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*J Immunol* 2001; 166:3724-3732; doi: 10.4049/jimmunol.166.6.3724

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Concomitant Inhibition of Janus Kinase 3 and Calcineurin-Dependent Signaling Pathways Synergistically Prolongs the Survival of Rat Heart Allografts

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The cytoplasmatic localized Janus tyrosine kinase 3 (Jak3) is activated by multiple cytokines, including IL-2, IL-4, and IL-7, through engagement of the IL-2R common γ-chain. Genetic inactivation of Jak3 is manifested as SCID in humans and mice. These findings have suggested that Jak3 represents a pharmacological target to control certain lymphoid-derived diseases. Using the rat T cell line Nb2-11c, we document that tyrophostin AG-490 blocked in vitro IL-2-induced cell proliferation (IC50 ~ 20 μM), Jak3 auto-phosphorylation, and activation of its key substrates, Stat5a and Stat5b, as measured by tyrosine/serine phosphorylation analysis and DNA-binding experiments. To test the notion that inhibition of Jak3 provides immunosuppressive potential, a 7-day course of i.v. therapy with 5–20 mg/kg AG-490 was used to inhibit rejection of heterotopically transplanted Lewis (RT1l) heart allografts in ACI (RT1b) recipients. In this study, we report that AG-490 significantly prolonged allograft survival, but also acted synergistically when used in combination with the signal 1 inhibitor cyclosporin A, but not the signal 3 inhibitor, rapamycin. Finally, AG-490 treatment reduced graft infiltration of mononuclear cells and Stat5a/b DNA binding of ex vivo IL-2-stimulated graft infiltrating of mononuclear cells, but failed to affect IL-2Rα expression, as judged by RNase protection assays. Thus, inhibition of Jak3 prolongs allograft survival and also potentiates the immunosuppressive effects of cyclosporin A, but not rapamycin. The Journal of Immunology, 2001, 166: 3724–3732.

Selective immunosuppressive agents are needed both to mitigate organ transplant rejection and avoid serious side effect profiles, which include nephrotoxicity, neurotoxicity, and hyperlipidemia (1, 2). Therefore, elucidation of the novel molecular intermediates in T cell activation and proliferation may provide insights into the design of new therapeutic strategies. T cell activation represents a coordinated cascade of molecular events triggered by engagement of the TCR complex (TCR/CD3) via specific Ags (signal 1), which are then amplified by the co-stimulatory molecules B7-1/CD80 and CD40/CD154 (signal 2) that promote the synthesis and secretion of cytokines such as IL–2, which drive proliferation (signal 3) (3, 4). Present immunosuppressive strategies utilize cyclosporin A (CsA)4 or FK506 (tacrolimus) to inhibit calcineurin (CaN), a serine-threonine phosphatase critical for early G1–G1 phase transition of T cells (1, 4). The recent development of rapamycin (RAPA) has focused on the IL-2-driven response late in the G1 phase, via inhibition of the ubiquitously expressed serine-threonine kinase, mammalian target of RAPA (mTOR) (1, 5). Although the combination of RAPA with either CsA or tacrolimus acts synergistically in both experimental and clinical trials, they are associated with adverse reactions, including nephrotoxicities and hyperlipidemias (6, 7). Therefore, the present studies were based on the hypothesis that unique molecular targets can be identified in T cells, particularly those activated by T cell growth factors (TCGFs), including IL-2.

IL-2-mediated signals are propagated following the binding of ligand to the high affinity-conferring IL-2R α-chain (IL-2Rα) and heterodimerization of two members of the hemopoietin receptor superfAMILY, IL-2Rβ and IL-2Rγ (8–10). Cytokine engagement of this receptor triggers intermolecular transphosphorylation and activation of the receptor-associated protein tyrosine kinases, Janus kinase (Jak)1, which binds to IL-2Rβ, and Jak3, which binds to IL-2Rγ (11, 12). Immunosuppressive strategies designed to disrupt IL-2 or its receptors, IL-2Rα or IL-2Rβ, have achieved only limited success owing to the redundant and compensatory signaling pathways mediated by shared receptors recruited by other TCGFs (13–17). For example, while IL-2Rα exclusively binds IL-2, IL-2Rβ is recruited by either IL-2 or IL-15 (18). IL-2Rγ is more promiscuous, serving as a common receptor (γc) for multiple cytokines, including IL-2, IL-4, IL-7, IL-9, IL-13, and IL-15 (19). Thus, inactivation of the latter receptor would disrupt an entire family of TCGFs.

Jak3, which is primarily expressed in T and B cells, is activated through the γc and plays a critical role in T cell development and function (20, 21). In humans or mice, genetic inactivation of either the γc or receptor-associated Jak3 is manifested as the SCID (11, 12), infectious diseases (NIH R1-AI39026), and the Roche Foundation (862506002).
20, 21), apparently due to disruption of Jak3 substrates, including two transcription factors known as Stat5a and Stat5b (22). Stat5a/b gene-deficient mice are immunodeficient, although not as severely as γc or Jak3 knockouts, with their T cells unable to respond to the mitogenic effects of IL-2 (23). This immunodeficiency is presumably due to greatly reduced levels of several cell cycle proteins, including the cyclin-dependent kinase-6 and cyclins A, D2, D3, and E (24).

Despite the impressive results observed in these knockout mice, it was not clear whether inhibition of the Jak3/γc pathway produces similar defects in mature T cells or whether these deficits represent a developmental phenomenon, although reconstitution of T cells with the corresponding functional gene restores IL-2 responsiveness (25, 26). Moreover, treatment of wild-type mice with noncytolytic anti-γc mAbs extended the survival of pancreatic islet allografts (27). However, these studies did not examine whether increased graft acceptance was associated with impaired Jak3/Stat5 activity.

We showed previously that AG-490, a tyrosine phosphatase family member (B42) and derivative of benzylidene malononitrile (28, 29), inhibited Jak3 autokinase activity in PHA-activated human T cells (30). Moreover, AG-490 blocked the effect of γc cytokines IL-2, IL-7, IL-9, and IL-15 to activate Jak3 in T cells. In contrast, AG-490 failed to affect Ag-induced activation of tetanus toxoid-responsive human T cell clones, anti-CD3 mAb stimulation of Zap70 or p56Lck tyrosine phosphorylation, or expression of the IL-2Rα, β, or γ-chains (30, 31). Furthermore, Meydan et al. (32) found that AG-490 does not affect Lck, Lyn, Btk, Syk, Jak1, or Tyk2 kinase activity, but may exert effects on Jak2 activity. The present studies sought to investigate whether AG-490 achieves inhibition of Jak3 and its downstream substrates Stat5a/b in vivo and whether it prolongs allograft survival. We conclude that AG-490 inhibits Jak3 and a Stat5a/b serine kinase, in addition to interrupting cytokine-induced T cell proliferation and allograft rejection, but does not affect intragraft mRNA expression of the IL-2Rα chain. Finally, concomitant administration of AG-490 and the CaN antagonist CsA, but not the mTOR antagonist RAPA, acts synergistically to extend allograft survival.

Materials and Methods

Cell culture and treatment

The rat T cell line, Nb2-11c, originally developed by Peter Gout (Vancouver, Canada), was grown in RPMI 1640 with 10% FCS (Intergen, Purchase, NY; catalogue no. 1020-90), 2 mM l-glutamine, 5 mM HEPES (pH 7.3), and penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively), at 37°C in 5% CO2. Nb2 cells, which had been grown to a density of 1–1.5 × 106/ml, were incubated for 20–24 h in a lactate-free medium consisting of RPMI 1640 and 10% gelded horse serum (Sigma; catalogue no. H-1895) substituted for FCS, and were then adjusted to a density of 5 × 106 cells/ml and incubated at 37°C for 10 min with the appropriate cytokine. Freshly explanted human T lymphocytes purified by isoelectrophoresis (Ficoll; EM Science, Gibbstown, NJ) were PHA activated for 72 h, as previously described (30). Cells were rested and treated with varying concentrations of AG-490 (catalogue no. 658401; Calbiochem-Novabiochem, San Diego, CA), as described in the figure legends. All cells were then stimulated with 100 nM human rIL-2 (Hoffman-LaRoche, Basel, Switzerland) or TNF-α (B42) and derivative of benzylidene difluoride (Immobilon, catalogue no. IPVH 00010; Millipore, Bedford, MA), as previously described (12). Western blot analysis was performed with either pAbs or murine anti-phosphotyrosine mAbs (4G10, catalogue no. 05-321; Upstate Biotechnology) that had been diluted either 1/1000, respectively, or 1/5000 in blocking buffer for phosphoserine Stat5 rabbit polyclonal Abs corresponding to the mCR-1 kit according to the manufacturer’s recommendations (PharMingen, San Diego, CA). Hybridized RNA probes for Stat5a, Stat5b (aa 777–787), while phosphoserine Stat5 rabbit polyclonal Abs (pAbs) were generated against a phosphopeptide surrounding Stat5a (33). Proteins bound to Abs were captured by incubation for 30 min with protein A-Sepharose beads (Pharmacia, Piscataway, NJ), sedimented for purification, and eluted by boiling in 2× SDS sample buffer (20% glycerol, 10% 2-ME, 4.6% SDS, 0.004% bromophenol blue in 0.125 M Tris, pH 6.8) for 4 min. After separation of proteins on 7.5% SDS-PAGE under reducing conditions, proteins were transferred to polyvinylidene difluoride (Immobilon, catalogue no. PVDFH 00010; Millipore, Bedford, MA). Western blot analysis was performed with either pAbs or murine anti-phosphotyrosine mAbs (4G10, catalogue no. 05-321; Upstate Biotechnology) that had been diluted either 1/1000, respectively, or 1/5000 in blocking buffer for phosphoserine Stat5a/b mAbs, as previously described (12).

Western blot analysis

Frozen cell pellets were thawed on ice and solubilized in lysis buffer (10% cells/ml), as previously described (30). Depending on the experiment, supernatants were incubated rotating end over end for 2 h at 4°C with either 5 μg/ml polyclonal rabbit antisera raised against Jak3 (Upstate Biotechnology, Lake Placid, NY) or carboxyl termini of human Stat5a (aa 773–794) or Stat5b (aa 777–787), while phosphoserine Stat5 rabbit polyclonal Abs (pAbs) were generated against a phosphopeptide corresponding to the mCR-1 kit according to the manufacturer’s recommendations (PharMingen, San Diego, CA). Hybridized RNA probes for Stat5a, Stat5b (aa 777–787), while phosphoserine Stat5 rabbit polyclonal Abs (pAbs) were generated against a phosphopeptide surrounding Stat5a (33). Proteins bound to Abs were captured by incubation for 30 min with protein A-Sepharose beads (Pharmacia, Piscataway, NJ), sedimented for purification, and eluted by boiling in 2× SDS sample buffer (20% glycerol, 10% 2-ME, 4.6% SDS, 0.004% bromophenol blue in 0.125 M Tris, pH 6.8) for 4 min. After separation of proteins on 7.5% SDS-PAGE under reducing conditions, proteins were transferred to polyvinylidene difluoride (Immobilon, catalogue no. IPVH 00010; Millipore, Bedford, MA), as previously described (12). Western blot analysis was performed with either pAbs or murine anti-phosphotyrosine mAbs (4G10, catalogue no. 05-321; Upstate Biotechnology) that had been diluted either 1/1000, respectively, or 1/5000 in blocking buffer for phosphoserine Stat5a/b mAbs, as previously described (12).

Histopathologic evaluation

At day 7 posttransplant, heart allografts derived from recipients receiving drug treatment were diced and pieces placed in Bouin’s fixative (Poly Scientific R&D, Bay Shore, NY), sectioned, and stained with hematoxylin, as described earlier (35, 36).

Analysis of spleen effector molecules

Spleens were harvested aseptically from recipient rats for RNA isolation to quantitate IL-2Rα expression by RNase protection assay (RPA). Briefly, 20 μg of total RNA was hybridized to 32P-labeled probes corresponding to the mCR-1 kit according to the manufacturer’s recommendations (PharMingen, San Diego, CA). Hybridized RNA probes were denatured and electrophoresed on a 5% polyacrylamide gel, then dried, and exposed to x-ray film, as previously described by our group (31). Quantitation of IL-2Rα expression was normalized against GAPDH housekeeping gene product using an Expression 633 scanner (Epson, Long Beach, CA), with each sample divided by the ratio of vehicle-treated samples from two sets of spleen transplants denoted as 1 or 2. For IL-2 activation of Stat5a/b molecules, spleens were pressed through a sterile wire mesh screen with a disposable syringe plunger into complete medium (RPMI 1640 supplemented with 50 μM, 2-ME, and 10 mM HEPES). Released cells were collected and centrifuged (1000 × g for 5 min) to remove cell debris, while contaminating RBCs were removed by incubating splenocytes in Orthomune Lysing Reagent (Ortho, Raritan, NJ) and centrifuged (1000 × g for 5 min). Cells were then washed three times, resuspended in 1 ml of complete medium at a final concentration of 5 × 107 cells/ml, and subjected to cytokine stimulation with IL-2 (100 nM), as described earlier (31).

EMSA

Nuclear extracts from cytokine-treated and untreated splenic or Nb2-11c cells were isolated, as previously described (30). For the EMSA analysis
(34), 1 μg of 32P-labeled oligonucleotide corresponding to either the β-casein promoter (5'-agattttctaggaattcaatcc-3') or NF-κB DNA-binding element (5'-agttaggaagctctccag-3'), labeled with [32P]dATP (fill-in reaction), was incubated with 5 μg of nuclear extracted protein and preincubated with 1 μl of either normal rabbit serum or anti-sera specific to Stat5a or Stat5b (above) or p50/p65 pAbs (Santa Cruz Biotechnology, Santa Cruz, CA) for NF-κB, separated on polyacrylamide gels (5%), dried, and exposed to x-ray film (X-Omat; Kodak, Rochester, NY), as described previously (30).

Median effect analysis

Organ allograft survival rates are presented as MST ± SD, with comparison among groups performed by Gehan’s survival test. The interaction between AG-490 and CsA was evaluated by the median effect analysis (37, 38). Computer software was used to calculate combination index (CI) values, which when <1 showed synergistic, >1 antagonistic, or = 1 additive interactions, respectively (38).

Results

AG-490 blocks IL-2-mediated growth of rat T cells

To investigate the role of Jak3 in rat T cell function, we initiated a series of experiments to test the in vitro effects of AG-490 on proliferation of the Nb2-11c cell line (39). Nb2 cells were treated with varying concentrations (0.5–100 μM) of AG-490 or control tyrphostin, AG-9, for 16 h and then stimulated in the absence or presence of 1 nM IL-2. As shown in Fig. 1A, ascending concentrations of AG-490 abolished IL-2-inducible [3H]thymidine incorporation with an IC50 of ~20 μM. In contrast, the inactive analogue AG-9 did not exhibit a significant inhibitory effect even at concentrations as high as 100 μM. At this time point (16 h), inhibition of rat T cell growth did not appear to be due to induction of apoptosis since cell viability was typically >85% based on annexin V analysis and trypan blue dye exclusion staining (data not shown). AG-490 treatment of non-Jak3-expressing Jurkat cells, a model for nonactivated T cells, at concentrations of as high as 100 μM failed to inhibit actively growing cells (30). Thus, AG-490 inhibits IL-2-induced proliferation of Jak3-expressing rat T cells.

AG-490 inhibits Jak3 activation and Stat5a/b tyrosine and serine phosphorylation in rat T cells

Catalytically active Jak3 is required for IL-2-driven tyrosine phosphorylation of Jak1 and Stat5a/b (12, 23, 24, 40, 41). Moreover, since Stat5a/b gene-deficient mice are unable to proliferate in response to IL-2, we examined whether AG-490 inhibits IL-2-induced activation of Stat5a/b via Jak3. For this analysis, Nb2-11c cells were treated with ascending concentrations of AG-490 (0–100 μM) for 16 h and then stimulated for the final 10 min with 100 nM IL-2. Lysed cells were immunoprecipitated with Jak3, Stat5a, or Stat5b, and then Western blotted with anti-phosphotyrosine Abs (lanes a–f, Fig. 1B). Tyrosine phosphorylation of all three signaling molecules displayed sensitivity to AG-490 treatment. In four separate experiments, Jak3 typically showed a loss of tyrosine phosphorylation between 25 and 50 μM AG-490, as shown in this representative experiment.

Next, we examined the effects of AG-490 on IL-2-induced serine phosphorylation of Stat5a/b. As previously shown, IFN-α/β- or IFN-γ-induced serine phosphorylation of Stat1α, and IL-6-mediated serine phosphorylation of Stat3, were found to be necessary for maximal nuclear translocation, DNA-binding, transcriptional activation, and cell cycle progression (42–44). Our previous results revealed that IL-2 and prolactin induced rapid phosphorylation of a serine residue located within a conserved Pro-Ser-Pro motif in Stat5a/b (33, 45, 46). To determine whether AG-490 also blocks Stat5a/b serine kinase activity in rat T cells, Nb2 cells were first pretreated for 16 h with 50 μM AG-490 and stimulated with

FIGURE 1. AG-490 inhibits IL-2-induced proliferation and Jak3 and Stat5a/b activation in the rat T cell line Nb2-11c. A. Proliferation of quiescent rat T cells (5 × 10^5 cells/well) in the absence or presence of IL-2 (1 nM) was examined following treatment with vehicle or increasing concentrations of AG-490 or AG-9 (ordinate) for 16 h at 37°C. Cells were then pulsed with [3H]thymidine (0.5 μci/200 μl) for 4 h and incorporated radiolabeled probe plotted on the abscissa expressed as % inhibition of nondrug-treated IL-2-responsive model for nonactivated T cells, at concentrations of as high as 100 μM failed to inhibit actively growing cells (30). Thus, AG-490 inhibits IL-2-induced proliferation of Jak3-expressing rat T cells.
AG-490 disrupts IL-2 activation of Stat5a/b DNA binding from rat T cell nuclear extracts

Jak3-regulated Stat5a/b tyrosine phosphorylation is required for dimerization, nuclear translocation, and gene transcription (22). To test the notion that AG-490 blocks IL-2-induced cell proliferation by Stat5a/b inactivation, DNA-binding experiments were performed with a Stat5a/b probe. For this study, 5 µg of nuclear extracted protein isolated from AG-490 (50 µM)-treated Nb2-11c cells was incubated with a 32P-labeled β-casein probe corresponding to the Stat5a/b DNA-binding prolactin-response element. In addition, a supershift of Stat5a/b/DNA probe complexes with anti-Stat5a/b sera was performed to verify the identity of the Stat5a/b complex (Fig. 2A). IL-2-stimulated control Nb2 cells displayed a single DNA-binding complex (lane b; indicated by arrow) that could be supershifted with either anti-Stat5a (lane c) or anti-Stat5b (lane d) pAbs, or completely supershifted with both pAbs (lane e). In contrast, normal rabbit sera alone failed to supershift the complex (lane f). However, equivalent protein (5 µg/lane; lanes g–l) obtained from AG-490-treated Nb2-11c cells displayed greatly reduced Stat5a/b DNA-binding efficiency. Since Stat5a/b is critical for IL-2-mediated cell cycle progression, we infer that the loss of IL-2-inducible T cell proliferation may be, at least in part, caused by the disruption of Stat5a/b activation.

To test the selectivity of AG-490 for Jak3/Stat5a/b activation, we examined the non-Jak3-mediated activation of TNF-α-induced NF-κB. Since Nb2-11c cells were only weakly responsive to TNF-α, we used human PBL stimulated with TNF-α. As shown in Fig. 2B, 50 µM AG-490 did not affect the TNF-α-mediated activation of p50/p65 components of NF-κB (lanes f–j) compared with untreated controls (lanes a–e). In contrast, 50 µM AG-490 completely inhibited IL-2-induced Stat5a/b DNA binding in IL-2-treated PBL, as shown earlier (30). These experiments revealed that AG-490 selectively blocks activation of the crucial Jak3 substrates, Stat5a/b, but not TNF-α-activated NF-κB.

Inhibition of Jak3 prolongs rat heart allograft survival. ACI (RT1+) recipients rejected LEW (RT11) heart allografts at an MST of 8.8 ± 0.8 days. Daily i.p. injections for 7 days with either 5, 10, or 20 mg/kg AG-490 alone significantly extended allograft survival in a dose-dependent fashion (Fig. 3). In contrast, the inactive analogue of AG-490, AG-9, was ineffective (8.7 ± 1.2 days; n = 4; not shown). Although 2.5, 5, 10, or 20 mg/kg/day CsA alone delivered p.o. for 3 days extended survivals (groups 5–8), the addition of 5, 10, or 20 mg/kg AG-490 produced significantly better results than CsA alone (groups 2–4). CI values of 0.43–0.98 suggested synergistic interactions (groups 9–17). In contrast, treatment of recipients p.o. for 7 days with 2 or 3 mg/kg RAPA in combination with 5, 10, or 20 mg/kg AG-490 produced only additive or even mildly antagonistic effects, as revealed by CI values of 0.97–1.42 (groups 22–27). These results demonstrate that inhibition of Jak3 by AG-490 blocks allograft rejection in a synergistic fashion with the CaN inhibitor CsA, but not the mTOR inhibitor RAPA.
Inhibition of Jak3 reduces graft damage and mononuclear cell infiltration. For histological examination, heart allografts obtained from recipients that had received daily injections of vehicle, 5 or 20 mg/kg AG-490 were examined on day 7 of postgrafting after staining with hematoxylin-eosin (Fig. 4). Untreated heart allografts displayed severe damage with myocardial necrosis and acute inflammatory infiltrate (Fig. 4A). In contrast, heart allografts from recipients treated with a minimally protective dose of AG-490 (5 mg/kg) displayed only mild intimal arteritis and graft damage, but showed prominent infiltration into the epicardium, myocardium, and endocardium (Fig. 4B). Increasing the dosage of AG-490 to 20 mg/kg completely blocked allograft rejection without any signs of myocardial damage to vessels or muscle fibers, with only mild infiltration of mononuclear cells (Fig. 4C). Thus, selective inhibition of Jak3 blocks graft damage and reduces infiltration of mononuclear cells and granulocytes.

In vivo inhibition of Jak3 inhibits IL-2 signaling, but not IL-2Rα chain expression in rat T cells. To analyze the maturation status of T cells, we measured expression of the early activation marker, IL-2Rα (47). For this analysis, we used irradiated (900 rad) LEW spleen transplants in ACI hosts as a source of the large numbers of graft-infiltrating cells (GICs) required for sensitive RPA and EMSA assays. GICs isolated on day 7 postgrafting from splenic graft hosts treated with AG-490 (20 mg/kg) and/or CsA (10 mg/kg) were used to isolate total RNA to assess IL-2Rα mRNA expression by RPA (Fig. 5). RNA extracts from either DMSO (lanes...
a and b)- or AG-490 (lanes e and f)-treated hosts showed similar expression of IL-2Rα mRNA, in contrast to grafts from either CsA-treated (lanes c and d) or CsA/AG-490-treated (lanes g and h) hosts, which displayed reduced transcripts as normalized against GAPDH using densitometry (Fig. 5B).

Finally, we examined the in vivo effect of AG-490 on Jak3/Stat5a/b signaling. The GICs obtained from spleen transplants were cultured for 10 min in the absence or presence of IL-2. Extracts from neither naive spleens (lanes a and b) nor spleen allografts from AG-490-treated hosts (lanes c and d) showed IL-2-inducible Stat5a/b-binding activity (Fig. 6A). In contrast, nuclear extracts isolated from spleen allografts of vehicle-treated hosts (lanes e–k) showed that IL-2 potently induced Stat5a/b DNA binding to the β-casein probe (Fig. 6). The identities of both transcription factors were verified by supershift analysis using pAbs to Stat5a (lane g), Stat5b (lane h), both pAbs (lane i), or pan-Stat5 (lane j), but not a normal rabbit serum control (lane k). The results presented in Fig. 6 are representative data from three independent transplantation and EMSA experiments. Finally, a treatment set (n = 3) for CsA alone or in combination with AG-490 also failed to show IL-2-inducible Stat5a/b DNA-binding activity (data not shown). These results demonstrate that AG-490 inhibits the IL-2-inducible Jak3/Stat5 signaling pathway of lymphocytes in vivo.

Discussion

There is an urgent need for the development of agents that selectively inhibit molecules unique for signal 3 pathways of T and B lymphocytes activated by TCGFs. These agents may block clonal expansion of T cells without affecting other cells. Direct targeting of a single γc cytokine has shown little therapeutic promise owing...
to a high degree of signaling redundancy due to the shared recruitment and activation of key effector molecules that drive T cell proliferation: Jak1, Jak3, Stat5a/b, etc. This has been demonstrated in vivo, as mice engineered to be defective in IL-2, IL-2/IL-4, or IL-2Rαβ continue to show relatively normal immune responses (13–17). Undoubtedly, a more effective strategy seeks to block the γc pathway, thereby disrupting Jak3, a critical intermediate in this cascade (27). In this study, we demonstrate that AG-490 potency and selectively inhibits γc/Jak3-dependent signaling pathways, including downstream Stat5a/b activation and subsequent T cell proliferation. In contrast, signal 1/2 pathways were not affected; both cytokine production and IL-2Ra expression remain largely unchanged following AG-490 treatment (30). In this study, we document that in vivo inhibition of Jak3-dependent signaling pathways alone blocks allograft rejection and that AG-490 acts in a synergistic fashion when used in combination with the CaN inhibitor CsA, but not with the mTOR inhibitor, RAPA.

Jak3 is an essential signaling intermediate for the development and function of mature monocytes, T and B cells, as well as NK cells (20, 21). Indeed, retroviral-mediated introduction of this enzyme into Jak3-deficient mice restores normal T cell development (48). Although the understanding of the signaling pathways activated by Jak3 (directly or indirectly) is incomplete, they clearly mediate signals via Stat5a/b critical to regulate genes necessary for cellular proliferation. Moriggl et al. (23) showed that Stat5a/b-deficient T cells failed to respond to the mitogenic effects of IL-2. As shown in this study, AG-490 abolished Jak3-mediated Stat5a/b tyrosine and serine phosphorylation (Fig. 1B), thereby preventing subsequent Stat5a/b dimerization via their SH2 domains and Stat5a/b translocation to the nucleus and gene transcription (Fig. 2).

Stats have been shown to profoundly impact immune activity. Stat1-deficient mice are highly sensitive to viral or bacterial infection because they fail to respond to IFN-γ or IFN-α (49, 50). Mice devoid of Stat4 or Stat6 display losses of Th1 or Th2 cell function, respectively (51, 52). Furthermore, T cells obtained from mammary adenocarcinoma-bearing mice or HIV-infected patients, which are both severely immunocompromised, lacked detectable levels of both Stat5a and Stat5b proteins, while other Stats were expressed normally (53, 54). Similarly, T cells isolated from Stat5a/b-deficient mice fail to respond to the mitogenic effects of IL-2 (23). These same T cell lines display reduced protein levels of key cell cycle messengers, cyclin-dependent kinase-6 and cyclins A, D2, D3, and E, suggesting that Stat5a/b may selectively regulate the expression of genes involved in cell cycle progression (23). Although these genes were not investigated in this study, our results would suggest that targeted inhibition of the γc cytokine-mediated Jak3/Stat5 pathway represents a convergence point by which TCGF-driven T cell clonal expansion may be inhibited.

In vivo therapy with AG-490 alone (but not the inactive analogue AG-9) produced moderate prolongation of allograft survival (Fig. 3). Histological examination of allografts from AG-490-treated hosts showed reduced intragraft cell infiltration of mononuclear cells without myocyte damage (Fig. 4, B and C). Our in vivo results suggest that AG-490 blocks signal 3 and may prolong allograft survival by inhibiting proliferation of alloreactive T cells. Lack of clonal expansion would prevent generation of a sufficient number of effector T cells, CTLs, and delayed-type hypersensitivity T cells, to mediate allograft destruction (56). However, recent work by Constantin et al. (57) suggests AG-490 may affect “lymphocyte homing,” thereby reducing the number of effector T cells to the site of the graft. Nonetheless, evidence provided by both groups supports the conclusion that this observation is dependent on the signal 3 pathway since we failed to observe effects of AG-490 on TCR activation of Zap70 or p56Lck tyrosine kinases, while Constantin found normal Ca2+ mobilization after TCR cross-linking in the presence of drug (57). In support of these conclusions, we also found no inhibition of de novo expression of IL-2Rα, in addition to continuously expressed IL-2Rβ and γc (19).

Our studies suggest that AG-490 promotes allograft acceptance by reducing Jak3 kinase activity and subsequent T cell proliferation. However, could these observations be the result of this drug inhibiting other cell signaling pathways? Previous evidence showed AG-490 does not affect lymphocytic tyrosine kinases Lck, Lyn, Btk, Syk, Src, Zap70, or p56Lck tyrosine kinases, while Constantin found normal Ca2+ mobilization after TCR cross-linking in the presence of drug (57). In support of these conclusions, we also found no inhibition of de novo expression of IL-2Rα, in addition to continuously expressed IL-2Rβ and γc (19).

T cell activation undergoes two phases: 1) Ag-driven (signal 1/2) activation and cytokine-driven (signal 3) T cell clonal expansion. Disruption in the kinetics and/or strength of these signals would prevent full T cell activation, thereby leading to T cell clonal anergy (59) or apoptosis (60). Delivery of signal 1/2 without signal 3 to T cell clones has been shown to induce T cell anergy (55, 61), and recent evidence suggests that blockade of signal 3 by γc mAbs (signal 3) results in...
apoptosis of all-reactive T cells and long-term islet allograft survival (27). However, to date, RAPA is the only effective, clinically approved signal 3 inhibitor. This agent inhibits the 256-kDa serine/threonine kinase, mTOR (also known as FK506-binding protein 2-rapamycin-associated protein and receptor-activated factor of transcription), and subsequently blocks IL-2-mediated distant signals, resulting in T cell clonal anergy (5–7, 62). As shown by several groups, mTOR plays a key role in regulating cell cycle progression in response to a number of stimuli (63), by direct and indirect regulation of a number of translational regulatory proteins, including p70S6 kinase (64–66), eukaryotic elongation factor 2 (67), eukaryotic initiation factor 4E-binding proteins (68), and eukaryotic initiation factor 4G (69). The wide-ranging regulation of these proteins affected by RAPA may explain the non-T cell-dependent toxicities associated with the drug. However, since the discretely expressed Jak3 is most likely an upstream regulator of mTOR in T cells, AG-490 or other Jak3 inhibitors should ultimately result in less overt toxicities.

Our previous studies showed that a combination of CsA and RAPA acts synergistically to prolong organ allograft survivals in animal models and in humans (6, 70). Rats treated i.v. with combinations of therapeutic doses of RAPA (0.04–0.8 mg/kg/day) and of CsA (0.5–2 mg/kg/day) displayed potent synergistic interactions on heart and kidney allograft survivals, as documented by CI values of 0.001–0.2 (70). Previously, we showed that subtherapeutic doses of CsA sufficient to inhibit expression of IL-2 mRNAs at the graft site would act synergistically in vivo with RAPA to block T cell clonal expansion. We propose a similar model to explain the synergistic effects of CsA in combination with AG-490. Although p.o. CsA (0.5–2.5 mg/kg) with RAPA (1–8 mg/kg) produced potent synergism, CsA with RAPA displayed exaggerated pharmacokinetic interactions, leading to marked increases of each drug’s level in whole blood and in tissues (7). Thus, analysis of CsA and AG-490 blood levels may be necessary to determine potential pharmacokinetic interactions.

In conclusion, Jak3 inhibition limits T cell clonal expansion both in vitro and in vivo. Considering the restricted expression of Jak3 to lymphoid compartments, pharmacological blockade of Jak3 should result in fewer adverse effects than those currently associated with RAPA and other immunosuppressants. Moreover, as demonstrated in this study, the synergy between CsA, which blocks the TCR-mediated Gs-Gi transition, and AG-490, which targets Jak3 and disrupts the TCGF-inducible Gs-Phosphorylated signals, should permit the delivery of pharmaceutica l compounds to subtoxic levels and thus minimize drug-associated toxicities.

Acknowledgments
We thank Dr. Lihua Wang for technical assistance on EMSAs, Jerry Ku for RPA analysis, and Neelam Tejpal for T cell proliferation assays.

References

The Journal of Immunology
3731

Downloaded from http://www.jimmunol.org/ by guest on April 13, 2017

Published online 13 April 2017

http://www.jimmunol.org/doi/10.4049/jimmunol.168.7.3731

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