pinl to interact specifically with IRAKI.

The binding of Pinl to IRAKI was somewhat surprising because Pinl interacts only with specific pSer/Thr-Pro motifs and there is little known about Pro-directed phosphorylation of IRAKI in TLR signaling. Consequently, we decided to define the Pinl binding region and site(s) in IRAKI. Structurally, IRAKI consists of an N-terminal death domain, a ProST-rich undetermined domain (UD) and a central kinase domain, with a C-terminal tail (Fig. 13a). To avoid interference of endogenous IRAKI, we expressed FLAG-IRAK1 or its mutants in IRAKI null (II A) 293 cells, followed by Pinl binding assay. Under overexpression conditions, IRAKI, but not its K239S kinase-dead (KD) mutant, was auto-activated independently of TLR stimulation, as indicated by the characteristic mobility shift (Fig. 2d, f, 13b), as shown previously. Importantly, IRAKI, but not its KD mutant, interacted with Pinl (Fig. 2f, g, 13b). Furthermore, deletion of the UD, abolished Pinl binding (Fig. 13b). Thus, Pinl binds to kinase active IRAKI, possibly through autophosphorylation sites in the UD.

To directly examine this possibility in vivo, we co-expressed FLAG tagged KD-IRAK1 with or without WT-IRAK1 in IRAKI -deficient cells, followed by analyzing Pinl binding specifically to KD-IRAK1. As shown previously, KD-IRAK1 did not show the characteristic mobility shift and failed to interact with Pinl when it was expressed alone (Fig. 2e left). However, when co-expressed with WT-IRAK1, KD-IRAK1 showed the mobility shift and, importantly, also bound to Pinl (Fig. 2e right), suggesting that Pinl binds to autophosphorylated IRAKI. To confirm that Pinl directly binds to IRAKI, we performed Far-Western blotting analysis using WT and KD IRAKI and GST-Pinl WW domain.

Indeed, Pinl bound only to the slower mobility shifted and presumably activated form of WT IRAKI, but there was no binding between Pinl and KD IRAKI (Fig. 2f). Finally, to confirm the binding of Pinl to the active form of IRAKI, we performed Pinl binding assay using mouse embryonic fibroblasts (MEFs) stably infected with WT and KD IRAKI in the presence or absence of TLR7 activation. Pinl bound to the active form of WT IRAKI, but not KD IRAKI confirming that Pinl predominately binds to activated IRAKI (Fig. 2g). Taken together, these results indicate that upon TLR ligation, IRAKI is activated by receptor recruitment and autophosphorylates on the pSer-Pro motifs, which in turn recruits Pinl to act on IRAKI.
To identify the IRAKI phosphorylation site(s) responsible for Pinl binding, we mutated each of the six possible Pinl binding pSer/Thr-Pro motifs in the UD of IRAKI to Ala, and assessed their binding to Pinl from retrovirally infected cells. Although the mutation of S110, S163 or S196 had little effect on Pinl binding, the mutation of S131, S144 or S173 alone to Ala considerably reduced IRAKI activation and Pinl binding, which was further reduced when all three sites were mutated together (Fig. 2h), indicating that phosphorylation of these sites participate in regulating IRAKI activation and Pinl binding. To confirm the phosphorylation status of these three sites, we used a two-step purification procedure to isolate IRAKI using sequential FLAG immunoprecipitation and GST-Pinl pulldown, followed by LC-MS analysis. Both S131 and S144 were indeed phosphorylated (Fig 14a, b). However, both trypsin and chymotrypsin digestions repeatedly failed to produce any peptides covering the region surrounding S173 (Fig 14e), possibly due to the numerous proline and hydrophobic residues in this region. This prompted us to generate phospho-specific antibodies against phosphorylated S173 of IRAKI. The resulting anti-pS173 antibodies specifically recognized activated WT IRAKI, but not its S173A point mutant, even when it was highly overexpressed and activated using transient transfection, confirming that S173 in the UD of IRAKI is indeed phosphorylated (Fig. 15a). Moreover, S173 phosphorylation of IRAKI was significantly induced in R848- or CpG-stimulated human peripheral blood mononuclear cells, as determined by flow cytometric (Fig. 2i) and Western blot (Fig. 15b) analysis using pS173-specific IRAKI antibodies. Taken together, these results indicate that upon TLR activation, S131/144/173-Pro motifs in the UD of IRAKI are not only phosphorylated in cells, but also are largely responsible for Pinl binding.

Pinl binds and isomerizes each of the phosphorylated-S13/S144/S173-Pro motifs in the UD of IRAKI. The Pinl WW domain and PPIase domains have been shown to bind and isomerize specific pSer/Thr-Pro motifs in its substrates, respectively. To measure the Pinl interaction with each of the implicated pSer-Pro motifs in IRAKI, we employed two-dimensional (2D) NMR spectroscopy to monitor the changes in the $^{15}$N-WW domain induced by titration with phosphopeptide ligands. In a 2d $^{15}$N-$^1$H HSQC spectrum of a protein (Fig. 3a), each backbone NH group is represented by a peak, whose position reflects the chemical environment of that NH bond. Ligand binding to the protein is detected by changes in peak positions (fast exchange) or by the appearance of new peaks (slow exchange) as ligand is added. WW binding at each distinct IRAKI site was measured using phosphopeptides containing residues 126-136 (pSer131-P132), 140-150 (pSer131-P132), and 157-180 (pSer173-P174) of IRAKI. The WW domain bound to each phosphopeptide and exhibited fast exchange kinetics, as demonstrated by changes in peak position, in each of the three titration experiments (Fig. 3a). Quantitative analysis of the change in chemical shift as a function of peptide concentration (Fig. 3b) yielded dissociation constants ($K_d$) of 220 ± 15 μM, 120 ± 12 μM, and 260 ± 75 μM for the 126-136 (pSer131-P132), 140-150 (pSer131-P132), and 157-180 (pSer173-P174) phosphopeptides, respectively. The IRAKI-Pinl interaction in the cell occurs as part of a multi-protein membrane-associated complex, suggesting the potential for significant binding enhancement due to avidity.
In order to determine whether Pinl catalysis occurs at each of these sites, the homonuclear 2D ROESY NMR experiment was used as we have previously reported. In the presence of a catalytic amount of Pinl, exchange crosspeaks between the cis and trans isomers of the pSer-Pro peptide bond were clearly observed for each peptide (Fig. 3c, top panels).

Conversely, in the absence of Pinl, exchange crosspeaks were missing (Fig. 3c, bottom panels). These results demonstrate that Pinl accelerates the cis-trans isomerization at each pSer-Pro motif, thereby confirming these sites as Pinl substrates.

**Pinl is essential for IRAKI activation upon TLR ligation.** Given that Pinl binds to and isomerizes multiple pSer-Pro motifs in IRAKI upon TLR activation, a key question is whether Pinl regulates IRAKI function in TLR signaling. Therefore, we examined the effects of Pinl KO on IRAKI activation in response to activation of various TLRs using Pinl WT and KO MEFs and pDCs. Although TLR7/9 ligation activated IRAKI in a time dependent fashion in both Pinl WT cells (Fig. 4a), as indicated by the mobility shift and increased kinase activity (Fig 4b), as previously described, there was no evidence for IRAKI activation in either assay in Pinl KO MEFs or pDCs (Fig. 4a, b) or in Pinl-silenced THPI cells using RNAi (Fig. 4c). Moreover, Pinl KO also completely abolished IRAKI activation in response to ligation of other TLRs including TLR2 and TLR4 (Fig. 16a, b). These effects were highly specific because Pinl KO did not affect activation of the IRAKI upstream kinase IRAK4 (Fig. 4b), or MAP kinases including ERKs, JNKs and p38 MAPKs upon TLR activation (Fig. 17).

Similar observations were made following LPS stimulation of macrophages (Fig. 18a). We also assessed the effects of Pinl deficiency on 1KB degradation following pDC stimulation with R848 and CpG or treatment of macrophages with LPS and did not see any obvious difference between Pinl WT and KO cells (Fig. 18b, c). To further confirm this effect of Pinl on IRAKI activation, we developed an assay to measure the kinase activity of IRAKI in cells utilizing the fact that IRAKI can phosphorylate the N-terminal 220 aa IRAKI fragment containing the UO in trans, as shown by the characteristic mobility shift after co-expression with WT IRAKI (Fig. 4d), as shown previously. As expected, exogenously expressed IRAKI in Pinl WT MEFs efficiently phosphorylated the IRAKI N-terminal fragment, inducing the characteristic mobility shift (Fig 4D). However, like KD IRAKI, WT IRAKI in Pin KO MEFs completely failed to induce any mobility shift of the N-terminal IRAKI (Fig. 4d). These results together indicate that Pinl is required for IRAKI activation.

To further demonstrate the importance of Pinl for the time dependent activation of IRAKI following TLR ligation, we overexpressed WT IRAKI and KD IRAKI in Pinl WT and KO MEFs using a retroviral expression system. Under overexpression conditions, WT-IRAK1 was partially activated, which was further activated upon TLR7 ligation in Pinl WT cells, as shown by the characteristic mobility shift (Fig. 4e), consistent with the findings that IRAKI activation is sensitive to IRAK protein levels. However, no IRAKI activation was observed in Pinl KO cells, even after stimulation (Fig. 4e), further confirming the role of Pin 1 in IRAKI activation. Importantly, KD IRAKI did not show any evidence of
activation following TLR ligation both in Pinl WT and KO cells (Fig. 4e). These results indicate that IRAKI fails to be activated in Pinl KO cells. To confirm that defective IRAKI activation in Pinl-null cells is specifically due to loss of Pinl and to examine the importance of Pinl binding and isomerase activities for IRAKI activation, we performed rescue experiments by re-expressing WT Pinl or its point mutants, W34A mutant (in the WW domain) or K63A mutant (in the catalytic domain), which fail to bind to or isomerase Pinl substrates, respectively. Re-expression of Pinl, but neither of its WW domain (W34A) nor catalytic domain (K63A) point mutants completely restored IRAKI activation in Pinl KO cells expressing IRAKI (Fig. 4f), reminiscent of IRAKI activation found in Pinl WT cells (Fig. 4e). Taken together, these results demonstrate an essential role for Pinl in IRAKI activation in TLR signaling.

Pinl bound to IRAKI and NF-κB, and Pinl KO abolished IRAKI activation, Ca²⁺ flux and NF-κB activation by IL-33. GST-Pinl pulldown showed that Pinl bound only to activated IRAKI in monocytes after IL-33 stimulation (Fig. 20A), similar to TLR ligation. IL-33 induced IRAKI activation in Pinl WT, but not KO MEFs (Fig. 20B). IL-33 induced Ca²⁺ flux in eosinophils derived from Pinl WT, but not KO BM (Fig. 20C), as described. Pinl also bound only to activated p65 NF-κB via the Ser254-Pro motif after IL-33 stimulation (Fig. 20D, E), as we have shown after cytokine stimulation. Moreover, IL-33 induced NF-κB activation in Pinl WT, but not Pinl KO cells (Fig. 20F), consistent with the fact that Ca²⁺ flux activates NF-κB.

Preliminary NMR results. To demonstrate feasibility of Pinl rate measurements, a single 15N label was incorporated at Ala173 in UΔ5 γ-180 (Fig. 20G) to monitor the nearby pSi γ,P motif using NMR studies. Because uncatalyzed cis-trans isomerization is a generally slow process (time constant ~ minutes), the equilibrium between cis and trans isomers yields two distinct peaks for residues (such as Ala175) whose chemical environment differs in the two isomer states. To investigate Pinl catalysis of the pSi γ,P motif, the two-dimensional 15N-1H Nzz exchange spectroscopy of [15N-Ala175UD57γ-180] (1 mM) in the presence of Pinl (17 μM) was used, as we have describe, revealing cis and trans conformations of the pSi γ,P bond (peaks labeled cc and tc) whose exchange is catalyzed by Pinl (Fig. 20H, inset). Cross-peaks (labeled ct and tc) demonstrate Pinl-catalyzed exchange between cis and trans isomers. The dependence of peak intensities on Nzz mixing time yields the cis-trans exchange rate. An example fitting of the Nzz data for 15N-Ala175-UD57γ-180 demonstrates the high quality of the data and fit (Fig. 20H), yielding a Pinl-catalyzed isomerization rate for the pSi γ,P peptide bond of kcat = 27 s⁻¹. Based on peak intensities in the absence of Pinl, the pSi γ,P trans:cis ratio is 85:15. Additionally, to demonstrate the feasibility of NMR studies on the intact IRAKI-UD, the 15N-1H fHSSQC spectrum of recombinant 15N-labeled IRAKI-UD y1–222 (IRAKI residues 101-222) (Fig. 20I) showed limited peak dispersion, characteristic of a disordered sequence, and will allow detection of conformational changes in the IRAKI-UD. This spectrum displays
several minor peaks that are attributed to the cis isomers of the various X-Pro peptide bonds, allowing residue-specific detection of changes in populations of cis and trans states.

**Pinl is essential for IRAKI-dependent IRF7- and IFN-a-mediated antiviral response in vitro.** Given that Pinl was required for activation of IRAKI we wondered whether Pinl regulates IRAKI mediated downstream signaling. Following TLR activation, IRAKI is recruited to the receptor complex via MyD88/IRAK4, where it is activated and released from the receptor complex. This allows transcription factors such as IRF7, the master regulator of IFN-a, to translocate into the nucleus where it activates IFN-a transcription, making IRAKI activation a key step in the TLR7/9 signaling cascade. Therefore, we examined whether Pinl KO affects the ability of IRAKI to transduce TLR signals.

To address whether IRAKI is still recruited to the TLR receptor complex in Pinl KO cells, we transfected HA-MyD88 into both Pinl WT and KO cells retrovirally expressing FLAGIRAK1, followed by immunoprecipitation with anti-HA antibodies and then immunoblotting with anti-FLAG antibodies. As shown previously, the activated form of IRAKI in Pinl WT cells was not readily found in the MyD88 immune complexes (Fig. 5a). However, IRAKI in Pinl KO cells formed a stable interaction with HA-MyD88 (Fig. 5a), presumably due to the fact that IRAKI is not fully activated in these cells (Fig. 4a, 5a). Thus it appears that IRAKI in Pinl KO cells is unable to dissociate from the receptor complex due to its lack of autophosphorylation, presumably retaining IRAKI at the receptor complex.

Given that Pinl is required for IRAKI activation and dissociation from the receptor complex, we examined whether Pinl affects IRF7 activation using Pinl knockdown and knockout. Pinl knockdown in THP1 cells using Pinl-RNAi not only abolished the IRF7 and TRAF6 interaction, as shown by Co-IP experiments (Fig. 5b), but also blocked IRF7 nuclear translocation in response to TLR 7/9 activation, as determined by subcellular fractionation followed by immunoblotting analysis (Fig. 5c, d). To further confirm these results, we immunostained for IRF7 in primary Pinl WT and KO pDCs after TLR7/9 ligation. Upon TLR activation, IRF7 translated to the nucleus in Pinl WT, but not Pinl KO pDCs (Fig. 5e). These results suggest that Pinl activates IRAKI to cause IRF7 nuclear translocation in response to TLR 7/9 stimulation.

This suggestion was further supported by our findings from IRAKI-mediated IRF7 functional assays. Specifically, Pinl KO abolished IRF7 reporter activity following TLR7/9 stimulation (Fig. 6a, b), and these defects were fully rescued by Pinl, but not its binding-inactive- or isomerase-defective mutant, as measured by IRF7 reporter activity and IFN-a production (Fig. 6c, d). To further investigate the role of Pinl and IRAKI kinase activity in IRF7 activation, we co-expressed MyD88, a Gal4-IRF7 reporter construct and various amounts of KD IRAKI in Pinl WT and KO MEFs. IRF7 activation in WT cells decreased as the amount of transfected KD IRAKI was increased. In contrast, IRF7 activation was consistently lower in Pinl KO cells and unaffected by the amount of KD IRAKI transfected (Fig. 6e). These results demonstrate that both Pinl and IRAKI kinase activity are necessary for activation of IRF7. These findings are consistent with the previous findings 1) that IRAKI, but not its KD mutant,
phosphorylates IRF7, 2) that IRAKI kinase activity is necessary for the transcriptional activity of IRF7, but not NF-kB, 3) that KD IRAKI inhibits MyD88-induced IRF7 activation in a dominant-negative manner, and 4) that inhibition of IRAK kinase activity with a synthetic inhibitor prevents IRF7 phosphorylation, but not NF-κB phosphorylation in CpG stimulated pDCs.

Moreover, the IRAKI mutations that prevented Pinl binding in retrovirally infected MEFs including S131A, S144A and S173A alone or together also decreased IRF7 promoter activation and IFN-α secretion similar to kinase-inactivating IRAKI mutation or Pinl KO (Fig. 6f, g). To confirm the importance of Pinl in IRAKI- and IFN-a- mediated antiviral activity, we performed plaque formation assays using GFP-expressing vesicular stomatitis virus (VSV). Specifically, L929 cells were infected with GFP-VSV and incubated with supernatants from Pinl WT and KO MEFs expressing IRF7 and IRAKI or its mutants, followed by GFP-positive plaque quantification. While supernatants from Pinl WT MEFs expressing WT IRAKI had potent antiviral activity, those from Pinl WT MEF expressing Pinl binding IRAKI mutants or KD IRAKI had little activity, similar to Pinl KO MEFs (Fig. 6h, i), consistent with IRF7 activity and IFNα production in these cells (Fig. 6f, g). Thus, disrupting the IRAKI activation by inhibiting Pinl or by preventing IRAKI from acting as a Pinl substrate drastically abrogates IRF7 activation, subsequent IFN-a production and antiviral response in vitro.

Pinl is required for type I interferon-mediated innate and adaptive immunity in vivo.

Given the essential role for Pinl on IRAKI-dependent antiviral cellular responses in vitro, we next examined the effects of Pinl KO in vivo using Pinl WT and KO mice. Following injection with R848 or CpG, robust IFN-a production could be observed in Pinl WT mice (Fig. 7a, b), as shown. In contrast, serum IFN-a levels in Pinl KO littermates were significantly reduced (Fig. 7a, b). When injecting mice with LPS or R848, the serum levels of IL-6 and IL-12p40 were significantly lower in Pinl KO mice, compared to WT controls, albeit not as dramatically as IFN-a levels (Fig. 19a-c). As the MyD88-IRF7 pathway has been shown to be essential for IFN-a production during MCMV infection, we next examined the effects of Pinl KO on systemic MCMV infection. Whereas IFN-a levels in Pinl WT animals peaked after 36 hours following MCMV infection, IFN-a induction was entirely suppressed in Pinl KO mice (Fig. 7c). Moreover, Pinl KO mice were much more vulnerable to systemic MCMV infection than their WT littermates, resulting in increased weight loss (Fig. 7d) and morbidity (Fig. 7e). These phenotypes are similar to those observed in IRF7 or MyD88 KO mice and further highlight the contribution of Pinl to the antiviral immune response in vivo.

Co-stimulation of TLR9 and CD40 induces CD8+ T-cell expansion in a pDC, IRF7 and IFN-a dependent manner, thereby playing a major role in regulation of adaptive immune responses. To study the effects of Pinl deficiency on adaptive immunity, we next investigated the effects of Pinl KO on the induction of antigen-specific CD8+ T-cell responses. As reported, treatment with ovalbumin and anti-CD40 alone did not induce specific CD8+ T-cell expansion, whereas co-inoculation of CpG-A complexed to DOTAP, a CD40 agonistic antibody and ovalbumin induced a strong expansion of antigen-specific
CD8+ T-cells in Pinl WT mice (Fig. 7f, left). In contrast, the ovalbumin-specific CD8+ T-cell response was greatly impaired in Pinl-deficient mice (Fig. 7f, right). Taken together, these results demonstrate a crucial role for Pinl in mediating both adaptive and innate immunity.

Pinl regulates upstream and downstream targets in TLR/IL-1R signal pathways in multiple cells in asthma based on our following results. 1) TLR7/9 activates Pinl, which in turn is critical for activation of IRAKI in TLR7/9 signaling in vitro and in vivo. 2) IL-33 activated Pinl and IRAKI, similarly to TLR stimulation. 3) Pinl KO almost fully abrogated Th2 cytokine production induced by IL-33 in mice and in vitro. 4) Pinl KO effectively suppressed asthma-like pathologies in mice induced by IL-33. 5) Pinl KO suppressed cytokine production induced by HDM or LPS.

Pinl is activated by IL-33 and Pinl KO suppressed IL-33-induced Th2 cytokine production and asthma-like phenotypes in mice. We found that IL-33 stimulation activated Pinl catalytic activity in THP-1 monocytes (Fig. 28A) and BM-derived eosinophils (Fig. 35B), with the extent of the activation being similar to that found in eosinophils in asthmatic airways. Importantly, Pinl KO abolished the ability of IL-33 to induce IL-6 production in mouse embryonic fibroblasts (MEFs) (Fig. 28B), which have been shown to express ST2. Moreover, Pinl KO significantly inhibited Th2 cytokine secretion in BAL fluids, lung inflammation and mucus hypersecretion induced by IL-33 (Fig. 28C-F). This phenotype is similar to what is observed in animals treated with soluble ST2 or IL-33 neutralizing antibody to inhibit IL-33 signaling in mouse asthma models.

Pinl KO reduced Th2 cytokine production and asthma-like phenotypes in mice after OVA challenge. To examine the role of Pinl on allergic asthma, we examined the effects of Pinl KO on OVA-induced mouse model of allergic asthma, as described. We found that Pinl KO significantly inhibited Th2 cytokine secretion, lung inflammation and eosinophilia in BAL fluids after allergen challenge (Fig. 29), which is consistent with the previous findings showing the requirement of Pinl for pulmonary eosinophilia and bronchial remodeling after allergen challenge. Of note, the effects of Pinl KO in the OVA asthma model (Fig. 29) were not as comprehensive as the i.n. IL-33 model (Fig. 28), which might be due to possibilities that OVA might be a broader allergen model than IL-33, that KO of ST2 has been shown to have more obvious effects in a short-term priming model of asthma and/or that Pinl might affect other pathways.

Pinl KO inhibited proinflammatory cytokine production induced by HDM or LPS. We found that Pinl KO potently suppressed LPS or HDM-induced IL-6 production in MEFs (Fig. 30A, C) and BM-derived mast cells generated using rmIL-3 (Fig. 30D). Pinl KO also significantly decreased TLR-induced IL-6 secretion from BM derived macrophages and myeloid dendritic cells (mDCs). Finally, Pinl KO reduced serum proinflammatory cytokine levels in mice after LPS i.p. (Fig. 30B).
**Pinl is activated in patients with systemic lupus erythematosus (SLE).** We asked whether Pinl is activated in immune cells from patients with SLE and we performed Pinl immunoblot analysis of lysates of peripheral blood mononuclear cells (PBMC) isolated from six active SLE patients (SLE Disease activity score (SLEDAI) >6) and six normal controls using antibodies recognizing specifically S16 or pS16 in the Pinl WW domain. We found that Pinl in PBMC from all healthy individuals examined existed in two forms with different mobility on SDS gels (Fig. 21A). What has attracted our attention is that the slower mobility form was absent in lysates from six SLE patients (Fig. 21A), suggesting that Pinl might be hypophosphorylated in SLE. Importantly we have previously shown that phosphorylation of Pinl on S16 in the WW domain (Fig. 22A, B) and S71 in the PPIase domain (Fig. 22C-E) inhibits its ability to bind and isomerizes its substrates, respectively (Fig. 22). To distinguish these phosphorylated forms of Pinl, especially in cells and tissues, we have generated phospho-specific antibodies that recognize pS16 or pS71 form of Pinl (Fig. 23A). Moreover, our X-ray structure of PKA phosphorylated Pinl has clearly revealed why phosphorylation of S16 and S71 inhibits its function. S16 is located at the center of the pSer/Thr-binding pocket of the WW domain and pS16 phosphate of formed H-bonds with Ser18 and Ser19, preventing the WW domain from interacting with Pinl substrates (Fig. 23B). S71 is located at the middle of the pSer/Thr-binding pocket of the PPIase domain and pS71 phosphate formed H-bonds with Arg69, preventing the substrate from entering the catalytic active site (Fig. 23C). To examine whether Pinl activity is inhibited in patients with SLE, we directly measured Pinl PPIase activity in PBMC lysates, as described. Pinl activity was significantly higher in SLE PBMC than normal PBMC (Fig. 21B), consistent with our immunoblot data (Fig. 21A). These results together, albeit preliminary, suggest that Pinl is activated in SLE patients.

**Pinl activity may be determined genetically.** Previously a leader in human SLE genetics, and we established an association between **PP2CA** polymorphisms and susceptibility to SLE in multiple ethnic groups (183) after we had established that PP2Ac is abnormally expressed in SLE T cells and contributed to abnormal T cell function. Pinl expression has been shown to be controlled by Pinl promoter SNPs (reduced by SNP rs2233678 (110-112, 181), increased by SNP rs2287839 (182) (Table 1)).

**Table 1. Frequency of Pin1 SNPs rs2233678 (G>C) and rs2287839 (G>C) in the normal population**

<table>
<thead>
<tr>
<th>SNP</th>
<th>Count (%)</th>
<th>GC Count (%)</th>
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<tbody>
<tr>
<td>rs2233678</td>
<td>794 (78.8%)</td>
<td>213 (21.2%)</td>
</tr>
<tr>
<td>rs2287839</td>
<td>110 (86.6%)</td>
<td>17 (13.4%)</td>
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</tbody>
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Besides monocytes and DCs, B cells and major T cell subsets also express Pinl. Pinl enzymatic activity, but not Pinl protein levels, increases in human PBMCs following stimulation with R-848 (TLR7) or CpG (TLR9). Since the number of different cell types in human blood samples is limited, we have now successfully established a highly sensitive icFACS-based method to quantify Pinl levels in cells using the non-phosphorylated Srl6-specific Pinl monoclonal antibody (mAb) that was conjugated with APC (Allophycocyanin). The labeled antibody was then used to immunostain splenic B cells with Pinl wild type (WT) and Pinl deficient (KO) mouse embryonic fibroblasts (MEFs) as controls, followed by detecting Pinl levels using a flow cytometer. Pinl mAb and isotope control mAb generated almost identical signals in Pinl KO MEFs or B cells (Fig. 24A). However, clear Pinl staining signals were detected in Pinl WT MEFs and B cells (Fig. 24Aa). Using this FACS-based quantitative method, we examined whether Pinl levels change upon stimulation with CpG. Indeed, Pinl levels in splenic B cells were dramatically increased upon activation of TLR9, which also led to activation of B cells (Fig. 24B) further supporting Pinl activation upon TLR activation as shown. To detect Pinl expression in various immune cells, we immunostained unstimulated splenocytes with labeled Pinl mAb and various immune cell markers, followed by FACS analysis. Pinl was detected in CD4+ and CD8+ T cells, dendritic cells (DC), macrophages, B cells and granulocytes (data not shown). Thus we have established quantitative approaches to detect Pinl in immune cell subsets, which we will apply to ask whether Pinl expression is comparable or altered in T (CD4+, CD8+, CD3+CD4-CD8-, CD69+), B (and subsets), pDCs and monocytes in patients with SLE. Flow cytometry requires fewer cells and we should be able to answer this question without any problems. We expect that at least several types of cells will have altered (increased) levels of non-phosphorylated Pinl. We have shown that besides monocytes and DCs, B cells and major T cell subsets also express Pin.

Activation of Pinl by PP2A in SLE. We have shown that in SLE T cells the message, protein and activity of PP2Ac is increased and is involved in their abnormal function. First, PP2Ac dephosphorylates pCREB and accounts for the decreased IL-2 production. Second, PP2Ac dephosphorylates the transcription factor Elf1 and decreases its ability to bind to the promoters of CD3zeta and FcRgamma genes causing suppression of the first and derepression of the second with significant repercussions in the composition of the CD3 complex in SLE T cells. Third, PP2Ac dephosphorylates (and activates) SPI, which binds to the promoter of IL-17A and promotes its expression. Our results show that PP2A not only efficiently dephosphorylated Pinl that was phosphorylated by PKA (Fig. 25A, B) but also fully restored its PPIase activity (Fig. 25C) in vitro. Furthermore, Pinl became dephosphorylated in
human PMBCs after TLR9 activation by CpG, which was reversed in cells pretreated with okadaic acid at a low concentration that selectively inhibits PP2A (5 nM) (Fig. 25D). Moreover, Pinl also was dephosphorylated in T cells isolated from CD2-PP2A transgenic mice that overexpresses PP2Ac in T cells developed in Dr. Tsokos laboratory, but not wild-type littermates (Fig. 25E). Therefore, it is reasonable to predict that increased PP2Ac activity may contribute to the dephosphorylation and activation of Pinl in SLE. Alternatively, because calcineurin (CaN) can also dephosphorylate Pinl in vitro (Fig 25) and CaN inhibitors may have a place in the treatment of SLE, we will consider it as a viable alternative to PP2Ac.

**Pinl conditional knockout in immune cells.** To address if Pinl in specific cell types contributes to the expression of autoimmunity in lupus prone mice, we recently generated Pinl conditional knockout (Pinl-CO) mice in B6 background that we have recently generated using the Cre- and loxP-mediated system (Fig. 26A) and confirmed conditional KO by crossing them with Nestin-Cre mice (Fig. 26B).

**HTS identification of novel Pinl inhibitors that blocked cytokine production induced using TLR9.** One of the challenges arising from the recent wealth of knowledge on TLR signaling is how to develop a strategy to inhibit specific arms of TLR mediated immune regulation while leaving other critical defensive nodes untouched. Significantly, we have uncovered that Pinl inhibition completely abrogates activation of IRAKI kinase, and fully suppresses type 1 IFN production, but with only a moderate effects on pro-inflammatory cytokine production in response to TLR7/9 activation. In addition, hydroxychloroquine, one of the most common used and effective drugs for treating SLE, inhibits stimulation of TLR9. These results suggest that inhibiting Pinl activity might allow selective inhibition of the type I IFN response while leaving other arms of the immune defense proficient. Such Pinl inhibitory approach might have advantages over conventional immunosuppressing strategies.

In this regard, recently, we identified specific and potent Pinl catalytic peptidic inhibitors and used them to establish a robust and sensitive FP-based HTS to screen approved drugs and NIH Chemical and Genomics Center, which led us to successfully identify all trans retinoic acid (ATRA) and four other Pinl inhibitors active in cells for further optimization including Cpd4 (Fig. 27A). Notably, ATRA has been shown to suppress SLE-related phenotypes in some lupus prone mouse models. Our preliminary results showed that ATRA and Cpd4 effectively competed Pinl catalytic peptidic inhibitors for binding to the Pinl active site (Fig. 27B) and inhibited its catalytic activity in vitro (Fig. 27C) and inhibited Pinl-dependent cancer cell growth (Fig. 27D) with a similar potency. Furthermore, ATRA also suppressed production of cytokines including IL6, IL12 and TNF-a from pDCs upon TLR9 activation by CpG (Fig. 27F-H). To confirm ATRA as a Pinl inhibitor, we also solved co-crystal structures of Pinl and ATRA and found that the carboxylic acid of ATRA formed H-bonds with R68 and K63, residues essential for Pinl to recognize the substrate phosphate group, while the other end of the molecule formed many hydrophobic interactions with residues critical for recognizing Pro in the substrate (Fig. 271).
Identification of trans-RA as a Pinl inhibitor. We developed a fluorescence polarization (FP)-based HTS using fluorescence labeled Pinl peptidic inhibitor, pTide to identify Pinl inhibitors. Our screening of selected compounds at the Harvard ICCB-L libraries identified the strongest hit to be cis-RA according to the Z-score (Fig. 31A). To confirm that RAs target Pinl, we examined cis-RA and trans-RA in vitro and in cells. Surprisingly, trans-RA displayed even more potent Pinl inhibition than cis-RA in FP assays (Fig. 31A), inhibiting Pinl PPlase assays (Fig. 31B), inhibiting cell growth (Fig. 31C) and reducing Pinl levels (Fig. 31D) in breast cancer cell lines SKBR3 and T47D, without any effects on normal breast cells (MCF-IOA), as expected from Pinl KD. Cellular Pinl activity was also reflected by cyclin D1 levels (Fig. 31D), a known Pinl biomarker. RAs did not alter Pinl mRNA levels, but increased Pinl protein turnover in cells, as determined by cycloheximide chase, which might explain their much higher potency in cells than in vitro. Finally, Pinl KO MEFs were much more resistant to trans-RA, but their drug sensitivity was fully restored by stable re-expression of Pinl, but not its inactive mutant (Fig. 31E).

Determining trans-RA structure-activity relationship [and co-crystal structure with Pinl].

To identify the essential moiety of trans-RA for Pinl binding, we tested commercially available retinoids for Pinl inhibition. Only those with a -COOH group inhibited Pinl, but those with -CHOH or -CHO or -COOCH3 groups were inactive (Fig. 32A). The carboxylic acid of trans-RA formed H-bonds with R68 and K63, residues essential for Pinl to recognize the substrate phosphate group, while the other end of the molecule formed many hydrophobic interactions with residues critical for recognizing Pro in the substrate (Fig. 32B).

Trans-RA inhibited IL-33-induced Pinl activation and cytokine secretion in eosinophils. To examine the effects of trans-RA on immune cells related to asthma, we stimulated BM-derived eosinophils with 100 ng/ml IL-33 and different levels of trans-RA. trans-RA induced Pinl degradation (Fig. 33A) and inhibited Pinl activation (Fig. 33B) and IL-6 secretion (Fig. 33C) induced by IL-33.

Proteinuria in NZBWF1 mice is significantly reduced by Pinl inhibitor ATRA. Our objective here was to determine if the Pinl inhibitor we have discovered, ATRA, offer a treatment benefit in preclinical models of SLE. To this end we have been treating NZBWF1 female mice for 3.5 months with controlled release ATRA pellets, which have been well established pharmacologically and which offer the advantage ease of delivery, reduced stress to subjects and controlled drug release. Each cohort has been pre-bleed and urine collected before the start of drug treatment. Subsequently, we have collected serum and urine samples monthly for evaluation of disease markers such as autoantibodies and proteinuria. We have analyzed each cohort for proteinuria at 3.5 months of ATRA and noticed that the
placebo cohort have significantly elevated urinary protein levels in comparison to ATRA treated mice (Figure 34).

**Cutaneous inflammation is reduced in Pinl KO mice.** We have compared the development of cutaneous inflammation induced by direct treatment with a TLR7 ligand formulated into a topical cream or injection of sera from lupus prone mice with active disease into both WT and KO Pinl mice followed by histological analysis. Both of these models have been proposed to recapitulate forms of human cutaneous lupus with TLR signaling believed to contribute to disease pathogenesis. Figure 35 shows representative images from H&E stained skin sections from Pinl WT and KO mice induced with either lupus sera or TLR7 ligand. We noticed that WT mice developed considerably more significant inflammation than Pinl KO mice for both types of inducer exemplified by thickening of the keratinocyte layer or hyperkeratosis (see arrows). Although we found inflammation in the Pinl KO mice as compared to non-treated Pinl KO the degree of keratinocyte thickening was considerably less than that identified in the Pinl WT mice. These findings are consistent with our previous findings that Pinl KO mice display a reduced TLR response.

**Other Embodiments**

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure that come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth, and follows in the scope of the claims.

Other embodiments are within the claims.
CLAIMS

What is claimed is:

1. A method of treating an immune disorder in a subject, comprising the step of administering to a subject in need thereof a therapeutically effective amount of a retinoic acid compound, wherein said subject is determined to have elevated levels of a Pinl marker prior to said administration.

2. A method of treating an immune disorder in a subject, comprising the steps of determining Pinl marker levels in a sample from said subject; and administering a therapeutically effective amount of a retinoic acid compound to said subject if said sample from said subject is determined to have elevated Pinl marker levels.

3. The method of claim 1 or 2, further comprising the administration of a second therapeutic compound, wherein said second therapeutic compound is an anti-inflammatory compound, anti-microbial compound, or anti-viral compound.

4. The method of claim 1, wherein said Pinl marker is reduced Ser71 phosphorylation of Pinl.

5. The method of any one of claims 1-4, further comprising determining Pinl marker levels in said sample after said administration of a retinoic acid compound.

6. The method of any one of claims 1-5, wherein said retinoic acid compound is selected from the group consisting of 13-cis-retinoic acid and all-trans-retinoic acid.

7. The method of any one of claims 1-5, wherein said retinoic acid compound is selected from the group consisting of retinol, retinyl acetate, retinal, and AC-55640.

8. The method of any one of claims 1-7, wherein said sample is selected from the group consisting of blood, urine, tissue biopsies, lymph, saliva, phlegm, and pus.

9. The method of any one of claims 1-8, wherein said elevated Pinl marker level is due to an inherited trait or a somatic mutation.

10. The method of claim 3, wherein said second therapeutic compound is selected from the group consisting of corticosteroids, NSAIDs, COX-2 inhibitors, biologies, small molecule immunomodulators, non-steroidal immunophilin-dependent immunosuppressants, 5-amino salicylic acid, DMARDs, hydroxychloroquine sulfate, and penicillamine.
11. The method of claim 3, wherein said second therapeutic compound is selected from the group consisting of microtubule inhibitors, topoisomerase inhibitors, platin, alkylating agents, and anti-metabolites.

12. The method of claim 3, wherein said second therapeutic compound is selected from the group consisting of 1-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, 9-β-hydroxy-ethoxy methylguanine, adamantanamine, 5-iodo-2′-deoxyuridine, trifluorothymidine, interferon, adenine arabinoside, protease inhibitors, thymidine kinase inhibitors, sugar or glycoprotein synthesis inhibitors, structural protein synthesis inhibitors, attachment and adsorption inhibitors, and nucleoside analogues such as acyclovir, penciclovir, valacyclovir, and ganciclovir.

13. The method of claim 3, wherein said second therapeutic compound is administered at a low dosage.

14. The method of claim 3 or 13, wherein said retinoic acid compound and said second therapeutic compound are formulated together.

15. The method of any of the preceding claims, wherein said immune disorder is related to increased susceptibility to infection.

16. The method of any one of the preceding claims, wherein said immune disorder is selected from the group consisting of acne vulgaris; acute respiratory distress syndrome; Addison's disease; adrenocortical insufficiency; adrenogenital syndrome; allergic conjunctivitis; allergic rhinitis; allergic intraocular inflammatory diseases, ANCA-associated small-vessel vasculitis; angioedema; ankylosing spondylitis; aphthous stomatitis; arthritis, asthma; atherosclerosis; atopic dermatitis; autoimmune disease; autoimmune hemolytic anemia; autoimmune hepatitis; Behcet's disease; Bell's palsy; berylliosis; bronchial asthma; bullous herpetiformis dermatitis; bullous pemphigoid; carditis; celiac disease; cerebral ischaemia; chronic obstructive pulmonary disease; cirrhosis; Cogan's syndrome; contact dermatitis; COPD; Crohn's disease; Cushing's syndrome; dermatomyositis; diabetes mellitus; discoid lupus erythematosus; eosinophilic fasciitis; epicondylitis; erythema nodosum; exfoliative dermatitis; fibromyalgia; focal glomerulosclerosis; giant cell arteritis; gout; gouty arthritis; graft-versus-host disease; hand eczema; Henoch-Schonlein purpura; herpes gestationis; hirsutism; hypersensitivity drug reactions; idiopathic cerato-scleritis; idiopathic pulmonary fibrosis; idiopathic thrombocytopenic purpura; inflammatory bowel or gastrointestinal disorders, inflammatory dermatoses; juvenile rheumatoid arthritis; laryngeal edema; lichen planus; Loeffler's syndrome; lupus nephritis; lupus vulgaris; lymphomatous tracheobronchitis; macular edema; multiple sclerosis; musculoskeletal and connective tissue disorder; myasthenia gravis; myositis; obstructive pulmonary disease; ocular inflammation; organ transplant rejection; osteoarthritis; pancreatitis; pemphigoid gestationis; pemphigus vulgaris; polyarteritis nodosa; polymyalgia rheumatica; primary adrenocortical
insufficiency; primary billiary cirrhosis; pruritus scroti; pruritis/inflammation, psoriasis; psoriatic arthritis; Reiter's disease; relapsing polychondritis; rheumatic carditis; rheumatic fever; rheumatoid arthritis; rosacea caused by sarcoidosis; rosacea caused by scleroderma; rosacea caused by Sweet's syndrome; rosacea caused by systemic lupus erythematosus; rosacea caused by urticaria; rosacea caused by zoster-associated pain; sarcoidosis; scleroderma; segmental glomerulosclerosis; septic shock syndrome; serum sickness; shoulder tendinitis or bursitis; Sjogren's syndrome; Still's disease; stroke-induced brain cell death; Sweet's disease; systemic dermatomyositis; systemic lupus erythematosus; systemic sclerosis; Takayasu's arteritis; temporal arteritis; thyroiditis; toxic epidermal necrolysis; tuberculosis; type-1 diabetes; ulcerative colitis; uveitis; vasculitis; and Wegener's granulomatosis.

17. The method of any one of the preceding claims wherein said immune disorder results from disregulation of Toll-like receptor signaling or type I interferon-mediated immunity.
Figure 1
Figure 9A

13-cis-retinoic acid (13cRA)  All-trans-retinoic acid (ATRA)
Figure 9C
Figure 10
### Figure 11

#### a

<table>
<thead>
<tr>
<th></th>
<th>B cells</th>
<th>CD4+ T cells</th>
<th>CD8+ T cells</th>
<th>mDC</th>
<th>pDC</th>
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</thead>
<tbody>
<tr>
<td>WT spleen</td>
<td>47.8</td>
<td>55.2</td>
<td>39.9</td>
<td>1.46</td>
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<tr>
<td>KO spleen</td>
<td>49.0</td>
<td>52.7</td>
<td>40.4</td>
<td>1.68</td>
<td>0.41</td>
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<tr>
<td>WT lymph nodes</td>
<td>29.1</td>
<td>54.1</td>
<td>33.1</td>
<td>1.03</td>
<td>n.d.</td>
</tr>
<tr>
<td>KO lymph nodes</td>
<td>26.0</td>
<td>63.1</td>
<td>31.3</td>
<td>0.03</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d.: not determined

#### b

- WT
- KO

#### c

- WT
- KO

TLR2  TLR4  TLR2  TLR4
Figure 12
Figure 15

(a) WT-IRAK1 173A-IRAK1 WT-IRAK1 173A-IRAK1
pIRAK1 ▶ ▶ ▶ ▶
IRAK1 ▶ ▶ ▶ ▶
IB: FLAG IB: pS173
IP: FLAG

(b) PBS R848 CpG
pIRAK1 ▶ ▶ ▶ ▶
IRAK1 ▶ ▶ ▶ ▶
Input IB: Total IRAK1
IB: Total IRAK1
GST GST PD
IB: p173-IRAK1
Figure 16

**a** Pam3CSK4

<table>
<thead>
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<th>Pin1-RNAi</th>
</tr>
</thead>
<tbody>
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<td>0</td>
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<td>30</td>
</tr>
</tbody>
</table>

**b** LPS

<table>
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<tr>
<th></th>
<th>Control</th>
<th>Pin1-RNAi</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15</td>
<td>30</td>
</tr>
</tbody>
</table>

- pIRAK1
- IRAK1
- IRAK4
Figure 17

**a**

<table>
<thead>
<tr>
<th>R848</th>
<th>Pin1 WT</th>
<th>Pin1 KO</th>
<th>(min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total ERK</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td>0 15 30 60</td>
</tr>
<tr>
<td>pERK</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>Total JNK</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>pJNK</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>Total p38</td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>p38</td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
<td></td>
</tr>
</tbody>
</table>

**b**

<table>
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<tr>
<th>CpG</th>
<th>Pin1 WT</th>
<th>Pin1 KO</th>
<th>(min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
<td>0 15 30 60</td>
<td></td>
</tr>
<tr>
<td><img src="image15" alt="Image" /></td>
<td><img src="image16" alt="Image" /></td>
<td></td>
<td></td>
</tr>
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<tr>
<td><img src="image23" alt="Image" /></td>
<td><img src="image24" alt="Image" /></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 18

(a) 

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Pin1 WT</th>
<th>Pin1 KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td><img src="image1" alt="Image of pERK" /></td>
<td><img src="image2" alt="Image of pERK" /></td>
</tr>
<tr>
<td>15</td>
<td><img src="image3" alt="Image of pERK" /></td>
<td><img src="image4" alt="Image of pERK" /></td>
</tr>
<tr>
<td>30</td>
<td><img src="image5" alt="Image of pERK" /></td>
<td><img src="image6" alt="Image of pERK" /></td>
</tr>
<tr>
<td>60</td>
<td><img src="image7" alt="Image of pERK" /></td>
<td><img src="image8" alt="Image of pERK" /></td>
</tr>
</tbody>
</table>

(b) 

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Pin1 WT</th>
<th>Pin1 KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td><img src="image1" alt="Image of IκBα" /></td>
<td><img src="image2" alt="Image of IκBα" /></td>
</tr>
<tr>
<td>5</td>
<td><img src="image3" alt="Image of IκBα" /></td>
<td><img src="image4" alt="Image of IκBα" /></td>
</tr>
<tr>
<td>15</td>
<td><img src="image5" alt="Image of IκBα" /></td>
<td><img src="image6" alt="Image of IκBα" /></td>
</tr>
<tr>
<td>30</td>
<td><img src="image7" alt="Image of IκBα" /></td>
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<td>90</td>
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</tbody>
</table>

(c) 

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Pin1 WT</th>
<th>Pin1 KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td><img src="image1" alt="Image of IκBα" /></td>
<td><img src="image2" alt="Image of IκBα" /></td>
</tr>
<tr>
<td>20</td>
<td><img src="image3" alt="Image of IκBα" /></td>
<td><img src="image4" alt="Image of IκBα" /></td>
</tr>
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<td><img src="image5" alt="Image of IκBα" /></td>
<td><img src="image6" alt="Image of IκBα" /></td>
</tr>
<tr>
<td>60</td>
<td><img src="image7" alt="Image of IκBα" /></td>
<td><img src="image8" alt="Image of IκBα" /></td>
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</tbody>
</table>

(d) 

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Pin1 WT</th>
<th>Pin1 KO</th>
</tr>
</thead>
<tbody>
<tr>
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<td>20</td>
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<td>40</td>
<td><img src="image5" alt="Image of Pin1" /></td>
<td><img src="image6" alt="Image of Pin1" /></td>
</tr>
<tr>
<td>60</td>
<td><img src="image7" alt="Image of Pin1" /></td>
<td><img src="image8" alt="Image of Pin1" /></td>
</tr>
</tbody>
</table>
Figure 19

(a) IL-6 Levels (pg/ml) vs. hours

(b) IL12p40 Levels (pg/ml) vs. hours

(c) IL-6 Levels (pg/ml) vs. hours

(d) IL12p40 Levels (pg/ml) vs. hours

Legend:
- □ WT
- ■ KO
Figure 20
Figure 21

A

<table>
<thead>
<tr>
<th>Control PMBC</th>
<th>SLE PMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

pS16-Pin1 Ab

S16-Pin1 Ab

B

Pin1 activity (A380 nm) vs. Time (second)

- NORMAL
- SLE
Figure 22

[Diagram of experimental results]

- Panel A shows gel images for different conditions.
- Panel B depicts the effects of various treatments on protein expression.
- Panels C through E present concentration-response curves for different Pin1 variants.

Concentration (EnM) vs. Response (units)
Figure 23
Figure 24
Figure 25
Figure 26

[Diagram showing genetic manipulation and expression patterns]
Figure 27

A
(NIH CGC libraries)
2,359,086
FP-HTS with green Pin1 probe
1,198
FP-HTS with red Pin1 probe
448
high quality hits
119
chemical families
45
Pin1 PPIase assays
Drug-like properties
15
Initial cell based assays
4 (including Cpd4)

B
IC50 [\text{mM}]

C
Pin1 activity

D
Cell growth [%]

E
Pin1-ATRA co-crystal

F
IL6 levels

G
IL12 levels

H
TNF-\alpha levels

ATRA Concentrations (\text{\mu M})

+ CpdG

+ CpdG

+ CpdG
Figure 28

A. IL33 activated Pin1

B. Pin1 KO reduced IL-6 in MEFs

C. Pin1 KO blocked Th2 cytokine in BAL

D. Pin1 KO reduced inflammation in lung

E. Pin1 KO reduced total cells in BAL

F. Pin1 KO reduced eosinophils in BAL
Figure 29
Figure 31

A. [Graph showing concentration vs. FP (mM)]

B. [Graph showing pin-1 activity (activity units) over time for Cis-RA and Trans-RA]

C. IC50s (μM) of cis-RA or trans-RA on suppressing cell growth

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>cis-RA</th>
<th>trans-RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF10A</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>SKBR3</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>T47D</td>
<td>0.04</td>
<td>0.01</td>
</tr>
</tbody>
</table>

D. [Western blot images showing protein expression levels for Pin-1, Cyclin D1, and β-tubulin for MCF10A and SKBR3 cell lines]

E. [Graph showing cell viability (%) over time for WT MEF and Pin1 K0 MEF in KO MEF and Pin1 in KO MEF]
**A. SAR studies**

<table>
<thead>
<tr>
<th>Retinoids</th>
<th>Carboxylic group</th>
<th>% of Pin1 inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTide</td>
<td>---</td>
<td>100</td>
</tr>
<tr>
<td>cis-RA</td>
<td>Yes</td>
<td>24</td>
</tr>
<tr>
<td>trans-RA</td>
<td>Yes</td>
<td>41</td>
</tr>
<tr>
<td>Retinol</td>
<td>No</td>
<td>7</td>
</tr>
<tr>
<td>Retinyl acetate</td>
<td>No</td>
<td>5</td>
</tr>
<tr>
<td>Retinal</td>
<td>No</td>
<td>0</td>
</tr>
<tr>
<td>AC-55649</td>
<td>Yes</td>
<td>18</td>
</tr>
<tr>
<td>β-carotene</td>
<td>No</td>
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</tbody>
</table>

**B. Pin1-trans RA co-crystal**

---

Figure 32
Figure 35
INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/039850

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/07 (2012.01)
USPC - 514/725

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61K 31/07, 203; A61P 37/00; C07K 14/705; G01N 33/50 (2012.01)
USPC - 514/725, 530/352, 702/19

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MicroPatent, FreePatentsOnline, Google Scholar

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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</thead>
</table>

Date of the actual completion of the international search 05 September 2012

Date of mailing of the international search report 03 OCT 2012

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-3201

Authorized officer: Blaine R. Copenhaver
<table>
<thead>
<tr>
<th>Box No. III</th>
<th>Observations where unity of invention is lacking (Continuation of item 3 of first sheet)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>This International Searching Authority found multiple inventions in this international application, as follows:</td>
</tr>
</tbody>
</table>

1. □ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. □ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. □ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.: 

4. □ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 

### Remark on Protest

- □ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- □ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- □ No protest accompanied the payment of additional search fees.