The Journal of Immunology

Kaempferol Inhibits IL-4-Induced STAT6 Activation by Specifically Targeting JAK3

Jose R. Cortes, Moises Perez-G, Maria D. Rivas and Jose Zamorano

J Immunol 2007; 179:3881-3887; ;
doi: 10.4049/jimmunol.179.6.3881
http://www.jimmunol.org/content/179/6/3881

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Kaempferol Inhibits IL-4-Induced STAT6 Activation by Specifically Targeting JAK3[1]

Jose R. Cortes, Moises Perez-G, Maria D. Rivas, and Jose Zamorano[2]

IL-4 is involved in several human diseases including allergies, autoimmunity, and cancer. Its effects are mainly mediated through the transcription factor STAT6. Therefore, investigation of compounds that regulate STAT6 activation is of great interest for these diseases. Natural polyphenols are compounds reported to have therapeutic properties in diseases involving IL-4 and STAT6. The aim of this study was to investigate the effect of these compounds in the activation of this transcription factor. We found that in hemopoietic cells from human and mouse origin, some flavonoids were able to inhibit the activation of STAT6 by IL-4. To identify molecular mechanisms, we focused on kaempferol, the compound that showed the greatest inhibitory effect with the lowest cell toxicity. Treatment of cells with kaempferol did not affect activation of Src kinase by IL-4 but did prevent the phosphorylation of JAK1 and JAK3. Further enzymatic analysis demonstrated that kaempferol blocked the in vitro phosphorylation activity of JAK3 without affecting JAK1, suggesting that it specifically targeted JAK3 activity. Accordingly, kaempferol had no effect on STAT6 activation in nonhemopoietic cell lines lacking JAK3, supporting its selective inhibition of IL-4 responses through type I receptors expressing JAK3 but not type II lacking this kinase. The inhibitory effect of kaempferol was also observed in IL-2 but not IL-3-mediated responses and correlated with the inhibition of MLC proliferation. These findings reveal the potential use of kaempferol as a tool for selectively controlling cell responses to IL-4 and, in general, JAK3-dependent responses. *The Journal of Immunology,* **2007, 179: 3881–3887.**

Interleukin-4 is a cytokine that regulates multiple biological processes during immune responses (1). It can regulate proliferation, differentiation, and apoptosis in lymphocytes but also in other cell types of hemological and nonhemological origin (2–4). One of the most important effects of IL-4 is the regulation of T cell differentiation. Thus, IL-4-driven Th2 cell differentiation is an important step during responses against parasitic infections (5). In contrast, alterations of IL-4 and its signaling machinery are believed to participate in the progression of diseases like autoimmunity, allergy, and cancer. The anti-inflammatory effect of IL-4 has been found to have a protective role in autoimmune diseases as demonstrated in models of rheumatoid arthritis (6). In contrast, findings from studies of murine models and humans demonstrate the promoting function of IL-4-signaling machinery in allergic diseases like asthma and some tumors (7, 8). These effects of IL-4 reveal its importance in disease and point to the IL-4-signaling machinery as a potential therapeutic target to investigate in these diseases.

The production of IL-4 is mainly restricted to a few cell types including T cells, mast cells, basophils, and activated eosinophils (1, 9). However, the effects of this cytokine are mediated by cell surface receptor complexes expressed in most types analyzed (9). Two types of IL-4R have been identified. The type I receptor consists of the common γ-chain and the IL-4Rα (10). The IL-4Rα confers specificity for IL-4 binding and signal transduction. The type II receptor also contains the IL-4Rα subunit, but in this case, the γ_c is substituted by the IL-13R α1 chain (11, 12). IL-4Rs lack enzymatic activity, but the binding of IL-4 provokes activation of JAK tyrosine kinases (9). Regarding type I receptors, it is believed that JAK1 interacts with the IL-4R α-chain and JAK3 with the γ-chain (9–12). For type II receptors, JAK2 and TYK2 have been proposed as interacting with the IL-13R α1 chain (12). There is no evidence that JAK3 associates with type II receptors. In addition to JAKs, IL-4 can also activate the tyrosine kinase Src. The importance of Src in IL-4 signaling is still not well-defined, but a previous study indicates that its activation is an earlier event during cell responses to IL-4 (13). Like comparable receptors, the activation of tyrosine kinases leads to phosphorylation of several intracellular signaling molecules (9). Among them, STAT6 plays a principal role in IL-4 signaling, demonstrated by the fact that mice lacking STAT6 show a phenotype similar to that of mice lacking the IL-4R (14). In these mice, IL-4-driven Th2 cell formation is severely impaired, with drastic effects in several diseases. Thus, mice lacking STAT6 fail to respond to parasitic infections (5), experience more aggressive autoimmune diseases (15), and are protected from allergic diseases and some tumors (16, 17). Therefore, the characterization of STAT6 inhibitors will be of great interest for these diseases.

Flavonoids are natural polyphenols found in a large variety of vegetable foods (18, 19). They are classified in several groups according to the substituents in their common three-ring nucleus (19). Among them, flavonols are likely the most consumed. The importance of these compounds is shown by the fact that they have been subject of therapeutic interest for long time (18, 19). Epidemiological and physiological studies have demonstrated that some flavonols and other natural polyphenols can influence the progression of cancer and inflammatory diseases including autoimmunity and allergy (18–21). Biochemical studies indicate that they can act

Unidad de Investigacion, Hospital San Pedro de Alcantara, Caceres, Spain

Received for publication February 16, 2007. Accepted for publication July 2, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by Fondo de Investigacion Sanitaria Grants 02/1150, and Junta de Extremadura Grant 2PR03A007. J.R.C. was supported by Fondo de Investigacion Sanitaria Grant 02/1150, M.P.-G. by the Fundacion Fernando Valhondo Calaff, and M.D.R. and J.Z. by the Subdireccion General de Investigacion Sanitaria, Exp. CA06/0110 and 99/3082.

[2] Address correspondence and reprint requests to Dr. Jose Zamorano, Unidad de Investigacion, Hospital San Pedro de Alcantara, Avenida Pablo Naranjo s/n, 10003 Caceres, Spain. E-mail address: jose.zamorano@ses.juntaex.es

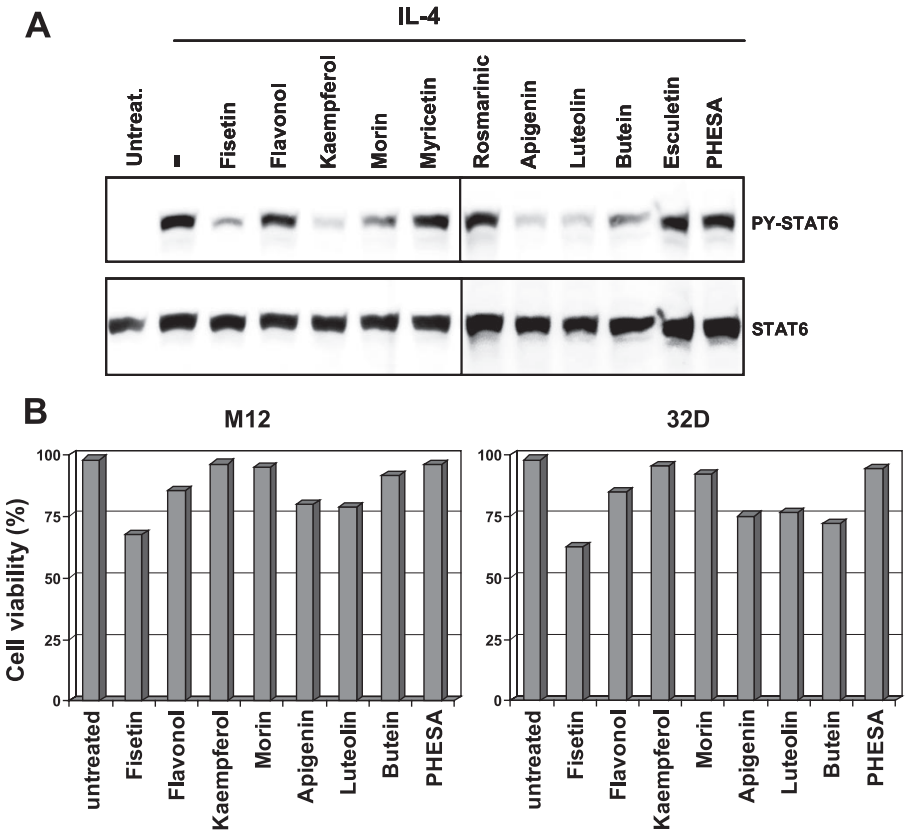

FIGURE 1. Effect of polyphenols in the phosphorylation of STAT6. *A*, M12 cells were treated with 40 μM of the indicated polyphenolic compounds for 1 h before stimulation with IL-4 for 15 min. Then, cell extracts were prepared and 5 μg of total protein was subjected to separation by SDS-PAGE. Phosphorylated STAT6 was first detected using a specific anti-STAT6 Ab that recognizes a domain surrounding phosphorylated Y641 (PY-STAT6, *upper blot*). Afterward, membranes were reprobed with a polyclonal anti-STAT6 Ab for total protein content (*lower blot*). *B*, Cells were incubated with 40 μM of the indicated compounds for 24 h. Then, cells were collected and viability was analyzed using the trypan blue exclusion method. Graphics are representative of triplicate experiments. PHESA, Phenethyl salicylate.

as antioxidants and inhibitors of cell-signaling proteins, including kinases and transcription factors (18–23). However, the molecular mechanisms underlying their biological effects have not been fully identified, suggesting the existence of undetermined molecular targets (18, 19, 24, 25). The fact that IL-4-driven STAT6 activation has been reported to play an active role in the progression of inflammatory diseases and cancer led us to investigate the effect of natural polyphenols in the activation of this transcription factor. Our data indicate that some of the tested compounds can block the activation of STAT6 by IL-4. Among these, kaempferol showed the highest inhibitory activity with the lowest cell toxicity. Further molecular studies indicated that kaempferol inhibited IL-4-induced STAT6 activation by specifically targeting JAK3 tyrosine kinase. This effect correlated with the inhibition of cell responses to IL-4 and other JAK3-dependent cytokines. These findings show a novel effect of some natural polyphenols that may be of therapeutic interest for diseases in which IL-4 and JAK3 are involved.

Materials and Methods

Cells and reagents

HL-60, U937, M12, and A1.1 were maintained in RPMI 1640 culture medium with glutamine, penicillin, streptomycin, and 10% FCS. 32D cells expressing IL-2Rβ have been previously described (26) and were maintained in the above medium supplemented with 10% WEHI-3-conditioned medium. The adherent cells used in this study were maintained in DMEM medium supplemented as above. Src8 is an embryonic fibroblast cell line obtained from Dr. X. Zhan (Holland Laboratory, American Red Cross, Rockville, MD) that has been previously described (27). The U4A mutant human fibrosarcoma cell line defective in JAK1—developed by Drs. I. M. Kerr (Imperial Cancer Research Fund, London, U.K.) and G. R. Stark (Cleveland Clinic Foundation, Cleveland, OH)—and U4A-expressing JAK1 (U4A/JAK1) were maintained as described (28). Primary BALB/c splenic cells were obtained by careful mincing of the spleens following incubation in lysis buffer (0.15 M NHCl, 1.0 mM KHCO and 0.1 mM EDTA (pH 7.2)) for 5 min to remove red cells. Cell viability was analyzed by using the trypan blue exclusion method. The study was approved by the Ethic Committee of Clinical Investigation (San Pedro de Alcantara Hospital, Caceres, Spain).

Anti-JAK1 and -JAK3 Abs were obtained from Upstate Biotechnology, RC20 anti-phosphotyrosine Ab from BD Biosciences, and STAT5, STAT6, phosphorylated STAT6, and Src Abs from Santa Cruz Biotechnology. All chemical reagents used in this study were purchased from Sigma-Aldrich and cytokines were obtained from R&D Systems.

Immunoprecipitation and immunoblotting

After the indicated culture conditions, cell pellets were treated with lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM NaF, 10 mM pyrophosphate, 1 mM PMSF, and protease inhibitor mixture) and clarified by centrifugation. The soluble fractions were then separated on a 7.5% SDS-polyacrylamide gel before transfer to a polyvinylidene difluoride membrane. Membranes were then probed with the indicated Abs. The bound Ab was detected using ECL (Pierce Biotechnology) in the LAS-3000 imaging system (Fuji Photo Film). Where indicated, proteins were immunoprecipitated with specific Abs followed with protein G-Sepharose as we have previously described (13). The washed precipitates were then analyzed by Western blot as above.

Kinase assays

JAK1, JAK3, and Src kinases were precipitated as described above. Precipitates were washed in kinase buffer (50 mM HEPES (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 50 μM Na_3VO_4, and protease inhibitors) and incubated in the presence of mentioned inhibitors. Enzymatic reaction was initiated with the addition of [γ-^{32}P]ATP (Amersham) and incubated for an 20 additional min. Samples were then separated on polyacrylamide gels, dried, and exposed to film. Radioactivity was detected using the FLA-5000 imaging system (Fuji Photo Film).

Analysis of CD23 expression

CD23 expression was analyzed on monocytic cells as previously described (13). PBMC isolated from blood samples by density gradient centrifugation were cultured for 30 h in the presence of indicated compounds. Then, cells were stained with FITC-conjugated anti-human CD23 Ab (Immunotech), and analyzed by flow cytometer (FACScan; BD Biosciences). Viable monocytic cells were selected using forward-scatter and side-scatter parameters.

Proliferation assays

Cell proliferation was determined using the MTT assay as previously described (29). Briefly, 32D cells expressing IL-2Rβ were grown in 96-well plates in the presence of the indicated cytokines and kaempferol for 24 h. Afterward, 20 μl of MTT (5 mg/ml) was added for additional 3 h. Formazan crystals were dissolved with 10% SDS and 0.01 N HCl solution before reading absorbance at 595 nm in a Tecan spectrophotometer. Relative cell proliferation is directly related to the absorbance detected at 595 nm (29).

Mixed lymphocyte culture

Stimulator and responder PBMC were isolated from unrelated donors although HLA typing was not performed. To culture, 4×10^5 responder cells (donor 1) were incubated with the same amount of mitomycin C-treated stimulator cells (donor 2) for 5 days in U-bottom microtiter plates. Where indicated, cells were treated with increasing amount of kaempferol. Afterward, MTT was added for additional 3 h and relative proliferation was determined by measuring absorbance at 595 nm as above.

Results

Inhibition of STAT6 activation by natural polyphenols

The fact that IL-4-driven STAT6 regulation has been implicated in diseases that natural polyphenols may influence (6–8, 15–25), led us to investigate whether these compounds affect STAT6 activation. To this end, cells were incubated with most common flavonols and related compounds for 1 h before stimulation with IL-4 (Fig. 1). To this end, we initially tested a concentration of 40 μM because this concentration has been reported to have biological effects in these compounds (Refs. 18–25 and references included). The data indicate that some tested compounds could inhibit IL-4-induced STAT6 phosphorylation. Among the flavonols tested, fisetin, kaempferol, and morin clearly inhibited STAT6 phosphorylation while flavonol and myricetin had little effect. Analysis of other polyphenols indicated that the most-related flavonoids apigenin, luteolin, and butein also could inhibit IL-4-induced STAT6 phosphorylation. In contrast, other less-related compounds like esculetin, rosmarinic acid, and phenethyl salicylate had little effect on the activation of STAT6 at the dose tested. To investigate further the molecular mechanisms involved in this inhibition, our efforts were focused on kaempferol because it showed the highest inhibitory activity with the least effect on cell viability. As shown in Fig. 1*B*, treatment of cells with kaempferol did not affect cell viability while other compounds that could inhibit STAT6 phosphorylation induced an important percentage of death in all cell lines analyzed. These data suggested that kaempferol may have a more specific effect on IL-4 signaling than the other compounds tested.

Kinetics analysis performed in several hemopoietic cell types indicated that kaempferol inhibited STAT6 phosphorylation in a dose-dependent way (Fig. 2). Thus, pretreatment of M12 cells with 40 μM kaempferol completely blocked the phosphorylation of STAT6 induced by IL-4, and a lower concentration of 10 μM could still produce a notable inhibition. A similar inhibitory effect of kaempferol was observed in primary lymphocytes (Fig. 2*B*) and in human and murine cell lines of hemopoietic origin such as A1.1, 32D, HL-60, and U937 (figures below and data not shown). This inhibitory effect of kaempferol correlated with its effect on cell responses to IL-4. As an example, kaempferol inhibited the ability of IL-4 to signal STAT6-dependent events like CD23 induction and more complex processes like protection from apoptosis. Thus, kaempferol inhibited the specific induction of CD23 expression by IL-4 on monocytic primary cells (Fig. 2*C*). In this case, 40 μM kaempferol completely abrogated the induction of CD23 and an important inhibition was observed at lower concentrations. Similar to other cell types shown in Fig. 1, treatment of primary splenic cells with kaempferol had little effect on spontaneous cell death.

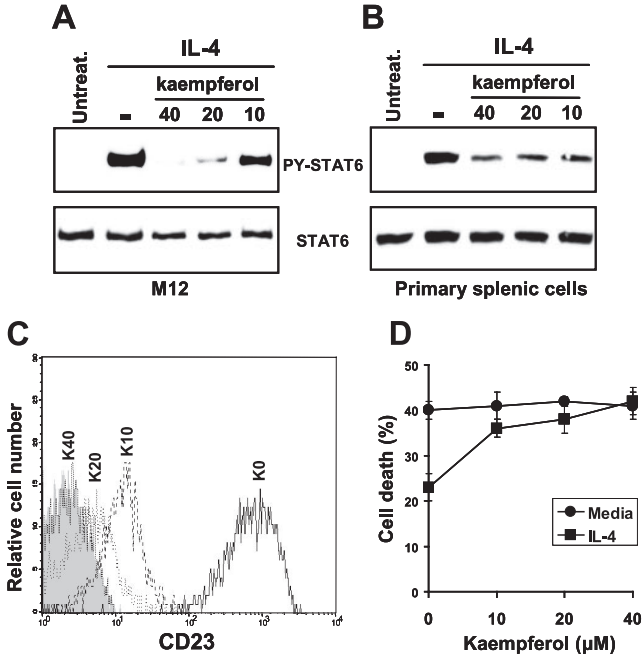

FIGURE 2. Kinetics of STAT6 inhibition by kaempferol. *A*, M12 cells were incubated with the indicated concentrations of kaempferol (micromoles) for 1 h before stimulation with IL-4 for 15 min. Then, cell extracts were separated by SDS-PAGE and immunoblotted with anti-phosphorylated STAT6 Ab to detect tyrosine-phosphorylated STAT6 (PY-STAT6; *upper blot*). Membranes were then stripped and reprobed with anti-STAT6 Ab (STAT6; *lower blot*). *B*, Murine splenic cells were incubated with the indicated amount of kaempferol for 1 h before treatment with IL-4. STAT6 was analyzed as above. *C*, PBMC were stimulated for 30 h with IL-4 in the absence (K0) or presence of 10 (K10), 20 (K20), and 40 μM (K40) kaempferol. As control, cells were cultured without treatments (filled histogram). Note that histograms from untreated cells and cells stimulated with IL-4 in the presence of 40 μM kaempferol (K40) overlaps. *D*, Mouse splenic cells were cultured in the presence (■) or absence (●) of IL-4 and various concentrations of kaempferol. After 48 h of culture, cell death was analyzed using the trypan blue exclusion method. Graphics are representative of two independent experiments.

However, the same amount of kaempferol inhibited the ability of IL-4 to prevent cell death in a dose-dependent manner (Fig. 2*D*). These data supported that kaempferol had an important inhibitory effect on cell responses to IL-4.

Kaempferol does not affect IL-4-driven Src activation

To investigate the mechanisms underlying the inhibition, we analyzed the effect of kaempferol in kinases involved in the activation of STAT6 because natural polyphenols have been found to modulate the action of this type of enzymes (18–23). To this end, we first analyzed Src activation by IL-4. Previous studies suggested that this kinase was involved in the earlier events that led to STAT6 phosphorylation (13). As shown in Fig. 3*A*, IL-4 induced the activation of Src, as demonstrated by the increasing autophosphorylation activity in Src precipitates from cells stimulated with IL-4. However, the activation of Src by IL-4 was not affected by pretreatment of cells with kaempferol at doses previously found to inhibit STAT6 activation. As control, treatment of cells with sodium salicylate inhibited src activation by IL-4 as previously demonstrated (13). These findings suggested that kaempferol exerted its effect independently of this kinase. This inference was further corroborated in the U4A cell line defective in JAK1. Previous studies indicated that a lack of JAK1 expression abrogated cell responses to IL-4, including STAT6 activation (30, 31). However,

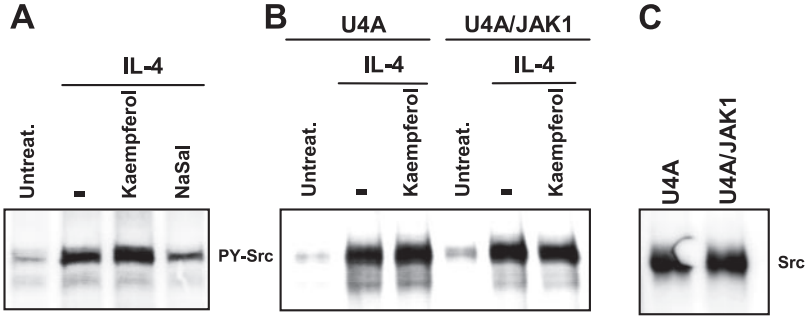

FIGURE 3. Kaempferol does not affect Src activation by IL-4. M12 (*A*), and U4A and U4A-expressing JAK1 (U4A/JAK1) cells (*B*) were treated or not with 40 μM kaempferol or 20 mM sodium salicylate (NaSal) before stimulation with IL-4. Then, cell extracts were incubated with anti-Src Ab followed by precipitation with protein G-Sepharose. The kinase activity of precipitates was analyzed by autophosphorylation using radioactive ATP. *C*, The same amount of cell extracts from U4A and U4A/JAK1 were immunoprecipitated with anti-Src Ab and separated by SDS-PAGE. Transferred protein was immunoblotted with anti-Src Ab to detect Src protein expression.

this outcome was not the case for Src because IL-4 could signal its activation in these cells (Fig. 3*B*). Thus, treatment of U4A cells with IL-4 promoted increased autophosphorylation activity of Src precipitates as compared with samples from unstimulated cells. In fact, the levels of Src phosphorylation induced by IL-4 were similar in U4A and U4A cells expressing JAK1 (U4A/JAK1), indicating that Src activation by IL-4 was independent on JAK1 expression. As control, the expression of Src was similar in both types of cells (Fig. 3*C*). In this case, preincubation of U4A cells with kaempferol also did not affect activation of Src by IL-4. Taken together, these data demonstrated that activation of Src by IL-4 was independent of JAKs and not affected by kaempferol.

Kaempferol inhibits JAK3 but not JAK1 activity

The importance of JAK1 and JAK3 in the activation of STAT6 by IL-4 in hemopoietic cells is well-documented (9–13). Therefore, we next investigated the effect of kaempferol in the activation of these kinases. In contrast to Src, we found that pretreatment of cells with kaempferol prevented tyrosine phosphorylation of JAK1 and JAK3 induced by IL-4 (Fig. 4). The amount of kaempferol required to inhibit the phosphorylation of these kinases was the same required to block STAT6 phosphorylation, supporting the idea that these effects were related.

To investigate whether JAKs were a direct target for kaempferol, we analyzed the effect of this compound on the in vitro activity of JAK1 and JAK3 (Fig. 5). The results indicated that the enzymatic activity of JAK3 but not JAK1 was inhibited by kaempferol. Thus, incubation of JAK1 immunoprecipitates with kaempferol did not affect the autophosphorylation activity of this enzyme (Fig. 5*A*). In contrast, the same concentration of kaempferol inhibited the in vitro activity of JAK3, as demonstrated by the lack of autophosphorylation (Fig. 5*B*). As control, the JAK inhibitor AG490 blocked the in vitro activity of JAK1 and JAK3, as previously reported (13).

Kaempferol inhibits STAT6 activation only in cells expressing JAK3

The above data suggested that kaempferol could inhibit the activation of STAT6 by IL-4 by targeting the kinase activity of JAK3. To investigate further, we analyzed the effect of kaempferol in several cell types expressing and lacking JAK3 (Fig. 6). To this end, we first analyzed the expression of JAK3 in several cell lines of hemopoietic and nonhemopoietic origin (Fig. 6*A*). Among the analyzed cells, 32D, Src8, and NIH3T3 expressed JAK3 as detected by specific immunoprecipitation followed by Western blotting. In contrast, the tumoral cell lines LOVO, SW480, MCF-7, HT-29, and HCT-15 lacked JAK3 expression. When activation was analyzed, we found that IL-4 could induce STAT6 phosphorylation in all cell lines regardless of JAK3 expression (Fig. 6*B*). This result can be explained by the presence of functional type II IL-4R in cells lacking JAK3 (9–12). However, when we analyzed the effect of kaempferol on the phosphorylation of STAT6 by IL-4, we found that it depended on the expression of JAK3. Thus, pretreatment of 32D, Src8, and NIH3T3 cells expressing JAK3 with kaempferol inhibited the ability of IL-4 to signal STAT6 phosphorylation. In contrast, kaempferol had no effect on the activation of STAT6 by IL-4 in LOVO, SW480, MCF7, HT-29, or HCT-15

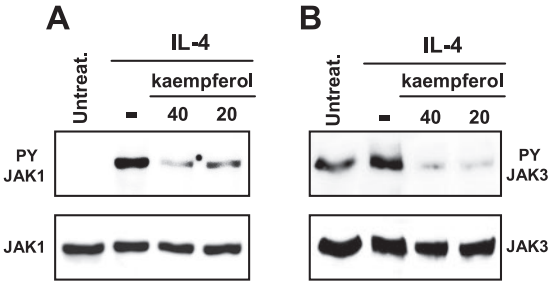

FIGURE 4. Kaempferol inhibits JAK1 and JAK3 phosphorylation by IL-4. M12 cells were incubated with kaempferol (micromoles) for 1 h before stimulation with IL-4 for 5 min. Cell lysates were treated with anti-JAK1 (*A*) or anti-JAK3 Abs (*B*) followed by protein G-Sepharose. Precipitates were separated by SDS-PAGE and immunoblotted with an antiphosphotyrosine Ab to detect tyrosine-phosphorylated kinases (*upper blots*). Membranes were stripped and reprobed with anti-JAK1 (*A*) or anti-JAK3 Abs (*B*; *lower blots*).

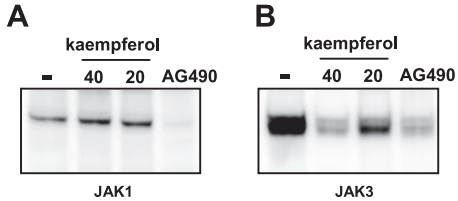

FIGURE 5. Kaempferol inhibits JAK3 but not JAK1 activity. M12 cell extracts were prepared from IL-4-stimulated cells and precipitated with anti-JAK1 (*A*) or anti-JAK3 (*B*). Precipitates were then incubated with nothing or the indicated amount of kaempferol (micromoles) or AG490 (50 μM) for 20 min. The kinase activity of precipitates was then analyzed by autophosphorylation using radioactive ATP.

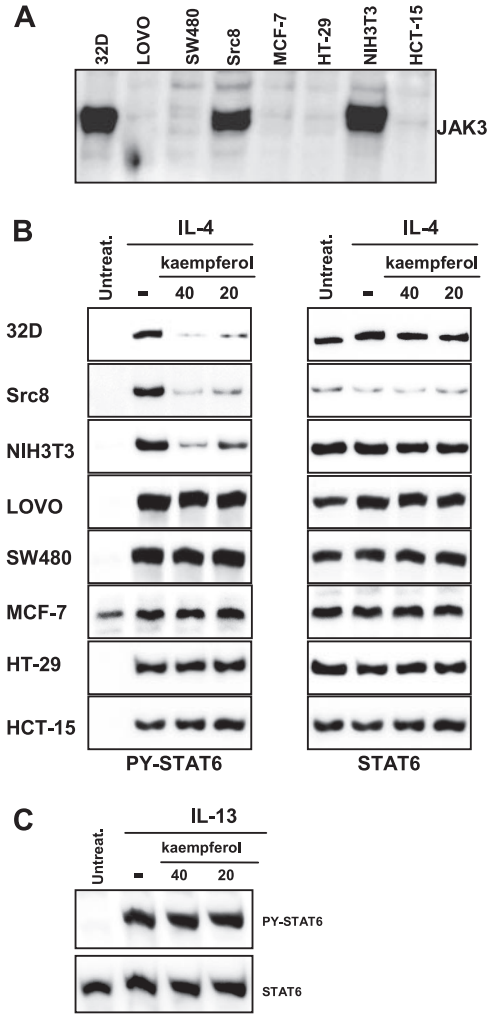

FIGURE 6. Kaempferol inhibits STAT6 phosphorylation in JAK3-expressing cells. *A*, Cells extracts prepared from the indicated cell lines were precipitated with a specific anti-JAK3 Ab followed by protein G-Sepharose. Precipitates were separated by SDS-PAGE and immunoblotted with anti-JAK3. *B*, The indicated cell lines were treated or not with kaempferol (μM) for 1 h before stimulation with IL-4 for 15 min. Then phosphorylated (PY-STAT6, *left blots*) and total STAT6 (STAT6, *right blots*) were determined as in Fig. 1. *C*, HT-29 cells were incubated with the indicated amount of kaempferol (micromoles) for 1 h before stimulation with IL-13 for 15 min. Phosphorylated and total STAT6 was determined as above.

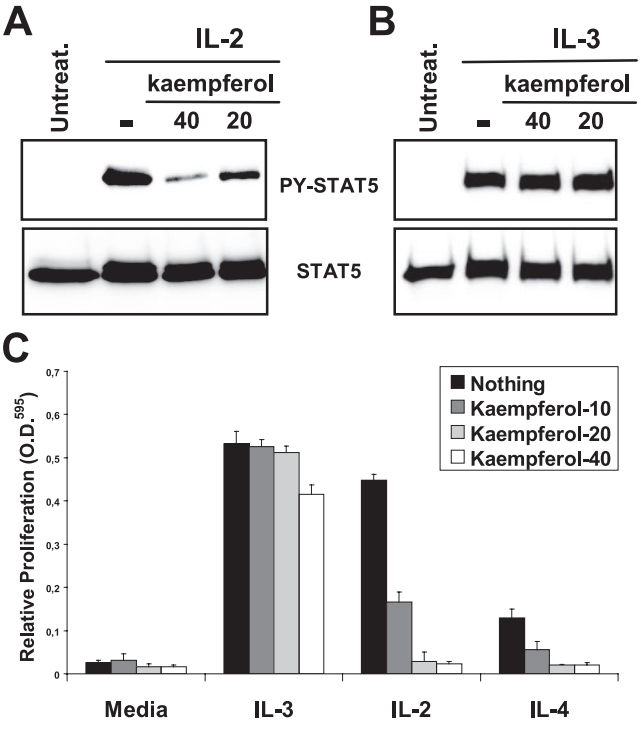

FIGURE 7. Effect of kaempferol in cell responses to IL-2 and IL-3. 32D cells expressing IL-2Rβ were treated or not with kaempferol (μM) for 1 h before the stimulation with IL-2 (*A*) or IL-3 (*B*) for 15 min. Then, STAT5 was precipitated from cell lysates using anti-STAT5 Ab followed by protein G-Sepharose. Precipitates were separated by SDS-PAGE and immunoblotted with an antiphosphotyrosine Ab to detect tyrosine phosphorylated (PY-STAT5, *upper blots*). Membranes were stripped and reprobed with anti-STAT5 Ab (STAT5, *lower blots*). *C*, Cells were incubated with nothing, IL-3, IL-2, and IL-4 in the absence or presence of the indicated amount of kaempferol (micromoles) for 27 h. To determine proliferation, samples were incubated with MTT during the last 3 h of culture. Relative proliferation represents the absorbance of samples at 595 nm. Values represent means ± SD of triplicate experiments.

tumoral cells lacking JAK3. Because these tumoral cells are expected to signal through type II IL-4Rs, these observations suggested that kaempferol could block IL-4 signaling by specifically inhibiting type I IL-4Rs containing JAK3. In accordance with these findings, kaempferol had little effect on the activation of STAT6 induced by IL-13, a cytokine that also signals through type II IL-4Rs (11, 12) (Fig. 6*C*). In this case, treatment of HT-29 cells with kaempferol also had no effect on the phosphorylation of STAT6 induced by IL-13. These findings supported that kaempferol had no inhibitory effect on type II IL-4Rs lacking JAK3.

Kaempferol inhibits JAK3-dependent but not -independent cytokines

The findings that kaempferol inhibits IL-4-induced STAT6 activation by targeting JAK3 suggested that this compound could affect other cytokines that require JAK3 for signaling. The results indicate that this was the case (Fig. 7). We found that treatment of 32D cells expressing IL-2Rβ with kaempferol prevented the phosphorylation of STAT5 induced by IL-2, a cytokine that signals through JAK3 (32) (Fig. 7*A*). In contrast, kaempferol did not affect the phosphorylation of STAT5 by IL-3, a cytokine that does not require JAK3 for signaling (33) (Fig. 7*B*). To investigate the biological consequences of these observations, we analyzed the effect of kaempferol in cell proliferation (Fig. 7*C*). As previously reported (34), IL-3 and IL-2 were able to induce proliferation in 32D cells expressing IL-2Rβ. However, whereas kaempferol had little effect in cell proliferation induced by IL-3, it completely blocked proliferation induced by IL-2. Thus, 40 and 20 μM kaempferol almost completely blocked cell proliferation induced by IL-2 and 10 μM inhibited proliferation by >60%. Kaempferol had a similar inhibitory effect when these cells were stimulated with IL-4 though this cytokine induced lower incorporation of MTT. In contrast, doses of 20 and 10 μM of kaempferol did not affect cell proliferation induced by IL-3 although higher concentrations had a little effect. Like in Fig. 1*B*, kaempferol did no affect cell viability under these experimental conditions. Taken together, these data support the hypothesis that kaempferol can specifically inhibit the signaling of JAK3-dependent cytokines.

Kaempferol inhibits mixed lymphocyte culture proliferation

JAK3 has been proposed as a therapeutic target for transplantation given its role in T cell regulation by specific cytokines (35, 36).

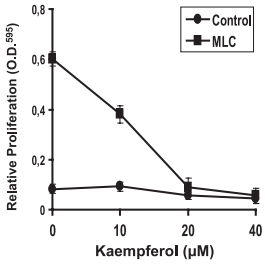

FIGURE 8. Inhibition of MLC reaction by kaempferol. Responder PBMC were cultured with mitomycin C-treated unrelated stimulator cells in presence or absence of the indicated amount of kaempferol for 5 days (■). Then, MTT was added for additional 3 h. Relative proliferation was determined by measuring the formation of formazan crystals at 595 nm. As control, responder cells were cultured alone (●). Values represent means ± SD of triplicate experiments.

The fact that kaempferol inhibited cytokine signaling by targeting JAK3 suggested that this compound may be useful in transplantation. To investigate it, we analyzed the effect of kaempferol on MLC. MLC is an in vitro test for the graft-vs-host-disease that measures allogeneic reactivity between lymphocytes. This reaction involves multiple cellular mediators including JAK3-dependent cytokines like IL-2 and IL-4 (37). The data found indicate that kaempferol prevented MLC proliferation in a dose-dependent manner (Fig. 8). Thus, treatment of MLC with 40 and 20 μM kaempferol prevented cell proliferation and an important inhibition was observed at lower doses. The inhibition of MLC proliferation was similar to JAK3, suggesting that these events were related. The fact that kaempferol inhibited cell proliferation on MLC suggests that it may be useful to prevent allogeneic responses.

Discussion

In this study, we found that some flavonoids can inhibit IL-4 activation of STAT6. These compounds include fisetin, kaempferol, morin, apigenin, luteolin, and butein. Previous studies have indicated that natural polyphenols can act as modulators of signal transduction pathways. Thus, they have been reported to inhibit a variety of enzymes and transcription factors including tyrosine kinases and NF-κB, in addition to having antioxidant properties (18–25). However, the molecular pathways underlying their biological effects are not fully understood, and some reports indicate that undetermined molecular targets must account for their effects (18, 19, 24, 25). The findings that some flavonoids can inhibit STAT6 activation may aid in understanding their effects in inflammatory diseases and cancer, in which IL-4 and STAT6 are involved (6–8, 15–17).

To investigate the molecular mechanisms underlying flavonoid inhibition, we focused on kaempferol because 1) it displays the highest inhibitory activity with the lowest cell toxicity and 2) it is one of the most abundant polyphenols (18, 19). These attributes may confer on it an important advantage over other inhibitors as potential modulators of IL-4 signaling. Our results indicate that kaempferol inhibits IL-4-induced STAT6 activation by specifically targeting the tyrosine kinase JAK3. This specificity of kaempferol for JAK3 is supported by the fact that it inhibited STAT6 activation only in cells expressing this kinase. As mentioned, IL-4 can signal through type I and II receptors (9). Type I requires JAK1 and JAK3 for signaling while JAK3 is substituted in the type II receptors by other JAKs (9–12). The fact that the inhibition induced by kaempferol is restricted to cells expressing JAK3 suggests that it may inhibit only type I IL-4Rs. This hypothesis was further supported by the fact that kaempferol did not affect activation of STAT6 by IL-13, a cytokine proposed to signal through type II IL-4R (11, 12). Therefore, kaempferol can be a useful tool for selectively inhibiting cell responses to IL-4 by blocking the type I IL-4R. Because type I receptors are mainly restricted to hemopoietic cells (9–12), kaempferol may not affect IL-4 and IL-13 signaling in other cell types as demonstrated in colon cancer cell lines. These findings provide molecular data to enhance our understanding of the beneficial effect of natural polyphenols in diseases involving these signaling molecules, such as the proven effectiveness of kaempferol in murine models of atopic dermatitis and anaphylaxis (38, 39).

It is remarkable that kaempferol inhibited IL-4 phosphorylation of JAKs without affecting the activation of Src kinase. These findings suggest that the activation of Src by IL-4 is independent of JAKs, which was further demonstrated in the U4A cell line lacking JAK1. In these cells, IL-4 could signal Src activation even though it appears to be unable to signal STAT6 activation (30, 31). Previous findings have suggested that Src activation precedes JAKs because inhibition of Src severely affected the activation of JAK1, JAK3, and STAT6 by IL-4 (13). These new findings support the hypothesis that JAKs act downstream and are not required for Src activation in IL-4 signaling.

The importance of kaempferol as a JAK3 inhibitor should not be restricted to IL-4 signaling. Kaempferol may also affect other cytokines that require JAK3 for signaling, such as IL-2, IL-7, IL-9, IL-15, and IL-21 (40). In fact, the data we found suggested that kaempferol inhibited IL-2 signaling to the same extent as IL-4. In this case, kaempferol was also able to inhibit IL-2-induced STAT5 phosphorylation and cell proliferation. In contrast, kaempferol had little effect on STAT5 phosphorylation and cell proliferation induced by IL-3, a cytokine that does not require JAK3 for signaling. Other authors have reported that kaempferol can reduce the constitutive phosphorylation of STAT3 in KNC differentiated colon cancer cells by undetermined mechanisms (41). Although it needs to be investigated, it is tentative to hypothesize that JAK3 activation may account for the constitutive phosphorylation of STAT3 in KNC cells.

The inhibitory effects of kaempferol raise the possibility that it would be useful in controlling T cell-dependent responses mediated by JAK3-dependent cytokines. The potential of JAK3 as a therapeutic target was evidenced by the findings that severe combined immune deficiency syndrome is caused by JAK3 abnormalities (35). This syndrome is characterized by a lack of T cell-dependent responses and a consequent profound immune suppression. Thus, the specific inhibition of JAK3 should provoke a T cell immune suppression that may be of enormous therapeutic value in lymphoproliferative diseases with JAK3 abnormalities (42, 43) and transplant rejection (35, 36). Accordingly, several groups have reported the characterization of synthetic JAK3 inhibitors (36, 43, 44). A common characteristic of these compounds is their similar size and planarity due to the presence of two adjacent aromatic rings. Comparative analysis indicates that kaempferol demonstrates a size and planar structure similar to those of some reported JAK3 inhibitors (43). Moreover, kaempferol contains a hydroxyl group in the 4′ position of the phenyl group proposed to be required to confer JAK3 inhibitory activity (43). As expected, some of these compounds have been reported to be effective in the prevention of organ allograft rejection (36). Accordingly, we have found that kaempferol can inhibit cell responses in MLC, suggesting that this compound could prevent allogeneic reactions. Taken together, this evidence point out that kaempferol can be a good candidate for investigations in transplantation and other JAK3-dependent diseases. In this regard, kaempferol may have several advantages over synthetically designed inhibitors: it (1) is a naturally available compound, (2) has low cell toxicity, (3) has already been reported to be effective in

murine models of atopic dermatitis and anaphylaxis (38, 39), and 4) prevents MLC proliferation.

In summary, our findings provide evidence that kaempferol could be a useful tool to selectively control cell responses to IL-4 and, by extension, to JAK3-dependent cytokines, and suggest a potential therapeutic use of kaempferol in diseases involving these proteins.

Disclosures

The authors have no financial conflict of interest.

References

1. Paul, W. E. 1991. Interleukin-4: a prototypic immunoregulatory lymphokine. *Blood* 77: 1859–1870.
2. Zamorano, J., H. Y. Wang, L. M. Wang, J. H. Pierce, and A. D. Keegan. 1996. IL-4 protects cells from apoptosis via the insulin receptor substrate pathway and a second independent signaling pathway. *J. Immunol.* 157: 4926–4934.
3. Lutz, M. B., M. Schnare, M. Menges, S. Rossner, M. Rollinghoff, G. Schuler, and A. Gessner. 2002. Differential functions of IL-4 receptor types I and II for dendritic cell maturation and IL-12 production and their dependency on GM-CSF. *J. Immunol.* 169: 3574–3580.
4. Yanagida, M., H. Fukamachi, K. Ohgami, T. Kuwaki, H. Ishii, H. Uzumaki, K. Amano, T. Tokiwa, H. Mitsui, and H. Saito. 1995. Effects of T-helper 2-type cytokines, interleukin-3 (IL-3), IL-4, IL-5, and IL-6 on the survival of cultured human mast cells. *Blood* 86: 3705–3714.
5. Urban, J. F., N. Noben-Trauth, D. D. Donaldson, K. B. Madden, S. C. Morris, M. Collins, and F. D. Finkelman. 1998. IL-13, IL-4Ra, and Stat6 are required for the expulsion of the gastrointestinal nematode parasite *N. brasiliensis*. *Immunity* 8: 255–264.
6. Finnegan, A., M. J. Grusby, C. D. Kaplan, S. K. O'Neill, H. Eibel, T. Koreny, M. Czipri, K. Mikecz, and J. Zhang. 2002. IL-4 and IL-12 regulate proteoglycan-induced arthritis through Stat-dependent mechanisms. *J. Immunol.* 169: 3345–3352.
7. Tomkinson, A., C. Duez, G. Cieslewicz, J. C. Pratt, A. Joetham, M. C. Shanafelt, R. Gundel, and E. W. Gelfand. 2001. A murine IL-4 receptor antagonist that inhibits IL-4- and IL-13-induced responses prevents antigen-induced airway eosinophilia and airway hyperresponsiveness. *J. Immunol.* 166: 5792–5800.
8. Prokopchuk, O., Y. Liu, D. Henne-Bruns, and M. Kornmann. 2005. Interleukin-4 enhances proliferation of human pancreatic cancer cells: evidence for autocrine and paracrine actions. *Brit. J. Cancer* 92: 921–928.
9. Nelms, K., A. D. Keegan, J. Zamorano, J. J. Ryan, and W. E. Paul. 1999. The IL-4 receptor: signaling mechanisms and biologic functions. *Annu. Rev. Immunol.* 17: 701–738.
10. Russell, S. M., A. D. Keegan, N. Harada, Y. Nakamura, M. Noguchi, P. Leland, M. C. Friedmann, A. Miyajima, R. K. Puri, W. E. Paul, and W. J. Leonard. 1993. Interleukin-2 receptor γ chain: a functional component of the interleukin-4 receptor. *Science* 262: 1880–1883.
11. Miloux, B., P. Laurent, O. Bonnin, J. Lupker, D. Caput, N. Vita, and P. Ferrara. 1997. Cloning of the human IL-13Ral chain and reconstitution with the IL4Ra of a functional IL-4/1L-13 receptor complex. *FEBS Lett.* 401: 163–166.
12. Hershey, G. K. 2003. IL-13 receptors and signalling pathways: an evolving web. *J. Allergy Clin. Immunol.* 111: 677–690.
13. Perez-G, M., M. Melo, A. D. Keegan, and J. Zamorano. 2002. Aspirin and salicylates inhibit the IL-4- and IL-13-induced activation of STAT6. *J. Immunol.* 168: 1428–1434.
14. Takeda, K., T. Tanaka, W. Shi, M. Matsumoto, M. Minami, S. Kashiwamura, K. Nakanishi, N. Yoshida, T. Kishimoto, and S. Akira. 1996. Essential role of Stat6 in IL-4 signalling. *Nature* 380: 627–630.
15. Chitnis, T., N. Najafian, C. Benou, A. D. Salama, M. J. Grusby, M. H. Sayegh, and S. J. Khoury. 2001. Effect of targeted disruption of STAT4 and STAT6 on the induction of experimental autoimmune encephalomyelitis. *J. Clin. Invest.* 108: 739–747.
16. Akimoto, T., F. Numata, M. Tamura, Y. Takata, N. Higashida, T. Takashi, K. Takeda, and S. Akira. 1998. Abrogation of bronquial eosinophilic inflamation and airways hyperreactivity in signal transducers and activators of transcription (STAT)6-deficient mice. *J. Exp. Med.* 187: 1537–1542.
17. Terabe, M., S. Matsui, N. Noben-Trauth, H. Chen, C. Watson, D. D. Donaldson, D. P. Carbone, W. E. Paul, and J. A. Berzofsky. 2000. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat. Immunol.* 1: 515–520.
18. Middleton, E. Jr., C. Kandaswami, and T. C. Theoharides. 2000. The effects of plant flavonoides on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* 52: 673–751.
19. Havsteen, B. H. 2002. The biochemistry and medical significance of the flavonoids. *Pharmacol. Ther.* 96: 67–202.
20. Knekt, P., J. Kumpulainen, R. Jarvinen, H. Rissanen, M. Heliovaara, A. Reunanen, T. Hakulinen, and A. Aromaa. 2002. Flavonoid intake and risk of chronic disease. *Am. J. Clin. Nutr.* 76: 560–568.
21. Shaheen, S. O., J. A. Sterne, R. L. Thompson, C. E. Songhurst, B. M. Margetts, and P. G. Burney. 2001. Dietary antioxidants and asthma in adults: population-based case-control study. *Am. J. Respir. Crit. Care Med.* 164: 1823–1828.
22. Lu, J., L. V. Papp, J. Fang, S. Rodriguez-Nieto, B. Zhivotovsky, and A. Holmgren. 2006. Inhibition of mammalian thioredoxin reductase by some flavonoids: implications for myricetin and quercetin anticancer activity. *Cancer Res.* 66: 4410–4418.
23. Chen, C. C., M. P. Chow, W. C. Huang, Y. C. Lin, and Y. J. Chang. 2004. Flavonoids inhibit tumor necrosis factor-α-induced up-regulation of intercellular adhesion molecule-1 (ICAM-1) in respiratory epithelial cells through activator protein-1 and nuclear factor-κB: structure-activity relationships. *Mol. Pharmacol.* 66: 683–693.
24. Lolito, S. B., and B. Frei. 2006. Dietary flavonoids attenuate tumor necrosis factor α-induced adhesion molecule expression in human aortic endothelial cells: structure-function relationships and activity after first pass metabolism. *J. Biol. Chem.* 281: 37102–37110.
25. Birrell, M. A., K. McCluskie, S. Wong, L. E. Donnelly, P. J. Barnes, and M. G. Belvisi. 2005. Resveratrol, an extract of red wine, inhibits lipopolysaccharide induced airway neutrophilia and inflammatory mediators through an NF-κ-independent mechanism. *FASEB J.* 19: 840–841.
26. Wang, H. Y., W. E. Paul, and A. Keegan. 1996. IL-4 function can be transferred to the IL-2 receptor by tyrosine containing sequences found in the IL-4 receptor a chain. *Immunity* 4: 113–121.
27. Soriano, P., C. Montgomery, R. Geske, and A. Bradley. 1991. Targeted disruption of the c-*src* proto-oncogene leads to osteopetrosis in mice. *Cell* 64: 693–702.
28. Muller, M., J. Briscoe, C. Laxton, D. Guschin, A. Ziemiecki, O. Silvennoinen, A. G. Harpur, G. Barbieri, B. A. Witthuhn, C. Schindler, et al. 1993. The protein tyrosine kinase JAK1 complements defects in interferon-α/β and -γ signal transduction. *Nature* 366: 129–135.
29. Denizot, F., and R. Lang. 1986. Rapid colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* 22: 271–277.
30. Chen, X. H., B. K. Patel, L. M. Wang, M. Frankel, N. Ellmore, R. A. Flavell, W. J. LaRochelle, and J. H. Pierce. 1997. Jak1 expression is required for mediating interleukin-4-induced tyrosine phosphorylation of insulin receptor substrate and Stat6 signalling molecules. *J. Biol. Chem.* 272: 6556–6560.
31. Daniel, C., A. Salvekar, and U. Schindler. 2000. A gain-of-function mutation in STAT6. *J. Biol. Chem.* 275: 14255–14259.
32. Miyazaki, T., A. Kawahara. H. Fujii, Y. Nakagawa, Y. Minami, Z. J. Liu, I. Oishi, O. Silvennoinen, B. A. Witthuhn, J. N. Ihle, and T. Taniguchi. 1994. Functional activation of Jak1 and Jak3 by selective association with IL-2 receptor subunits. *Science* 266: 1045–1047.
33. Reddy, E. P., A. Korapati, P. Chaturvedi, and S. Rane. 2000. IL-3 signalling and the role of Src kinases, JAKs and STATs: a cover liaison unveiled. *Oncogene* 19: 2532–2547.
34. Zamorano, J., H. Y. Wang, R. X. Wang, Y. Shi, G. D. Longmore, and A.D. Keegan. 1998. Regulation of cell growth by IL-2: role of STAT5 in protection from apoptosis but not in cell cycle progression. *J. Immunol.* 160: 3502–3512.
35. Pesu, M., F. Candotti, M. Husa, S. R. Hofmann, L. D. Notarangelo, and J. J. O'Shea. 2005. Jak3, severe combined immunodeficiency, and a new class of immunosuppressive drugs. *Immunol. Rev.* 203: 127–142.
36. Changelian, P. S., M. E. Flanagan, D. J. Ball, C. R. Kent, K. S. Magnuson, W. H. Martin, B. J. Rizzuti, P. S. Sawyer, B. D. Perry, W. H. Brissette, et al. 2003. Prevention of organ allograft rejection by a specific Janus kinase 3 inhibitor. *Science* 302: 875–878.
37. Nikolic, B., S. Lee, R. T. Bronson, M. J. Grusby, and M. Sykes. 2000. Th1 and Th2 mediate acute graft-versus-host-disease, each with distinct end-organ targets. *J. Clin. Invest.* 105: 1289–1298.
38. Oku, H., and K. Ishiguro. 2001. Antipruritic and antidermatitic effect of extract and compounds of *Impatiens balsamina L.* in atopic dermatitis model NC mice. *Phytother. Res.* 15: 506–510.
39. Ishiguro, K., Y. Ohira, and H. Oku. 2002. Preventive effects of *Impatiens balsamina* on the hen egg-white lysozyme (HEL)-induced decrease in blood flow. *Biol. Pharm. Bull.* 25: 505–508.
40. Yamaoka, K., B. Min, Y. J. Zhou, W. E. Paul, and J. J. O'Shea. 2005. Jak3 negatively regulates dendritic-cell cytokine production and survival. *Blood* 106: 3227–3233.
41. Nakamura, Y., C. Chang, T. Mori, K. Sato, K. Ohtsuki, B. L. Upham, and J. E. Trosko. 2005. Augmentation of differentiation and gap function by kaempferol in partially differentiated colon cancer cells. *Carcinogenesis* 26: 665–671.
42. Kirken, R. A., R. A. Erwin, L. Wang, Y. Wang, H. Rui, and W. L. Farrar. 2000. Functional uncoupling of the Janus kinase 3-Stat5 pathway in malignant growth of human T cell leukaemia virus type 1-transformed human T cells. *J. Immunol.* 165: 5097–5104.
43. Sudbeck, E. A., X. P. Liu, R. K. Narla, S. Mahajan, S. Ghosh, C. Mao, and F. M. Uckun. 1999. Structure-based design of specific inhibitors of Janus kinase 3 as apoptosis-inducing antileukemic agents. *Clin. Cancer Res.* 5: 1569–1582.
44. Brown, G., R. A. M. Bamford, J. Bowyer, D. S. James, N. Rankine, E. Tang, V. Torr, and E. J. Culbert. 2000. Naphthyl ketones: a new class of Janus kinase 3 inhibitors. *Bioorg. Med. Chem. Lett.* 10: 575–579.