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JAK3 Inhibition Significantly Attenuates Psoriasiform Skin Inflammation in CD18 Mutant PL/J Mice

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JAK3, a member of the Janus kinase family, is predominantly expressed in hemopoietic cells and binds specifically to the common γ chain of a subfamily of cytokine receptors that includes IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Previous studies suggest that this tyrosine kinase plays key roles in mediating T cell functions, and inhibition of JAK3 has been shown to prevent graft rejection and decrease the severity of arthritis in rodent models. However, the functions of JAK3 in the development of skin immune responses and diseases such as psoriasis have not been determined. CD18 mutant PL/J mice develop spontaneous T cell-dependent psoriasiform skin disease with several similarities to human psoriasis. In this study, we treated mice with established skin disease with R348, a small molecule inhibitor of JAK3, and observed a marked attenuation of skin lesions following 6 wk of treatment. Histological analyses revealed major reductions of both epidermal and dermal lesion severity scores in R348-treated CD18-deficient PL/J mice compared with vehicle controls, which was associated with decreased CD4+ T cell infiltration. In addition, systemic levels of IL-17, IL-22, IL-23, and TNF-α were significantly lower in mice receiving the compound, and T cells isolated from R348-treated mice also showed reduced phosphorylation of Stat5 after stimulation with IL-2. These findings suggest that small-molecule inhibitors of JAK3 may be useful in the treatment of inflammatory skin diseases such as psoriasis and strongly implicate JAK signaling events as important in the pathogenesis of this disease. The Journal of Immunology, 2009, 183: 2183–2192.

Cytokines induce cellular responses through binding to their receptors and inducing phosphorylation of cellular substrates. As the receptors themselves lack intrinsic kinase activity, signaling is achieved by association of the receptor with protein tyrosine kinases. JAK3, a member of the Janus family of tyrosine kinases (JAK1, JAK2, JAK3, and Tyk2), is constitutively associated with the common γ chain of the receptors of numerous cytokines, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (1). Upon cytokine binding to the receptor, the Janus kinases become activated, autophosphorylate, and transphosphorylate the receptor, leading to association of Stat proteins with the receptor (2–8). Tyrosine phosphorylation of the Stat proteins by JAK lead to dimerization and translocation of the complexes to the cell nucleus where they induce DNA transcription of specific target genes. Many of these cytokines that signal through JAK are critical for lymphocyte activation; therefore, selective inhibition of JAK represents an optimal strategy for immunosuppression and the treatment of many autoimmune disorders (9–11).

Selective small molecule JAK3 inhibitors have been shown previously to prevent graft rejection in transplantation models (12, 13), although in some instances, anemia and neutropenia were observed presumably due to the inhibition of JAK2 pathways (13, 14). In addition, the rat collagen-induced arthritis model, treatment with a specific JAK3 inhibitor resulted in a significant reduction in the severity of joint inflammation and prevented bone erosion when used prophylactically (15). We identified a novel small molecule kinase inhibitor, R333, showing potent inhibition of JAK1/3-dependent signaling pathways. The cell-based selectivity of R333 was described previously (16). R333 inhibits IL-2 signaling in T cells and IL-4 signaling in B cells with EC50s of 0.18 and 0.07 μM, respectively, and is a potent inhibitor of T cell proliferation in the MLR with an EC50 of 0.12 μM. R333 was less potent against Epo/JAK2-dependent signaling pathways in primary erythroblasts (EC50 of 0.84 μM) and was selective for JAK1/JAK3 when tested in various receptor tyrosine kinase signaling and proliferation assays (16). R333 is an ATP-competitive inhibitor of JAK3 kinase with a Ki of 16 nM. R348 is a prodrug of R333 demonstrating enhanced bioavailability and absorption of compound in vivo. R348 was found to prevent acute rejection in a rat heart heterotopic allograft model and synergized with tacrolimus to prolong cardiac allograft survival (16).

As JAK play a pivotal role in cytokine signaling for lymphocytes, we postulated that selective inhibition of JAK1/3 with R333/348 may be beneficial for the treatment of inflammatory disorders such as psoriasis, where T cells have been shown to play an active role in promoting skin inflammation (17, 18). To test this hypothesis, we evaluated the efficacy of R348 in treating psoriasisform skin disease in CD18 mutant PL/J mice, a well-established model of human psoriasis (19). These mice spontaneously develop a polygenic T cell-dependent inflammatory skin disorder with requirements for TNF-α and other cytokines for disease pathogenesis (19–22). Treatment of affected CD18 mutant PL/J mice with R348 resulted in a significant resolution of disease activity compared with vehicle controls. Mice treated for 6 wk with R348 showed a dose-dependent attenuation of both the clinical and histological signs of skin inflammation, including reduced epidermal thickening and decreased CD4+ and CD8+ T cell infiltration. In addition, a significant reduction in plasma levels of IL-17, IL-22, IL-23, and TNF-α levels was observed in R348-treated mice.
compared with vehicle-treated controls. RT-PCR results from the skin of animals treated with R348 indicated reduced IL-17F, IL-22, CXCL1 (KC), TNF-α, and CCL2 (MCP-1) compared with vehicle treatment. Finally, T cells isolated from the draining lymph nodes and spleens of mice after R348 administration showed reduced phosphorylation of Stat5 following stimulation with IL-2, confirming JAK1/3 inhibition in secondary lymphoid organs where important immune responses take place. These results suggest that small-molecule inhibitors of JAK1/3, as with R348, may be useful for the treatment of skin diseases such as psoriasis as well as other T cell-dependent inflammatory disorders.

Materials and Methods

R348 and R333

The structure and synthesis of the active compound R333 is disclosed in the patent application U.S. 2006-029311A1. In vivo, the prodru molecule of R348 is released in the gastrointestinal tract by an esterase, converting it into the active metabolite R333. R348 was formulated in 0.25% w/w hydroxypropyl methylcellulose, pH 9.0 solution (vehicle).

Western blotting

Human primary T cells were isolated from PBMC of healthy human volunteers. Cultured human erythroid progenitor cells were generated by culturing human CD34+ cells in StemPro complete (Invitrogen) with 10 ng/ml IL-3, 10 ng/ml IL-6, and 25 ng/ml stem cell factor for 9 days and stimulated with 10 U/ml erythropoietin on day 10. Various cell lines or primary cells were preincubated with serial dilutions of R348 for 40 min and stimulated with cytokines or growth factors as indicated for 5–20 min. Each agonist was individually titrated, and a time course of phosphorylation was performed as part of the assay optimization. The cells were spun down, washed in PBS, and resuspended in Tris-glycine SDS sample buffer. Western blots were performed according to standard protocols, using 8% Tris-glycine gels, Immobilon-P membrane, and ECL Western blot detection reagent. Primary Abs were purchased from Cell Signaling Technology or Santa Cruz Biotechnology. Membranes were reprobed with Abs recognizing the protein of interest to verify equal protein loading.

Whole-blood JAK3 and JAK2 assays

Peripheral whole blood from healthy volunteers was stimulated with human IL-2 (PeproTech) at 100 ng/ml (1000 U/ml) or hGM-CSF (PeproTech) at 5 ng/ml for 15 min at 37°C, and fixed with 1× BD Phosflow Fix Solution (BD Biosciences) for 10 min. Cells were then surface stained for CD3 (FITC-mouse anti-human CD3) and CD11b (PE-mouse anti-human CD11b; both from BD Biosciences) for 30 min. Cells were permeabilized in BD Phosflow Perm Buffer for 30 min, stained with pStat5 (Alexa 647-pStat5; BD Biosciences), and analyzed with FACSCalibur or FlowJo (Tree Star) to determine pStat5 signal in T cells (CD3+CD11b+) or granulocytes (CD15+/CD11b+). The percentage and geometrical mean of CD3+CD11b+ pStat5+ and CD11b+CD3+ pStat5+ cells were determined in triplicate for each drug concentration. The EC50 from each independent experiment was determined using nonlinear regression (curve fit) (GraphPad Prism 4.0).

Ex vivo splenocyte cytokine measurements

Spleens from R348 or vehicle treated homozygous or heterozygous CD18-deficient mice were removed following 6 wk of treatment. Single-cell splenocyte suspensions were generated from the spleens, and cell counts were determined by Cell-Dyn 7500 (Abbott Laboratories). Equal numbers of splenocytes were cultured in RPMI 1640 and 10% FBS, cells were counted and spun down. Culture media were collected and evaluated for cytokine production by Luminex or ELISA as described above.

Generation of ear lysates

Ear tissue from mice treated with vehicle or R348 (120 mg/kg) was harvested after 6 wk of treatment and snap-frozen in liquid nitrogen. Ears were homogenized under liquid nitrogen with radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors, centrifuged at 13,000 rpm, and the supernatant was collected to generate ear lysates. Ear lysates were normalized for equal protein concentration. Cytokines were measured using an equal amount of protein using methods described above, and the cytokine concentration was expressed as a percentage to the vehicle group.

Real-time TaqMan PCR arrays

Total RNA was isolated from ear tissue of vehicle or R348-treated mice (n = 8/group) by guanidinium thiocyanate-phenol-chloroform extraction using TRIzol reagent (Invitrogen), followed by column purification steps with RT² qPCR-Grade RNA Isolation Kit (PA-001; SABiosciences). RNA samples were then run on the Agilent Bioanalyzer. The integrity of RNA was assessed by analyzing both the 18s and 28s rRNA peaks and the RNA integrity number. RNA concentrations were measured using NanoDrop (ThermoScientific), and all samples had 260:280 ratios above 1.8 and 260:230 ratios above 1.7. Equal amounts of RNA (1 mg) were taken for all

Histopathology and immunohistochemical analyses

Ears and skin samples from CD18-deficient PL/J mice were either fixed in 10% neutral buffered formalin or frozen and embedded in OCT. Formalin-fixed ears and skin were processed and embedded in paraffin, sectioned at ~5 μm, and stained with H&E. The frozen specimens embedded in OCT were stained at ~5 μm and immunohistologically stained with Abs against the following mouse target Ags: CD4 (rat-anti-mouse CD4, clone H129.2, BD Pharmingen), CD8 (rat-anti-mouse CD8a, clone 53-6.7, BD Pharmingen), and F4/80 (rat-anti-mouse F4/80, clone C1-A3-1; Serotec). Briefly, endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide and blocked with 5% normal goat serum plus 0.05% casein. Slides were incubated with the primary Ab described above, followed by the secondary biotinylated goat anti-mouse IgG reac- tor Ab, and the addition of ABC Elite reagent (Vector Laboratories). Fi- nally, specimens were incubated in diaminobenzidine for 5 min, followed by hematoxylin counterstaining. Isotype control Abs were used to assess the overall level of nonspecific and background staining. All H&E and immunohistochemically stained slides were evaluated blindly by a veteri- nary pathologist. The H&E slides were scored semiquantitatively for the following parameters: inflammation, fibrosis, and hyperkeratosis. Immuno- histochemically stained slides were quantitatively evaluated for the following parameters: 1) percentage of positively stained cells to total cells in the dermis for each target Ag (CD4, CD8, and F4/80) measured in 15×400 fields; and 2) the average number of positively stained cells/250 mm of epidermis.

Cytokine measurements

Multiplex immunoassays were performed using a mouse cytokine/chemo- kine multiplex immunoassay kit from Millipore according to the manufac- turer’s protocol. Briefly, 10 μl of plasma/culture media/ear lysates from animals was diluted and assayed for 22 murine cytokines/chemokines, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17 (detects both IL-17A, and IL-17F), KC, MCP-1, G-CSF, GM-CSF, IFN-γ, IFN-γ-inducible protein-10, MIP-1α, RANTES, and TNF-α using specific Abs conjugated to beads and read on a Luminex 100 instrument (Luminex). The concentration of each cytokine/chemokine in each sample was determined by preparation of a standard curve using standard cytokine concentrations and by five parameter logistics in MasterPlex QT3.0 soft- ware (Mibabio). Results are expressed in picograms per milliliter. The detection levels of these Abs were between 1 and 5 pg/ml with little or no cross-reactivity among the Abs.

Generation and maintenance of transgenic mouse colonies

Murine IL-2 and IL-12 were determined using prepackaged Quantikine kits (M2200 and M2300) from R&D Systems, respectively, and following 1/2 dilutions of the samples.
samples, and reverse transcription was performed using RT
First Strand kit from SABiosciences. PCR were performed with customized RT
Profiler PCR arrays containing predispensed primer assays (SABiosciences) on the ABI 7900 using RT
SYBR Green/ROX qPCR Master Mix (SABiosciences). Each PCR contained cDNA synthesized from 10 ng of total RNA. The thermocycler parameters were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative changes in gene expression were calculated using the $\Delta\Delta C_{t}$ (threshold cycle) method (23, 24). Threshold cycle numbers $(\Delta C_{t})$ above 35 were considered below detection level. The housekeeping gene Rpl13a (Ribosomal Protein L13a) was used to normalize to the RNA amounts. Fold change values were calculated using the formula $2^{-\Delta\Delta C_{t}}$. There were three biological replicates in each group.

Immunophenotyping analysis
Splenocytes harvested from mice treated with vehicle or R348 for 6 wk were passed through a 70-μm filter, washed, and resuspended, followed by red cell lysis using PharmLyse (BD Biosciences). Cell counts were determined by CellDyon 3700. One million cells were aliquoted for surface staining, and purified rat anti-mouse CD16/32 Ab (Fc Block; BD Biosciences) was added to block nonspecific FcR binding. All Abs for immunophenotyping analysis were purchased from BD Pharmingen, including FITC-
CD25, PE-CD69, PerCP-CD4, allophycocyanin CD8, FITC-CD3ε, PE-
Gr1, and allophycocyanin NK1.1. A total of 50,000 events were collected per sample with the exception of the NK1.1 staining, where 200,000 events were collected on a FACS caliber and analyzed with CellQuest (BD Biosciences) and FlowJo (Tree Star) software. The percentage of cells positive for a given lymphocyte subset marker in various tissues was multiplied by the total splenocyte cell count to derive the total cell count for each white blood cell subset (i.e., total CD4+ cells = percentage of CD4+ cells × total cell count).

Statistics
Results are presented as the mean ± SEM for $n = 5–8$ mice/group, with the exception of the nondiseased heterozygotes $(n = 2–3)$ or unless noted otherwise. Statistical significance between groups were assessed by using the one-tailed unpaired Student $t$ test or one-way ANOVA using Prism (GraphPad) with Tukey’s or Bonferroni’s multicomparison test. Differences in clinical scores among the various treatment groups were assessed using a summary statistic, the slope of the repeated measures, as the data unit. An analysis of variables program was used with the statistical package S-PLUS (TIBCO). Pairwise comparisons were made between the dose treatments with a multiple comparisons program based on the Tukey method.

Results
R333 selectively inhibits JAK3 activity in human primary cells and whole-blood T cells
A novel small molecule inhibitor R333 was identified through screening for inhibitors of IL-2 and IL4 (JAK1/3)-dependent signaling. The cell-based selectivity of R333 was described previously (16). We further evaluated R333 potency and selectivity for inhibition of various tyrosine kinases in cells by measuring phosphorylation of the kinase itself or signaling proteins downstream to the kinase of interest. JAK1 and JAK3 activities cannot be differentiated from one another in cell-based assays due to the presence of both molecules downstream of $\gamma$ chain containing cytokine signaling pathways (reviewed in Ref. 25). R333 potently inhibited JAK1/JAK3-dependent Stat5 and Stat3 phosphorylation in primary T cells with an EC50 of $\sim0.2 \mu M$ following stimulation with IL-2 and IL-21, respectively, but was 5- to 10-fold less potent on JAK2-dependent Stat5 phosphorylation in primary T cells and in primary erythroblast cells (cultured human erythroid progenitor cells) following IL-12 and Epo stimulation, respectively (Fig. 1). In addition, R333 also inhibited Syk-dependent anti-IgM-induced Erk1/2 phosphorylation in Ramos B cells but not as potently as JAK1/3 activity in T cells. In contrast, R333 was less potent in the inhibition of TCR or VEGFR2 signaling pathways and much less potent on Fli3 activity (Fig. 1). These experiments and others (16)

Abbreviations used in this paper: Ct, threshold cycle number; qd, once daily orally.

FIGURE 1. The kinase specificity of R333 was evaluated by phospho-
blotting with human cell lines. Various human cell lines or primary T cells were treated with increasing concentrations of R333 (from 0.016 up to 10
$\mu M$) in 0.01% DMSO for 40 min and then stimulated with corresponding growth factors and stimuli as indicated for 5–10 min. Cell lysates were
separated on SDS-PAGE gels, and the Western blots were probed with Abs specific for phosphorylated kinase targets. Fli3 was immunoprecipitated and probed with anti-phosphotyrosine Ab. The left panel is displayed with Fli3 and without growth factor/cytokine treatment in the presence of 0.01%
DMSO only.

Peripheral whole blood collected from healthy volunteers was treated with serial dilutions of R333 ranging from 1 nM to 50 $\mu M$. Whole-blood cells were then stimulated ex vivo with IL-2 or GM-CSF at physiological levels, and pStat5 was analyzed in T cell (CD3+ or myeloid (CD11b+)-positive cells by intracellular staining to determine the extent of inhibition of JAK1/3 or JAK2 activity, respectively. Significant inhibition of IL-2-induced phosphorylation of Stat5 in T cells was initially observed at 500 nM, and a dose-dependent inhibition of Stat5 phosphorylation was demonstrated from 500 nM to 50 $\mu M$ R333 (Fig. 2). The EC50 of the inhibition of JAK3 activity in human peripheral blood T cells was determined to be 1 $\mu M$. For JAK2 activity, the EC50 value cannot be determined due to poor suppression of GM-CSF-induced Stat5 phosphorylation in granulocytes (Fig. 2). A modest inhibition of JAK2 activity was observed starting only at 25 $\mu M$. These results demonstrate that R333 is active in the inhibition of JAK1/3 signaling in human blood, whereas JAK2 activity was not inhibited from 500 nM to 10 $\mu M$, providing a wide therapeutic window (>50-fold) for JAK3 vs JAK2 activity in human whole blood. The 5-fold increase in EC50 activity from cellular assays to whole-blood assays is most likely due to albumin/protein binding of R333 (>95%) as cellular assays contain <10% serum albumin in the media. Whole-blood assays provide a more reliable estimate of systemic drug levels needed to achieve activity than the EC50 of cell-based or biochemical assays routinely used for compound profiling (26). Numerous in vivo pharmacokinetics and pharmacodynamics correlation experiments following oral administration of R348 in peripheral blood of rodents (Lewis rats or BALB/c mice)
FIGURE 2. The JAK3 vs JAK2 activity of R333 in human whole blood were determined by phosphoflow cytometry analysis. Upper panels. The percentage (left panel) and geometric mean (right panel) of CD3⁺CD11b⁺pStat5⁺ cells in human whole-blood lymphocytes following ex vivo treatment with R333 (and IL-2 stimulation). Lower panels. The percentage and geometric mean of CD3⁺CD11b⁺pStat5⁺ in human whole-blood myeloid/granulocytes upon treatment with R333 (and GM-CSF stimulation). Statistical significance from R333 treatment groups were compared with DMSO vehicle control using a t test. *, p < 0.05, **, p < 0.01, and ***, p < 0.001. These results were reproduced three times, and results were similar.

showed similar reproducible results with comparable EC₅₀ and with no inhibition of JAK2 (up to 20 μM) (data not shown).

Reversal of psoriasiform lesions in CD18 mutant PL/J mice

Previous studies have shown that treatment of rodents with specific JAK3 inhibitors can prevent graft rejection and decrease the severity of arthritis, although the efficacy of these compounds in restricting inflammatory responses in other diseases has not been explored (15, 16). To determine whether inhibition of JAK3-dependent cytokine signaling could suppress chronic skin inflammation, such as occurs in psoriasis, we treated CD18 mutant PL/J mice with R348, a prodrug of R333. These mice develop a T cell-dependent inflammatory skin disease with several similarities to psoriasis and serve as a well-established model of this disease (19–21, 27). Severely affected CD18 mutant PL/J mice were treated with R348 at 40, 80, 120, or vehicle once daily orally (qd) for a period of 42 days (6 wk) and scored weekly for clinical severity using the scoring system described in Materials and Methods. Significant decreases in mean cumulative severity scores, reflecting the combination of erythema, crust/scaling, and alopecia, were observed for all R348-treated groups compared with vehicle-treated mice (Fig. 3A). In addition, individual scores of erythema, crust and scaling, and alopecia were also significantly reduced upon treatment with R348 at all concentrations compared with vehicle controls (Fig. 3, B–D). Notably, three of five mice treated with R348 at 120 mg/kg had a complete clinical resolution of erythema, scales and crusts, showed hair regrowth following 3 wk of treatment, and remained free of psoriasiform disease during the rest of the treatment period of 6 wk (Fig. 3E).

Additional treatment studies were also performed on severely affected CD18 mutant PL/J mice to further analyze the effects of high-dose (120 mg/kg) R348 treatment. In these experiments, 11 of 21 (52%) affected mice dosed with R348 for 5–6 wk had complete clinical resolution of disease at the cessation of treatment (clinical score of 0; 0–9 scale), whereas another 9 of 21 (43%) had clinical scores of only 1. In contrast, the majority of vehicle-treated mice, 15 of 21 (71%) still had clinical scores of 6 or above at the end of the study (data not shown). Pharmacokinetics of R348 in PL/J male or female mice showed the maintenance of >1000 ng/ml or 2.34 μM plasma R333 (the EC₅₀ for JAK3 inhibition in whole blood is 1 μM) for approximately half of the day following a single administration of compound (data not shown).

H&E staining of skin sections (both ears and dorsal skin) from drug-treated animals showed a distinct dose-dependent decrease in the incidence and severity of psoriasiform changes compared with the vehicle-treated mice (Table I and Fig. 4A). The epidermal lesions in both ear and dorsal skin were evaluated for acanthosis, hyperkeratosis, basal cell proliferation, ulceration, and lymphoid exocytosis. The dermal lesions were evaluated for infiltration of lymphocytes, mononuclear cells, presence of neutrophils and mast cells, vascular density, and the extent of fibrosis. There was a dose-dependent decrease in the average global lesion scores in both the epidermis and dermis of the ear and dorsal skin. This decrease was characterized by diminished acanthosis and hyperkeratosis in the epidermis, as well as decreases in dermal leukocyte infiltration, dermal fibrosis, and vascular density (Table I and Fig. 4A). Histological comparisons of R348-treated CD18 mutant PL/J mice at the higher doses of 80 and 120 mg/kg demonstrated significant decreases in both dermal and epidermal lesion severity scores in the dorsal skin compared with vehicle controls (Table I). Statistically, there were no differences between the 120 mg/kg R348-treated skin vs nondiseased heterozygotes in terms of epidermis/dermis lesion scores, either from the ear or from the dorsal neck area (Table I). The individual global lesion scores for the ears were reduced in a dose-dependent fashion following R348 treatment (Fig. 4A).

CD4 and CD8 immunohistochemical staining of skin from treated vs nontreated mice revealed a statistically significant dose-dependent reduction of CD4⁺ and CD8⁺ T cell infiltration in the epidermis and dermis of skin from the ear (Fig. 4, B and C, upper panel) and dorsal neck area (Fig. 4C, lower panel) and a distinct decrease of macrophages (F4/80⁺ staining) in the dermis of R348-treated mice (Fig. 4B). There was a statistically significant reduction in CD4⁺ and CD8⁺ T cell infiltration into the epidermis in both the ear and dorsal skin. The extent of T cell infiltration in the
dermis was greater than the epidermis; R348 treatment also substantially reduced T cell infiltration into the dermis. Quantitative analysis for each marker (Fig. 4C) showed that small numbers of CD4\(^+\), CD8\(^-\), and F4/80\(^+\)-stained cells were present within the dermis region in mice treated with R348 as well as in nondiseased heterozygous mice.

**Table I.** Average epidermal, dermal and global lesion scores from the ear and dorsal skin of vehicle or R348 (40, 80, or 120 mg/kg)-treated animals following 6 wk of treatment\(^a\)

<table>
<thead>
<tr>
<th>R348</th>
<th>Vehicle</th>
<th>40 mg/kg</th>
<th>80 mg/kg</th>
<th>120 mg/kg</th>
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</thead>
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<tr>
<td>Ear</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Epidermis lesion score</td>
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<td>9.0</td>
<td>7.8</td>
<td>4.2**</td>
<td>2***</td>
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<tr>
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<td>10.6</td>
<td>8.4*</td>
<td>5.2***</td>
<td>2***</td>
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<td>Global lesion score</td>
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<td>19.6</td>
<td>16.2</td>
<td>9.4***</td>
<td>4***</td>
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<tr>
<td>Dorsal skin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epidermis lesion score</td>
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<td>6.4</td>
<td>2.0*</td>
<td>0.8**</td>
<td>0**</td>
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<tr>
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<td>7.6</td>
<td>4.4**</td>
<td>3.0***</td>
<td>2***</td>
</tr>
<tr>
<td>Global lesion score</td>
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<td>14.0</td>
<td>6.4**</td>
<td>3.8***</td>
<td>2**</td>
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</table>

\(^a\) Average epidermal, dermal and global lesion scores from the ear and dorsal skin of vehicle or R348 (40, 80, or 120 mg/kg)-treated animals after 6 wk of treatment. Dermis lesion score is a combination of the evaluation of six characteristics, each with a value (0 –3): lymphocytes, mononuclear cells, neutrophils, mast cells, vascular density, and fibrosis. Epidermal lesion score is evaluated for acanthosis, hyperkeratosis, basal cell proliferation, ulceration, and lymphoid exocytosis, each with a score of 0–3 (n = 5 for each group). Global lesion score is the combination of dermal and epidermal lesion scores. Skin pathology was evaluated in two other independent studies with similar results. \(^*, p < 0.05; **, p < 0.01; and ***, p < 0.001 in one-way ANOVA.\)

**FIGURE 3.** Psoriasiform lesions in CD18-deficient homozygous PL/J mice were reversed following treatment. Affected mice were treated with one of three different doses of R348 (40, 80, and 120 mg/kg) or vehicle daily for 6 wk. The clinical severity of skin disease was evaluated weekly using the scoring system described in Materials and Methods. A, The average of total clinical scores (0–9) from each treatment group. B–D, The average scores for erythema, crust/scaling, and alopecia, respectively (n = 5, Tukey HSD all-pairwise comparisons test for scores of each group, ***, p < 0.01, and ***, p < 0.001). Clinical pictures of a representative mouse at days 0, 21, and 42 posttreatment with R348 (120 mg/kg) are shown in E.
Comparative analyses of cytokine expression

Initial characterization of the nontreated homozygous-affected CD18-deficient mice showed increased systemic (plasma) concentrations of IL-17, IL-22, IL-23, KC, and TNF-α compared with the levels seen in heterozygous CD18 mice. We found that plasma levels of these cytokines were significantly reduced in mice treated 6 wk with 120 mg/kg R348 compared with vehicle controls (Fig. 5A). Interestingly, most of the cytokine concentrations were reduced in R348-treated mice, with the exception of IL-23, which showed a slight increase.

**FIGURE 4.** The skin sections of CD18-deficient mice treated with R348 or vehicle for 6 wk were evaluated by H&E staining and immunohistochemistry. A, Paraffin sections of ears (upper panels) or dorsal neck skin (lower panels) of treated PL/J mice were stained with H&E, examined by light microscopy, and evaluated by a blinded veterinary pathologist. Histopathology of each dose was examined in two independent experiments with similar results. a and d, Vehicle-treated animal; b and e, 120 mg/kg R348-treated animal; and c and f, noninjected heterozygous animal. Arrows point to hyperkeratosis. Double-headed arrows show acanthosis. Note the thickened dermis in a, with infiltrates of lymphocytes, macrophages, and neutrophils. For all images, bar = 100 μm, magnification is ×100. B, Frozen OCT sections of ears were stained with anti-CD4, CD8, and F4/80 to evaluate CD4⁺, CD8⁺, and macrophage infiltration in the dermis and epidermis of the ear skin following either vehicle (upper panels) or R348 treatment at 120 mg/kg (lower panels). CD4 stained: a and d; CD8 stained: b and e; and F4/80 stained: c and f. Arrows point to cells positively stained for CD4, CD8, or F4/80. The scale bar is 100 μM, and the magnification is ×400. C, CD4- and CD8-positive cells were enumerated in the epidermis as the number of positive cells per 250 mm width of section (left panels), or for dermis, the percentage of positive-stained cells per 1000 cells counted under the microscope (right panels). The analyses are from the ear skin (upper panels) and the dorsal neck skin (lower panels). The vehicle and R348-treated homozygous mice were statistically compared with a one-tailed Student t test using prism. *, p < 0.05 and **, p < 0.01.
were reduced following R348 treatment, including IL-17, IL-22, KC, MCP-1, and TNF-α (n = 5 for homozygous mutant mice; *, p < 0.05, and **, p < 0.01 with one-way ANOVA and Bonferroni posttesting with multiparameter comparison). B. Splenocytes from animals treated with either vehicle or R348 following 6 wk of treatment were isolated and stimulated with anti-CD3 and anti-CD28 for 5 days, and cytokines were measured decreased to levels comparable to nondiseased heterozygous mice. Cytokine expression analyses in CD4+ T cells isolated from the lymph nodes and spleens of mice treated for 6 wk with either 120 mg/kg or vehicle were performed and revealed a decreased frequency and number of cells expressing either IFN-γ or IL-17 in R348-treated mice compared with vehicle controls (data not shown). Cytokine transcript levels in ear tissue were determined using real-time quantitative TaqMan PCR. Among the cytokines/chemokines evaluated, the most prominent changes in transcript levels in R348-treated mice were observed for IL-19, CXCL1 (KC), IL-1β, IL-17F, IL-22, TNF-α, and CCL2 (MCP-1) (Table II). In addition, JAK3, IL-23A (p19), Syk, IL-6, IL-12B (p40), and IL-20 transcript levels were also lower in the R348-treated group compared with vehicle controls; however, the average cycle number was >35, which precluded accurate comparisons between groups.

Splenocytes isolated from CD18-deficient mice treated for 6 wk with R348 were cultured ex vivo following stimulation with anti-CD3 and anti-CD28 for 5 days, and cytokines were measured

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Vehicle</th>
<th>R348</th>
<th>Mean fold change</th>
<th>p Value</th>
</tr>
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<tbody>
<tr>
<td>IL-19</td>
<td>26.70</td>
<td>33.15</td>
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<td>CXCL1</td>
<td>29.92</td>
<td>34.65</td>
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<td>IL-1β</td>
<td>23.30</td>
<td>26.93</td>
<td>−10.57</td>
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<td>IL-17F</td>
<td>28.50</td>
<td>31.74</td>
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<td>TNF-α</td>
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<td>34.06</td>
<td>−7.34</td>
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<td>28.90</td>
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<td>CCL2</td>
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<td>JAK3</td>
<td>32.30</td>
<td>&gt;35</td>
<td>−</td>
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<tr>
<td>IL-23A</td>
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<td>&gt;35</td>
<td>−</td>
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</tr>
<tr>
<td>Syk</td>
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<td>&gt;35</td>
<td>−</td>
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<td>IL-6</td>
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<td>&gt;35</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>IL-12B</td>
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<td>−</td>
<td></td>
</tr>
<tr>
<td>IL-17A</td>
<td>34.50</td>
<td>&gt;35</td>
<td>−</td>
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<td>RPL13α</td>
<td>17.61</td>
<td>17.75</td>
<td>1.05</td>
<td>0.3343</td>
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* RNA from treated mice ears was extracted, reverse transcribed, and used to evaluate for gene expression using real-time TaqMan PCR methods. The mean of the Ct before normalization is displayed. Cts over 35 are shown as >35, and fold changes are not displayed as conventional cutoff for limit of detection is set at 35. Changes in gene expression were evaluated through the Ct method and normalized to the housekeeping gene Rpl13a. Mean fold of regulation was derived by comparing the R348-treated gene expression profile to the vehicle-treated profile (n = 8). The p values were determined using a Student t test. This experiment was performed twice, and a representative result is shown.
Table III. Absolute cell counts of leukocyte subpopulations in spleen following 6-wk R348 treatment (×10⁶)

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Vehicle (μg/kg)</th>
<th>R348 (120 μg/kg)</th>
<th>Heterozygotes</th>
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<tr>
<td>Lymphocytes</td>
<td>63.8 ± 21.7</td>
<td>45.4 ± 11.4</td>
<td>40.0 ± 17.3</td>
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<tr>
<td>CD4⁺CD8⁻</td>
<td>26.3 ± 9.2</td>
<td>17.5 ± 4.6</td>
<td>12.4 ± 5.5</td>
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<tr>
<td>CD4⁺CD8⁺</td>
<td>8.6 ± 3.0</td>
<td>5.7 ± 1.5</td>
<td>5.4 ± 2.4</td>
</tr>
<tr>
<td>CD4⁺CD8⁻CD19⁺</td>
<td>17.8 ± 5.9</td>
<td>16.3 ± 4.1</td>
<td>17.6 ± 7.6</td>
</tr>
<tr>
<td>CD4⁺CD8⁺Gr1⁻CD19⁺</td>
<td>5.3 ± 1.4</td>
<td>3.4 ± 0.9</td>
<td>3.0 ± 1.7</td>
</tr>
<tr>
<td>CD3⁻NK1.1⁺</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.0</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>WBC</td>
<td>73.9 ± 22.9</td>
<td>50.4 ± 11.9</td>
<td>43.6 ± 18.6</td>
</tr>
</tbody>
</table>

*Leukocyte subpopulations in spleen following 6-wk treatment of R348. Average total cell count (×10⁶) ± SE (n = 8 for vehicle, R348 treatment, n = 3 for heterozygotes). Similar phenotyping experiments were performed in three other independent studies, and results were similar.

3 The online version of this article contains supplemental material.

FIGURE 6. Immunophenotypical analysis of spleen cells following R348 treatment of 6 wk. Individual spleen cell suspensions were generated from spleens of treated mice and stained for surface markers that specify distinct subpopulations (i.e., CD4⁺, CD8⁺, CD25⁺, and CD69⁺) (n = 5). These evaluations were performed in four independent experiments and results were similar, **, p < 0.01 with one-way ANOVA and Bonferroni posttesting with multiparameter comparison.

Immunophenotyping of spleen cells from R348-treated CD18 mutant PL/J mice

We next examined whether the high dose of R348 treatment of CD18 mutant PL/J mice resulted in changes or alterations in both the frequency and/or the overall numbers of different leukocyte subpopulations. These studies were performed on splenocytes isolated from vehicle-treated or R348-treated mice. Surface marker immunophenotyping studies showed several specific changes in cell populations following treatment, mainly a decrease in CD4⁺ and CD8⁺ T cells as well as Gr1⁺ granulocytes (Table III). Lymphocyte counts following treatment were reduced compared with vehicle treatment and were closer in numbers to the nondiseased heterozygote animals (Table III). Within the T cell subpopulation, a decreased percentage of CD4⁺/CD25⁺, CD8⁺/CD25⁺, CD4⁺/CD69⁺, and CD8⁺/CD69⁺ cells were observed (Fig. 6). The CD4⁺/CD25⁺Fox3⁺ subpopulation was also decreased, most likely due to the target inhibition of the α subunit of the IL-2R. In addition, the percentage of CD4 and CD8 memory/effector populations (CD4⁺/CD44⁺CD62L⁻ and CD8⁺/CD44⁺CD62L⁻) were slightly decreased, whereas CD4⁺/CD44⁻/CD62L⁺ or CD8⁺/CD44⁺CD62L⁺ naive cells were increased following R348 treatments (supplemental Fig. 2).

Reduction of IL-2 induced Stat5 phosphorylation in draining lymph nodes and spleen

To determine both the distribution of R348 and the overall level of JAK1/3 inhibition in secondary lymphoid organs in CD18 mutant PL/J mice receiving this compound, we analyzed the overall frequencies of phospho-Stat5-positive T cells following IL-2 stimulation of spleen and draining lymph node cells collected after 6 wk of continual treatment. Cell suspensions were generated from these organs and restimulated with exogenous murine IL-2. IL-2 stimulation of phospho-Stat5 was higher in lymph node cells compared with the splenocytes, presumably due to the higher IL-2R expression in the draining lymph node cells (Fig. 7). A significant reduction of ~50% of CD3⁺pStat5⁺ cells was observed in the R348-treated mice compared with the vehicle-treated control mice at the end of the study. No exogenous compound was added to these cultures during the experiment. Interestingly, high levels of IL-17 were found in the culture media from cells isolated from vehicle-treated diseased animals. These levels were at least 5-fold higher than from cells isolated from naive PL/J or C57BL/6 mice that were stimulated with anti-CD3/28 and the Th17-polarizing cytokines IL-23, IL-6, and TGF-β (data not shown). Cells from R348-treated mice or from nondiseased heterozygotes showed significantly reduced IL-17 levels (Fig. 5B). In addition, IL-22, MCP-1, KC, and IFN-γ levels were also decreased but not to the same extent as IL-17. In contrast, no significant changes of IL-10 were seen. In addition to IL-10, other Th2 cytokines such as IL-4, IL-5, IL-9, and IL-13 were either not changed or had a trend of increase following R348 treatments (supplemental Fig. 1). The total cell counts remained similar between treatment groups (data not shown). To determine local cytokine concentration in the skin, we generated ear protein lysates from mice treated with either vehicle or R348 at 120 μg/kg and evaluated them for local cytokine concentration. These results demonstrated a substantial dose-dependent decrease of IL-17, IL-22, MCP-1, KC, and IFN-γ levels, in plasma, in secondary lymphoid organs, and in the skin, which may be one of the major mechanisms by which this compound promotes resolution of psoriasiform skin lesions in CD18 mutant PL/J mice.
FIGURE 7. Reduction of IL-2 pStat5 in draining lymph node cells and splenocytes from R348-treated mice. Single-cell suspensions of splenocytes were stimulated ex vivo with 20 ng/ml murine IL-2. Cells were subsequently surface stained with CD3 and stained intracellularly for phosphorylation of Stat5 to determine JAK3 activity in these cells following oral administration of 80 mg/kg R348 for 6 wk (n = 8). Similar results were seen in two other independent experiments. **, p < 0.01, and ***, p < 0.001 with one-way ANOVA and Bonferroni posttesting with multiparameter comparison.

We identified R333 as a small molecule inhibitor of JAK1/3-dependent signaling, an important intracellular signaling cascade for many different cytokine receptors on T cells. This compound is selective, with activities on JAK1/JAK3 in cellular assays with EC \textsubscript{50} values of 0.2 \textmu M. In human whole-blood assays, R333 inhibited JAK1/JAK3 activities as assessed by reduced Stat5 phosphorylation following IL-2 stimulation of CD3 \textsuperscript{+} T cells with an EC \textsubscript{50} of 1 \textmu M. More importantly, R333 did not inhibit GM-CSF stimulation of Stat5 phosphorylation in the granulocyte population mediated by JAK2 until concentrations of R333 were increased to 25 \textmu M. The EC \textsubscript{50} of JAK2 inhibition will likely be >50 \textmu M because the JAK2 inhibition was <50\% at this concentration in whole blood, increasing the window of JAK3 vs JAK2 activity to >50. This wide window of JAK3 vs JAK2 activity minimizes the risk of JAK2-associated adverse events because JAK2 is critical for erythropoiesis and granulopoiesis, and blocking of its activity may cause development of anemia and neutropenia, which was observed in patients treated with CP-690,550, a potent JAK3 inhibitor (14). Mice treated with R348 for 6 wk at 120 mg/kg qd did not develop anemia or neutropenia (data not shown).

In the current study, we evaluated the biological activity and efficacy of R348, a bioavailable prodrug of R333, in treating established psoriasisform skin disease in CD18-deficient PL/J mice. R348 dose-dependently reversed the clinical and histological manifestations of the disease upon 6 wk of qd treatment, with the higher doses producing a near-complete resolution of disease. Regrowth of hair and complete resolution of crusts and scales were apparent in most animals treated with higher doses of R348 following 3–4 wk of treatment. Histological evaluation of skin sections in mice receiving this compound showed distinct and prominent reductions of both epidermal proliferation (acanthosis and hyperkeratosis) and leukocyte infiltration. In addition, immunohistochemical staining of skin sections with different leukocyte-specific Abs demonstrated significant reductions in the overall numbers of infiltrating CD4 \textsuperscript{+} and CD8 \textsuperscript{+} T cells in R348-treated mice compared with vehicle controls.

Phenotypic analysis of spleen leukocyte populations after 6 wk of R348 treatment showed a reduction of CD4 \textsuperscript{+} and CD8 \textsuperscript{+} T cells, which is consistent with JAK3 deficiency (28–30). The most prominent reduction observed was in the CD25 \textsuperscript{+} T cells in the CD4 \textsuperscript{+} and CD8 \textsuperscript{+} populations, which may be due to a decrease of IL-2R expression resulting from inhibition of IL-2 signaling. CD18 mutant PL/J mice treated with 120 mg/kg R348 for 6 wk retained >70\% of their lymphocytes, compared with the vehicle group (and similar to the heterozygote mice with no disease), suggesting skin disease resolution correlated with a reduction of activated T cells rather than a general depletion of CD4 \textsuperscript{+} T cells, which has been previously shown to significantly lessen the severity of dermatitis in this model (21).

Our findings also demonstrate a substantial reduction of systemic and local Th17-related cytokines such as IL-23, IL-17, IL-22, and others in chronically affected CD18-deficient PL/J mice following treatment with R348. Splenocytes isolated from treated animals were cultured in vitro, and a significant reduction of IL-17 was also observed. Consistent with the systemic cytokine results, splenocytes from the chronically affected CD18-deficient mice expressed high levels of IL-17, which was at least 10- to 15-fold higher than that observed in the nondoxygenated heterozygous mice. Using quantitative RT-PCR analyses to measure cytokine and other inflammation-related gene transcript levels, we found that treated mice showed over a 5-fold decrease in numerous cytokine/chemokine transcripts, including IL-17F, IL-22, IL-1β, and TNF-α. One possible hypothesis to explain how R348 suppresses skin inflammation in this model is that the JAK1/3 inhibitor acts in secondary lymphoid organs to suppress signaling of cytokines such as IL-2, IL-7, IL-15, and IL-21, which are important in T cell proliferation, differentiation, and activation (31). The inhibition of JAK3 activity may as a result reduce proinflammatory cytokine production leading to both decreased epidermal proliferation and leukocyte infiltration into the skin. Alternatively, R348 may directly inhibit the differentiation of naive spleen cells into Th17 cells. However, as R333 inhibits both JAK1 and JAK3 (and eventually JAK2 at higher concentrations), a wide range of cytokines important in the Th1 and Th17 differentiation/maintenance, such as IL-2, IL-15, IL-21, IL-6, IL-22, IFN-γ, and IL-23, may be targeted that cumulatively results in the suppression of inflammatory responses in vivo.

These results also demonstrate for the first time the up-regulation of Th17-related cytokines, such as IL-22 and IL-17, both in terms of transcript levels and protein expression in the CD18-deficient PL/J mice, consistent with the association of Th17 pathway and psoriasis (32, 33). More recently, IL-17 and IL-22 have been shown to be associated with psoriasis-like disease in mice and human psoriasis (32, 34, 35), and serum IL-17 and IL-22 levels are reduced following efficacious treatment in patients with psoriasis (36).

Interestingly, in the RT-PCR analysis, the reduction of IL-19 was the most prominent with >75-fold decrease in message levels. IL-19 has been shown to be important for keratinocyte proliferation and is associated with the pathology of psoriasis (37–39), although the exact nature of the interplay of IL-19 with other Th17/Th1 cytokines involved in immune responses is not clear (17, 40).

In summary, our findings in the CD18 mutant PL/J mouse model suggest that inhibition of JAK1/3 by R333/R348 can lead to the amelioration of chronic T cell-dependent skin inflammation and demonstrate for the first time that a selective small molecule kinase inhibitor can effectively treat psoriasisform disease and result in a reduction of systemic cytokines related to Th17 pathways. Furthermore, they implicate JAK1/3-dependent pathways in the development of psoriasis. Although biologic therapies such as anti-TNF-α and anti-IL-12 and have been proven to reduce...
Psoriasis Area and Severity Index scores in human psoriasis patients (41–44), we believe novel small molecule inhibitors that target JAK1/3 may provide affordable alternatives to costly biological therapies for diseases such as psoriasis (moderate to severe forms) over classic immunosuppressors such as cyclosporin or FK506 that target a broader spectrum of cells compared with JAK3 inhibitors.

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Disclosures
Hong Ren, Sylvia Braselman, Vanessa Taylor, Donald Payan, Elliott Grossbard and Polly Pine are all employees and stockholders of Riged Pharmaceuticals Inc. Betty Chang and Feifei Zhao are stockholders of Rigel Pharmaceuticals. Daniel Bullard and Xiaodong He have nothing to declare.

References