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Targeting Janus kinase 3 in the treatment of leukemia and inflammatory diseases

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Summary

Janus tyrosine kinases (JAKs) are cytoplasmic protein tyrosine kinases that play a crucial role in the initial steps of cytokine signaling. JAK3, a member of JAK kinase family of four (JAK1, JAK2, JAK3 and TYK2), is abundantly expressed in lymphoid cells. JAK3 has been found to initiate signaling of interleukin (IL)-2, IL-4, IL-7, IL-9, IL-13 and IL-15. Indispensable role of JAK3 in lymphocyte development and function has been revealed recently. Because of the involvement of JAK3 in T cell activation and proliferation, and the documented genetic evidence for the role of JAK3 in autoimmune or transplant-induced inflammatory disorders, the selective targeting of JAK3 in T cells may potentially be clinically beneficial in T cell-derived pathologic disorders. In this review we discuss inhibitors of JAK3 as a new class of immunomodulatory agents with immunosuppressive, anti-inflammatory, anti-allergic, and anti-leukemic properties. Preclinical data from multiple experimental model systems of autoimmune diabetes, allergy, solid organ transplantation, pancreatic islet transplantation and bone marrow transplantation are discussed in the context of the clinical need for new immunomodulatory agents with such properties.

Key words: Janus kinase 3 (JAK3) • JAK3 inhibitors • type 1 diabetes • allogeneic transplantation • allergy

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INTRODUCTION

Janus tyrosine kinases (JAKs) are cytoplasmic protein tyrosine kinases (PTK) that play pivotal roles in the initiation of cytokine-triggered signaling events by activating through tyrosine phosphorylation the signal transducers and activators of transcription (STAT) proteins. The JAK family of kinases consists of 4 known members – JAK1¹⁴³, JAK2^{38, 143}, JAK3¹²³ and TYK (tyrosine kinase)²⁵⁷ – ranging in size from 110 to 140 kDa. Among the 7 conserved JH regions in the JAKs, there are two kinase-like domains. The JH1 domain is a functional catalytic domain, while the JH2 is a pseudokinase domain. The name Janus kinase is taken from the mythological Roman god and refers to the fact that JAKs have “two faces” consisting of the tandem kinase and pseudokinase domains. Initially, the important function of JAKs in cytokine signaling was shown using mutant cell lines that failed to respond to interferons (IFNs)^{90, 137}. Subsequent studies have demonstrated that JAKs are activated by many other cytokines as well (Table 1)⁶³. The physical association between JAKs and cytokine receptors was first reported for JAK2 and the erythropoietin as well as growth hormone receptors^{3, 145}. The binding of a cytokine to its receptor activates the receptor-associated JAKs, presumably through autophosphorylation on regulatory tyrosine residues. Activated JAKs then phosphorylate the cytoplasmic domains of the cytokine receptors, generating docking site(s) for STATs^{64, 84}. STATs are then phosphorylated by JAKs on a conserved tyrosine residue at their C-terminus. Subsequently, the STATs form stable homodimers and heterodimers by interactions

between the SH (Src homology) 2 domain of one STAT protein and the phosphotyrosine of another before translocation to the nucleus⁴³, where they influence transcription of target genes by binding to specific regulatory sequences²³. Seven STAT proteins have been identified in mammalian cells (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6). All interferons and type I cytokines can activate one or more STATs with varying degrees of selectivity (Table 1).

JAK3 KINASE

In contrast to the relatively ubiquitous expression of JAK1, JAK2 and TYK2 kinases in many cells, JAK3 has more restricted and regulated tissue expression. It is expressed constitutively at high levels in natural killer (NK) cells and thymocytes and is inducible in T cells and B cells^{35, 51, 113, 131}. JAK3 plays an important role in normal lymphocyte development and function, as evidenced by numerous studies. Initially, mutation of the common γ chain (γ_c) of the interleukin (IL)-2 receptor, which specifically associates with JAK3, was discovered to be the molecular basis of certain X-linked severe combined immunodeficiency (X-SCID) cases^{62, 93, 105}. The association of the IL-2 γ_c with JAK3 suggested that mutations of the latter might also cause SCID. Subsequently, some patients with autosomal recessive SCID were found to have JAK3 mutations^{13, 70, 106}. JAK3^{94, 97, 127} as well as γ_c knockout mice^{16, 26} were generated and found to have an immunodeficient phenotype. While JAK3-deficient humans have profoundly decreased numbers of T cells with normal or increased numbers of B

Table 1. JAKs and STATs activated by cytokines

Cytokines which receptors share:	JAKs	STATs
γ_c IL-2, IL-7, IL-9, IL-15 IL-4	JAK1, JAK3	STAT5a, STAT5b, STAT3 STAT6
β_c IL-3, IL-5, GM-CSF	JAK2	STAT5a, STAT5b
Homodimeric receptors growth hormone prolactin thrombopoietin erythropoietin	JAK2	STAT5a, STAT5b, STAT3 STAT5a, STAT5b
gp130 IL-6, IL-11, OSM, CNTF, LIF, CT-1	JAK1, JAK2, TYK2	STAT3
IFN receptors IFN- α , IFN- β IFN- γ IL-10	JAK1, TYK2 JAK1, JAK2 JAK1, TYK2	STAT1, STAT2 STAT1 STAT3

OSM – oncostatin M, CNTF – ciliary neurotrophic factor, LIF – leukemia inhibitory factor, CT-1 – cardiotrophin-1, GM-CSF – granulocyte-macrophage colony-stimulating factor.

cells, JAK3-deficient mice exhibit relatively normal numbers of peripheral T cells, but lack B cells^{16, 26}.

JAK3 specifically associates with the γ c receptor of IL-2 receptor, and is activated by the cytokines IL-2, IL-4, IL-7, IL-9, IL-13 and IL-15 that share the γ c receptor⁶³ as well as the more recently described cytokine IL-21³⁶. Recently, a more complex immunoregulatory role of JAK3 has been suggested because of indications that JAK3 is involved in the IL-2 production by T cells¹²⁶. Moreover, recent data have demonstrated that JAK3 is physically associated with the T cell receptor (TCR)/CD3 complex and plays an important role in TCR-mediated signaling events and T cell activation¹³⁰. The association of JAK3 with the TCR/CD3 machinery as well as with the IL-2 receptor complex emphasizes a crucial role of this kinase in the regulation of both early T cell activation¹³⁰ and cell growth^{94, 97, 127}. Genetic inactivation of JAK3 in recipients' T lymphocytes has been shown to prevent rejection of mouse pancreatic islet allografts¹⁸, suggesting the crucial role of JAK3 in the outcome of allotransplantation. Studies using a murine allogeneic bone marrow transplantation (BMT) model confirmed the JAK3 in donor T cells as a key mediator of graft-versus-host disease (GVHD)²⁰. *Jak3*^{-/-} BM/splenocyte allografts were not able to induce fatal GVHD across the major histocompatibility complex (MHC) barrier²⁰. As will be documented in this paper, *Jak3*^{-/-} mice are also protected from development of autoimmune diabetes induced by low-dose streptozotocin (STZ), suggesting the crucial role of the JAK3 in development of the autoimmune diabetes (Fig. 1A and B). Because of this dual involvement of JAK3 in T cell activation and proliferation, the abundant expression of JAK3 in lymphoid cells, and the documented genetic evidence for the role of JAK3 in autoimmune or transplant-induced inflammatory disorders, the selective targeting of JAK3 in T cells may potentially be clinically beneficial in T cell-derived pathologic disorders. This possibility prompted us to rationally design specific inhibitors of JAK3 by structure-based drug design¹¹⁸.

INHIBITORS OF JAK3

Several JAK inhibitors have been identified in recent years, but most are general PTK inhibitors and are not specific for the JAK family or for an individual kinase protein within the JAK family. For example, cytotaricin B was reported to inhibit the JAK/STAT pathway by inhibiting STAT5 phosphorylation without affecting JAK2 phosphorylation¹⁴⁷. Other non-specific JAK inhibitors include dimethoxyquinazoline inhibitor of JAK3 and epidermal growth factor

receptor (EGFR) – WHI-P97¹¹⁸, and the octylamino undecyl dimethylxanthine derivatives CT-2576 and CT-5589¹⁴⁰. The leflunomide metabolite (LFM) inhibited lymphocyte specific protein (LCK)⁷⁶, EGFR^{32, 79} as well as JAK3¹¹⁴. Besides JAK³⁰, staurosporine inhibits v-SRC (sarcoma)⁹¹, LYN (Lck/Yes related kinase)⁸⁰, SRC⁵⁹, insulin receptor kinase (IRK) kinase and EGFR¹⁷. The compound 6, a pyridone containing tetracycle, inhibits JAK3, JAK2, TYK2 and JAK1¹²⁸. Although tyrphostin derivative AG-490 was reported to be a specific JAK2 inhibitor⁸¹, more recent studies showed that AG-490 could also inhibit JAK3¹³⁹. However, its specificity for the JAK family of tyrosine kinases was demonstrated by its inability to inhibit other tyrosine kinases such as the thymic epithelial cytokine (TEC) family Bruton's tyrosine kinase (BTK) kinase, the ZAP/SYK family SYK kinase, and the SRC family SRC, LYN and LCK kinases⁸¹.

Only two compounds, the dimethoxyquinazoline derivatives JANEX-1 (also known as WHI-P131) and JANEX-2 (also known as WHI-P154) have been demonstrated to be selective inhibitors of JAK3 within the JAK family¹¹⁸. The structure-based design of those specific inhibitors of JAK3 kinase was reported recently¹¹⁸. The lead compound JANEX-1 [4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline], also known as WHI-P131, inhibited JAK3 but not JAK1 or JAK2. Similarly, the ZAP/SYK family tyrosine kinase SYK, the TEC family tyrosine kinase BTK, the SRC family tyrosine kinase LYN, and the receptor family tyrosine kinase IRK were not inhibited by JANEX-1¹¹⁸. It is described that JANEX-1 was very well tolerated by mice and monkeys and effective plasma concentrations of JANEX-1 could be achieved at non-toxic dose levels¹³⁴. Recent report claims that PNU156804, an analogue of the undecyl-prodigiosin, is a selective JAK3 kinase inhibitor¹¹⁶. However, PNU156804 inhibited JAK2 as well, albeit to a lesser degree than JAK3¹¹⁶.

TARGETING JAK3 KINASE BY SPECIFIC INHIBITORS TO ALTER T CELL RESPONSES *IN VITRO*

In vitro studies have confirmed the effectiveness of targeting JAK3 with inhibitors such as JANEX-1 and JANEX-3 to suppress mitogen (PHA and concanavalin A)^{20, 21}, IL-2- (Cetkovic-Cvrlje and Uckun, unpublished observations), and allo-antigen-induced proliferation of mouse T cells²¹. Kinase assays performed on mouse (C57BL/6) nylon wool-purified T cells revealed that IL-2-induced JAK3 phosphorylation was suppressed with JAK3 kinase inhibitor JANEX-1 (Fig. 2). Activated JAK3 is required for IL-2-driven tyrosine phosphorylation of JAK3 substrates

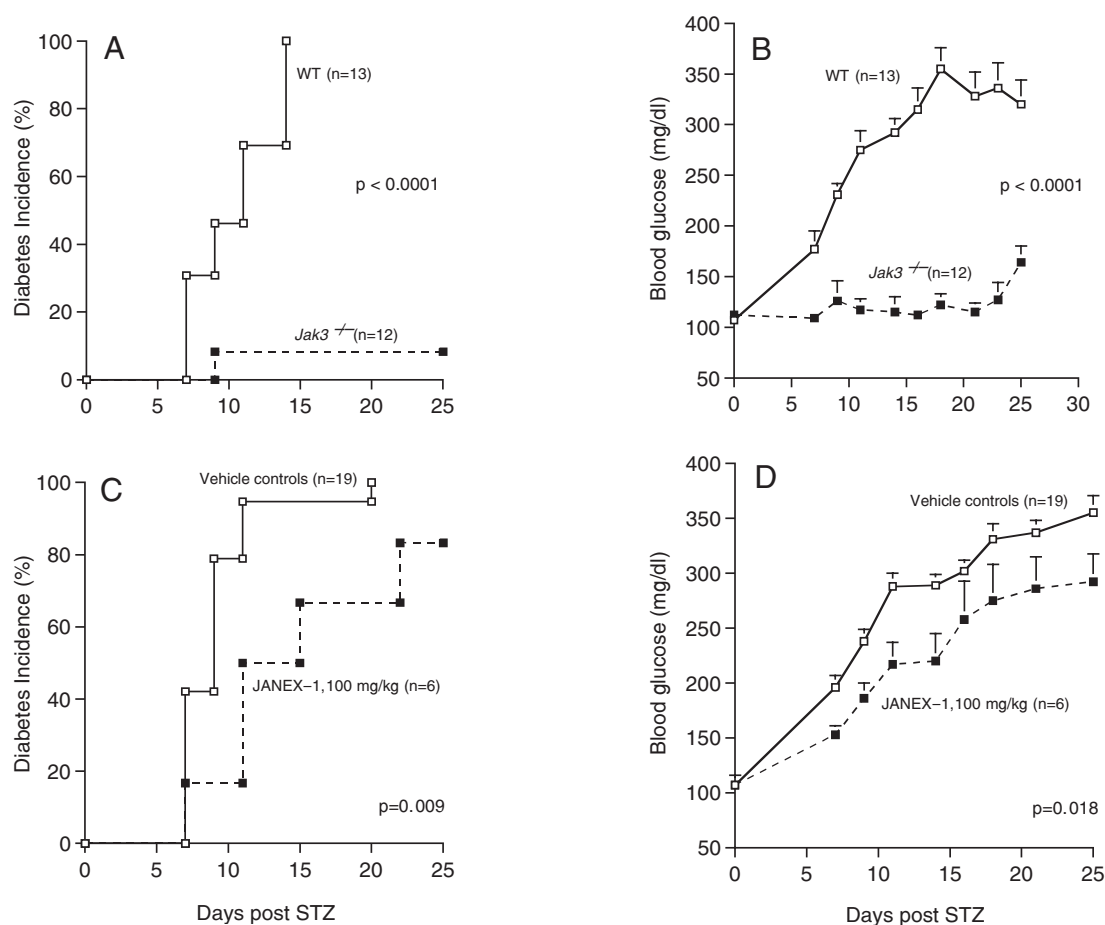


Figure 1. Targeting JAK3 genetically (**A, B**) and chemically (**C, D**) prevents development of autoimmune diabetes induced by multiple low doses of streptozotocin (STZ). Diabetes incidence (**A, C**) was followed in *Jak3*^{-/-} and wild-type (WT; **A**), and JANEX-1- and vehicle-treated C57BL/6 (**C**) mice during the 25-day-period following the first injection of STZ. Blood glucose level (mg/dl; **B, D**) was measured in *Jak3*^{-/-} and WT (**B**), and JANEX-1- and vehicle-treated C57BL/6 (**D**) mice during the 25-day-period after the first injection of STZ. JANEX-1 was injected intraperitoneal, daily in a dose of 100 mg/kg/day (**C, D**). Control mice received a vehicle (10% DMSO in PBS; **C, D**). STZ was administered in a dose of 40 mg/kg/day during the first 5 days. Statistical differences obtained by life table analysis (log-rank; **A, C**) and by the ANOVA test of repeated measurements (**B, D**). $p < 0.05$ was considered as statistically significant.

STAT3⁴⁸ and STAT5a/b^{86, 87}. Figure 2 shows that IL-2 failed to induce STAT3 and STAT5a phosphorylation in JANEX-1 (but not vehicle)-treated T cells, confirming JANEX-1 is an effective inhibitor of JAK3-mediated tyrosine phosphorylation of STAT5a in IL-2 stimulated mouse T cells. Similar data, regarding the inhibition of mitogen-induced proliferation of T cells and inhibition of JAK3-STAT5 signal transduction pathway by JANEX-1, were generated by using T cells obtained from diabetes prone NOD mice, as well¹⁹. AG-490, an inhibitor of JAK2 and JAK3, was found to effectively inhibit IL-2-induced proliferation of mouse T cell lines D10 and CTLL-2¹³⁹, rat T cell line Nb2-11c⁶, and human T cells⁵⁴. Human T cell responses to allo-antigen in mixed lymphocyte culture were suppressed by AG-490 as well¹⁰⁷. It was also demonstrated that AG-490 potentially inhibit JAK3 autophosphorylation and activation of its key substrates STAT5a and STAT5b in rat⁶ and human⁵⁴ T cells. PNU156804, a

JAK3 and JAK2 inhibitor, was found to block IL-2 proliferation of human T cells⁸⁸, and IL-2-induced activation of JAK3-STAT5 pathway.

JAK3 is associated with the CD3/TCR complex and plays an important role in signaling events linked to the TCR¹³⁰. Pharmacologic targeting of JAK3 with WHI-P154¹¹⁸ impaired many of the TCR-triggered proximal signaling events, resulting in defective nuclear binding of transcription factors essential for the generation of a productive T cell response¹⁰⁸. WHI-P154 impaired the TCR-triggered phospholipase C (PLC)- γ 1 phosphorylation, Ca²⁺-mobilization and caused a significant delay and premature termination of extracellular-signal regulated kinase (ERK) phosphorylation¹⁰⁸. In conclusion, JAK3 inhibitors are effective anti-T cell agents and hence show therapeutic potential for T cell-derived disorders such as autoimmune diseases, allergy, GVHD or organ rejection.

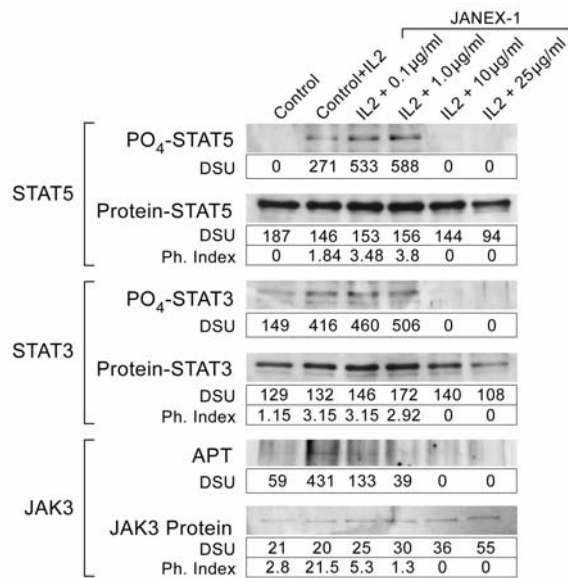


Figure 2. JANEX-1 inhibits IL-2-induced tyrosine phosphorylation of JAK3, STAT3 and STAT5a in purified T cells from C57BL/6 mice. T cells were isolated by nylon wool purification from the spleens of C57BL/6 mice and cultured for 3 h in the presence of vehicle (control) or PHA ± addition of JANEX-1 (0.1–25 µg/ml). IL-2 (50 ng/ml) was added to the PHA-treated cultures for 10 min and JAK3, STAT3 and STAT5a phosphorylation was determined as previously detailed^{19, 71, 118}. DSU – densitometry units, Ph. Index - phosphorylation index, APT – anti-phospho tyrosine.

TARGETING JAK3 *IN VIVO* IN DIFFERENT EXPERIMENTAL MODELS OF AUTOIMMUNE DISEASE AND TRANSPLANTATION

GVHD

GVHD is a donor T cell-initiated highly complex pathologic condition that frequently follows allogeneic BMT, especially in the context of a MHC disparity. The major target organs associated with clinical signs of GVHD include the skin, liver and gastrointestinal tract. Severe GVHD, which is associated with significant morbidity and mortality⁹⁶, remains a major obstacle to a more successful outcome of allogeneic BMT^{39, 60, 95}. However, allogeneic BMT remains a promising strategy in salvage of chemotherapy-resistant leukemia patients^{14, 103}. Much of the therapeutic potential of this procedure in treatment of leukemia patients relates to the graft-versus-leukemia (GVL) effect of alloreactive T cells in the donor BM graft. While some data indicate that the GVL effect is associated with a development of GVHD, and that allo-reactive donor T cells may mediate both the desired GVL effect and the undesired GVHD, the other results, obtained from clinical

as well as experimental studies, suggest that GVL can be at least partially separated from GVHD^{1, 24, 29, 46, 47, 56, 109, 122, 132, 133, 136, 138}. Contemporary methods for GVHD prophylaxis, including *ex vivo* T cell depletion of marrow grafts^{77, 78, 100}, use of positively selected CD34⁺ hematopoietic precursor cells⁸, and systemic immunosuppression (cyclosporine, corticosteroids and methotrexate)⁹⁵, are associated with an increased risk of relapse in leukemia patients undergoing BMT², which has generally been attributed to loss of the GVL function of the marrow allografts. Hence, novel anti-GVHD agents, which spare the GVL function of the marrow allografts, are urgently needed for effective prevention of GVHD after BMT without facilitating the recurrence of leukemia.

Because of the potency of the JAK3 inhibitor JANEX-1 in suppression of immunological responses *in vitro*²¹, and a key role of JAK3 in the induction of GVHD *in vivo* (observed by using *Jak3* knockout mice as a donors of BM)²⁰, we studied the ability of JANEX-1 to prevent the development of GVHD *in vivo* using a mouse model of allogeneic BMT. Notably, daily treatment with 60 mg/kg of JANEX-1 from the day of BMT prolonged the median survival of allogeneic recipients by attenuating the severity of GVHD²¹. Moreover, JANEX-1 treatment did not affect the engraftment of allogeneic BM stem cells. A novel GVHD prevention regimen was developed that employs JANEX-1 in combination with the standard immunosuppressive agent methotrexate. The combination regimen JANEX-1 (60 mg/kg/day) plus methotrexate (10 mg/m²) was markedly more effective in attenuation of GVHD post allogeneic BMT than JANEX-1 or methotrexate alone (mean survival time was >85 days and proportion of recipients surviving day 85 post-BMT was 70±10% in JANEX-1 plus methotrexate group, compared with 56 days and 19±7% in the JANEX-1-treated, and 63 days and 25±11% in the methotrexate-treated group of mouse recipients). The long-term survival of recipients treated with a combination regimen was not due to poor engraftment of donor cells, indicating that the attenuation of GVHD in JANEX-1 plus methotrexate-treated recipient mice was not attributed to a lack of donor cell engraftment with concomitant autologous recovery. JANEX-1²¹ as well as JANEX-3²⁰ resulted in significant improvement of survival post allogeneic BMT when treatment was initiated after onset of GVHD, indicating that JAK3 inhibitors may be useful not only for prevention of GVHD, but for GVHD treatment as well.

As mentioned above, the ideal anti-GVHD agent should spare the GVL function of the allografts. Therefore, the next study was designed to evaluate

the effects of GVHD prophylaxis with the JAK3 inhibitor JANEX-1 on the GVL function of marrow allografts in mice undergoing BMT after being challenged with an otherwise invariably fatal dose of BCL-1 leukemia cells. Detailed studies, using pathologic and histopathologic examinations, immunophenotyping, and adoptive transfer experiments aimed at identifying residual BCL-1 leukemia cells in allo-transplanted F1 recipients, provided strong experimental evidence that GVHD prophylaxis using JANEX-1 does not impair the GVL function of the allografts and consequently contributes to an improved post-BMT survival outcome of the recipient mice¹³⁵. In that study the JAK3-negative BCL-1 leukemia cells were used in order to avoid the anti-leukemic activity of JANEX-1 against JAK3-positive leukemia cells as a confounding factor in the evaluation of its effects on the GVL function of marrow allografts. JANEX-1 was otherwise characterized as potential anti-leukemic agent, by showing that it induced apoptosis in the JAK3-expressing leukemia cell lines DAUDI, RAMOS, LC-19, NALM-6, MOLT-3, and HL-60 (but not in the JAK3-negative BT-20 breast cancer, M24-MET melanoma, or SQ20B squamous carcinoma cell lines) in a concentration-dependent fashion¹¹⁸. Therefore, the anti-leukemic activity of JANEX-1 against JAK3-expressing human leukemia cells¹¹⁸ may further enhance its ability to attenuate the severity of GVHD without increasing the risk of relapse post-BMT in clinical settings. Even though the GVHD prophylaxis with JANEX-1 significantly improved the survival outcome of BCL-1-challenged F1 mice undergoing allogeneic BMT and prevented leukemic deaths, more than half of the mice died of GVHD¹³⁵. Therefore, it was explored whether the survival outcome of BCL-1-challenged F1 mice could be further improved by using a combination of JANEX-1 plus methotrexate for GVHD prophylaxis. Notably, the combination regimen resulted in 100% survival of allotransplanted mice challenged with an otherwise invariably fatal dose of BCL-1 leukemia¹³⁵, confirming the preserved anti-GVL effect and the most effective anti-GVHD action of the combination regimen JANEX-1 plus methotrexate.

Taken together, these data provide strong experimental evidence that targeting JAK3 in alloreactive donor lymphocytes with a chemical JAK3 kinase inhibitor may attenuate the severity of GVHD without impairment of GVL function of the allografts.

TYPE 1 DIABETES

Insulin-dependent (type 1) diabetes mellitus ensues from the selective aggression of autoreactive T cells

against insulin-secreting β cells of the islets of Langerhans. NOD mice, an animal model of type 1 diabetes^{11, 52}, spontaneously develop insulin-dependent autoimmune diabetes at 12–30 weeks of age which is caused by severe lymphocytic infiltration of the islets (insulitis) and progressive destruction of islet β cells¹²⁵. Both CD4⁺ and CD8⁺ T cells play a role in the immunopathogenesis of type 1 diabetes¹⁴⁶. Since a long prodromal period precedes the onset of clinical symptoms in NOD mice¹⁴⁹, this model provides an excellent opportunity to evaluate immunomodulatory protocols aimed at prevention or treatment of autoimmune diabetes. The immunologic interventions which interrupt the pathogenic process of spontaneous disease in NOD mice can be grouped in several categories based on their presumed mechanism of action: immunosuppression (administration of immunosuppressive drugs and different antibodies against molecules involved in antigen presentation/recognition or costimulation); tolerance induction [administration of a number of putative β cell autoantigens, such as insulin, glutamic acid decarboxylase (GAD65 and GAD67) and heat-shock protein (HSP65)]; immunostimulation (administration of BCG, CFA); anti-inflammatory activity (administration of nicotinamide, superoxide dismutase, vitamin E)⁶¹. The extensive list of immunosuppressive drugs used in NOD mice include: cyclosporin, cyclosporin A (CsA), deoxyspergualin, azathioprine, sodium fusidate, rapamycin, FK506, LZ8 and linomide^{5, 15, 33, 53, 58, 83, 85, 92, 117}. However, while indiscriminate and broad-spectrum immunosuppression can prevent the development of type 1 diabetes in NOD mice^{5, 49, 85}, and may be of benefit in humans⁷², it also has substantial adverse effects⁸². Therefore, a more selective and tailored immunomodulation, aimed at restoring the immune balance without interfering with the immune defense system would be more beneficial.

Multiple cytokines are thought to participate in the T cell-mediated autoimmune destruction of β cells¹⁰¹. An abundance of the cytokines IL-2 and interferon (IFN)- γ which are produced by islet-infiltrating T helper (Th)1 subset of the T cell compartment relative to the cytokines produced by the Th2 cells, such as IL-4 and IL-10 is implicated in the diabetogenic β cell destruction in type 1 diabetes^{67, 101, 129}. Targeting key signal transduction molecules, such as the members of the JAK family that are responsible for several of the cytokine-receptor mediated biochemical events in T cells, may provide a unique opportunity for the prevention of T cell-mediated autoimmune β cell destruction and development of type 1 diabetes.

JANEX-1 suppresses the mitogen as well as antigen

responses of T cells and attenuates the severity of T cell-dependent GVHD in a BMT model²¹. Therefore, we hypothesized that JANEX-1 treatment could also prevent the development of autoimmune type 1 diabetes which is a T cell-mediated pathologic condition as well. This hypothesis was tested using the NOD mouse model of type 1 diabetes¹⁹. Notably, JANEX-1 (50 and 100 mg/kg) was able to prevent spontaneous autoimmune diabetes development in the NOD mice. It is found that “early” (initiated at 5 weeks of age) prophylactic treatment with JANEX-1 prevented diabetes development in NOD females without significant side effects. Moreover, “late” prophylactic treatment, initiated at 10 weeks of age, when diabetes occurs in a small number of NOD females and most of the normoglycemic animals show clear signs of insulinitis, was effective in diabetes prevention, as well. This would suggest that the inhibition of JAK3 kinase with JANEX-1 is able to reverse an active and destructive autoimmune process, a potentially important observation which emphasizes the potential of JANEX-1 in the treatment of autoimmune disorders⁴. However, in contrast to chronic daily treatment, short-term JANEX-1 administration during the early prediabetic phase (5–8 weeks of age), did not result in prevention of diabetes, indicating the need of continued exposure to JANEX-1¹⁹. This observation is in agreement with the finding that the inhibition of NOD T cell antigen responses requires the continued presence of JANEX-1, and *in vivo* treatment with JANEX-1 does not impair the recall antigen (nominal antigen or autoantigen GAD65) response or induce tolerance¹⁹. This observation is also consistent with the previous reports that continuous treatment with the immunosuppressive drugs CsA³¹ and rapamycin⁵ might be needed for diabetes prevention in NOD mice. The prevention of the adoptive transfer of diabetes by splenocytes from diabetic donors to NOD-*scid* recipients by JANEX-1 confirmed the ability of JANEX-1 to inhibit the action of T cell effectors involved in the pathogenesis of insulinitis and autoimmune diabetes¹⁹.

What could be the possible mechanism of JANEX-1 action in the context of the Th1/Th2 paradigm? T cells from JANEX-1 treated NOD mice showed a differential modulation of their cytokine secretion profile with significantly increased release of the Th2 cytokine IL-10 before as well as after stimulation with mitogen or antigen. IL-10 has emerged as a potential key cytokine in a protection of type 1 diabetes in NOD mice^{99, 148}. IL-10 induces T cell anergy and therefore may play an important role in the induction and maintenance of antigen-specific T cell tolerance³⁴. There are several studies showing that admin-

istration of exogenous IL-10 is able to prevent development of insulinitis or diabetes onset in NOD mice^{98, 152}. Although data reported by Cetkovic-Cvrlje et al.¹⁹ support the general concept that IL-10 response (Th2) is protective in the development of destructive insulinitis in NOD mice, these preliminary findings should be interpreted with due caution and final conclusions regarding the role of IL-10 in the JANEX-1-mediated prevention of autoimmune diabetes should be deferred until results from larger and more definitive studies become available. Although the exact and complete details of the molecular mechanism of JANEX-1 action on the autoimmune process have yet to be deciphered, generalized immunosuppressive effects were ruled out, because flow cytometric analysis of splenic leukocytes, performed in NOD mice after 20 weeks of treatment with JANEX-1, revealed that drug treatment reduced neither the percentage nor the absolute cell number of T cells, CD4⁺ and CD8⁺ T cell subsets, or B cells in NOD mice¹⁹.

Another mouse model, multiple low-dose STZ-induced diabetes (MLDS), is widely used as a model for type 1 diabetes, as well^{68, 69}. STZ is a β cell toxin, which is directly toxic to β cells at high doses¹⁰². However, multiple injections of low doses of STZ cause partial damage to islet β cells and thereby trigger a mononuclear islet infiltration which finally results in the loss of more than 80–90% of β cells in certain susceptible strains of mice¹⁰⁴. Although STZ could induce a modest increase of blood glucose due to its toxic action on β cells, this effect is not sufficient to cause disease without a T cell-mediated immune response²⁸. Numerous previous studies have shown that the development of diabetes in the MLDS model is indeed a T cell-dependent process^{10, 28, 40, 41, 55}. Here we show the JANEX-1 effect on the development of MLDS diabetes in C57BL/6 mice. Indeed, a significant inhibition of diabetes development (Fig. 1C), as well as lower glycemic values throughout the entire experimental period (Fig. 1D), were found in JANEX-1-treated mice compared with controls. These data, together with the described protective role of JAK3 deficiency in diabetogenesis of JAK3-deficient mice (Fig. 1A and B), point to JAK3 kinase as an essential factor in the development of experimental STZ-induced autoimmune diabetes.

In summary, JAK3 is identified as a new potential molecular target in the prevention of autoimmune diabetes. These studies provide experimental evidence that targeting JAK3 with JANEX-1, a potent and specific inhibitor of JAK3 kinase, prevents the spontaneous as well as chemically-induced development of autoimmune diabetes in mice. Because of its

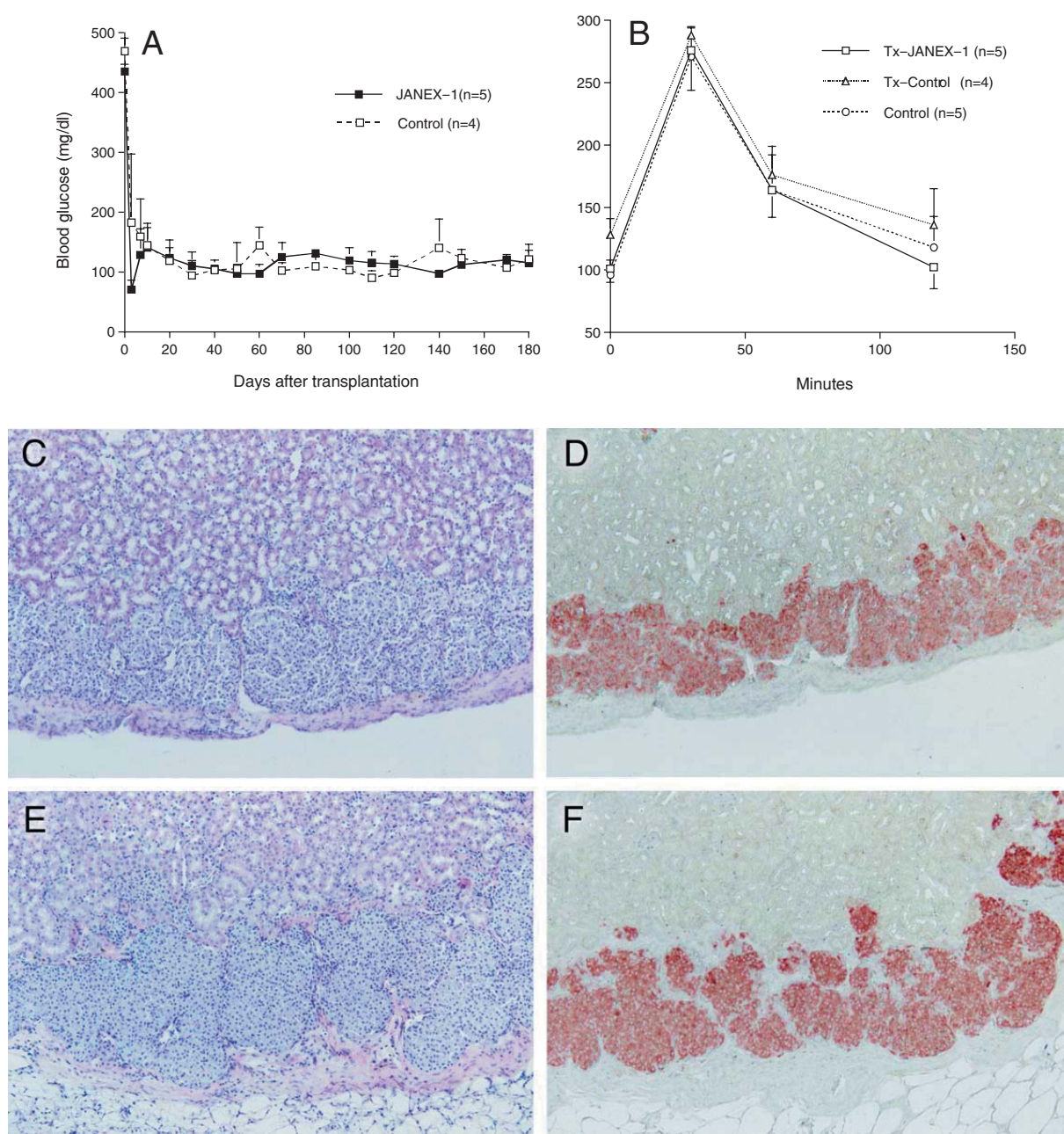


Figure 3. JANEX-1 does not exhibit diabetogenic effects in a syngeneic islet transplantation setting. Diabetic C57BL/6 mice were transplanted with 400 syngeneic islets under the kidney capsule and treated intraperitoneal with 50 mg/kg, daily, of JANEX-1 or vehicle (10% DMSO in PBS) for 180 days. Non-fasting blood glucose level (mg/dl) of JANEX-1-treated and control recipients were followed for 180 days (A); intraperitoneal glucose-tolerance test (B) of the same recipients were performed on day 180 post syngeneic islet transplantation. Non-transplanted C57BL/6 mice (control) were used as controls for transplanted JANEX-1- (Tx-JANEX-1) and vehicle-treated (Tx-control) recipients in glucose-tolerance test (B). Hematoxylin and eosin (C, E) and immunostaining for insulin (D, F) of syngeneic islet grafts obtained from JANEX-1- (E, F) or vehicle-treated (C, D) recipients on day 180 post-transplantation.

in vivo potency and lack of systemic toxicity, JANEX-1 shows significant clinical potential as a new target-specific immunosuppressive agent for the prevention of type 1 diabetes. Further development of chemical inhibitors of JAK3 such as JANEX-1 may provide the basis for effective treatment modalities against human type1 diabetes.

Allogeneic transplantation

T cells play a central role in acute allograft rejection³⁷. So far, complete activation of T cells requires three sequential signals^{50, 141}. Signal 1, delivered by antigens that engage the TCR, is followed by signal 2, delivered by cognate ligands on the antigen-pre-

senting cells (B7-CD28 and CD40-CD154 interactions). Signals 1 and 2 are critical for the synthesis and secretion of IL-2, which together with other T cell growth factors such as IL-4, IL-7, IL-9, IL-13 and IL-15, deliver a signal 3 through cytokine receptors. Therefore, these T cell-stimulatory cytokines, including IL-2¹⁵⁰, IL-4⁸⁹, IL-7, and IL-15⁶⁶, promote allograft rejection by augmenting the activation and proliferation of alloreactive T cells. As mentioned before, these cytokines utilize the same IL-2 receptor γ c as an essential signal transducing component in their multichain receptor complexes¹¹⁹. Immunosuppressive strategies designed to disrupt a particular cytokine or its receptor have achieved only limited success owing to redundant and compensatory signaling pathways mediated by shared receptors recruited by other cytokines^{66, 110, 115, 121, 144}. The γ c receptor is more promiscuous, serving as a common receptor for multiple cytokines mentioned above¹²⁴. As JAK3 is activated through the γ c receptor, inactivation of the JAK3 could disrupt an entire family of cytokines. Thus, the inhibition of not a single cytokine, but rather of a common intermediary signaling molecule, such as JAK3, may represent an ideal strategy for the prevention of allograft rejection. Interestingly, blocking the γ c receptor by using a monoclonal antibody was shown to prevent islet allograft rejection⁶⁵, suggesting that targeting the γ c-triggered signaling events, e.g. JAK3 activation, may produce similar inhibitory effects in T cell activation. Current immunosuppressive regimens have relied on calcineurin-, e.g. signal 1-inhibitors (CsA and FK506). However, ubiquitous expression of calcineurin in many different tissues contributes to several adverse side effects including nephrotoxicity and neurotoxicity^{7, 142}. JAK3 is abundantly expressed in lymphocytes, but not ubiquitously in other tissues. Therefore, JAK3 is an ideal target with the desired selectivity in blocking T cell function and allograft rejection.

Beta cell replacement by pancreas transplantation for type 1 diabetes mellitus can induce an insulin-independent normoglycemic state¹²⁰. Advances in immunosuppressive regimens, organ procurement techniques, and isolation techniques have promoted the development of pancreatic Langerhans islet transplantation as a new treatment modality for insulin-dependent diabetic patients which offers a chance of good glycemic control without major surgical risks^{9, 12}. A recent transformation in the outcome of clinical islet transplantation for diabetes has galvanized a research focus on transplantation¹¹². Shapiro et al.¹¹¹ demonstrated that islet transplantation can result in insulin independence with excellent metabolic control when glucocorticoid-free immuno-

suppression is combined with the infusion of an adequate islet mass.

It was described recently that JAK3-deficient recipients were not able to reject islet allografts¹⁸. Therefore, the specific JAK3 kinase inhibitor JANEX-1 was tested in a context of islet allotransplantation in mice. It was shown that JANEX-1 (50 mg/kg from the day of transplantation until the day of rejection) exhibited significant activity as an anti-rejection drug. Interestingly, the combination of JANEX-1 (50 mg/kg) and CsA (20 mg/kg) was more effective than either agent alone in prolongation of islet allograft survival. This report was the first one about the immunosuppressive activity of a specific JAK3 inhibitor in islet allotransplantation¹⁸. However, there are several reports about the successful use of JAK3 kinase inhibitors in experimental heart transplantation. Thus, it is shown that typhostin AG-490, originally described as a JAK2 kinase inhibitor, inhibits JAK3 as well, and induces prolongation of rat allogeneic heart transplants survival⁶. AG-490 was found to potentiate the immunosuppressive effects of CsA, but not rapamycin, in the same allogeneic heart transplant model⁶. Another report reveals that PNU156804, which is twice as efficient in inhibiting JAK3 than JAK2 kinase, prolongs heart allograft survival, as well, and acts synergistically/additively with CsA/rapamycin¹¹⁶.

Besides the common side effects of conventional immunosuppressants, it is currently well documented that standard immunosuppressive drugs cause post-transplant diabetes mellitus after islet transplantation^{44, 45}. The diabetogenic activities of the immunosuppressants, such as steroids¹⁵¹, CsA²⁷ and FK506^{27, 42}, preclude them from being optimal immunosuppressants for pancreatic islet transplantation. New drugs without or with fewer diabetogenic properties will be especially beneficial to clinical islet transplantation. Therefore, the effects of long-term (6 months) JANEX-1 treatment on the function and morphology of syngeneic-grafted islets were studied to evaluate potential diabetogenic properties of such treatment. As it is shown in Fig. 3A, C57BL/6 mice transplanted by syngeneic islets and treated by JANEX-1 exhibited no differences in nonfasting blood glucose level at all time points during the 6-month observation period compared with vehicle-treated control recipients. JANEX-1 did not induce glucose intolerance (Fig. 3B), and immunohistochemical analysis of the grafted islets after 6 months of treatment revealed a level of insulin expression (Fig. 3F) comparable to that of the vehicle-treated control grafts (Fig. 3D). Therefore, this study clearly indicates that JANEX-1 has no diabetogenic property *in vivo*.

In conclusion, targeting JAK3 in alloreactive T cells by chemical inhibitors could be an useful strategy in preventing islet and heart allograft rejection. A specific JAK3 kinase inhibitor such as JANEX-1 could prevent islet allograft rejection without the diabetogenic side effects reported for other immunosuppressive drugs. Considering the restricted expression of JAK3 to lymphoid compartments, the pharmacological inhibition of JAK3 should provide fewer adverse effects than those currently linked to the conventional immunosuppressants. Moreover, the synergy between the conventional immunosuppressive drugs and JAK3 blockers should allow the delivery of drugs at subtoxic levels and therefore minimizing the adverse effects of conventional immunosuppressants.

ALLERGIES

Mast cells and basophils have been implicated in the pathogenesis of asthma particularly in the acute phase (early phase of the allergic reaction), through the release of inflammatory mediators such as histamine and leukotrienes that exhibit their effects on target organs, particularly smooth muscles²². These reactions can be reversed or prevented by drugs that target mast cells or basophils or their released mediator²⁵. It was recently discovered that mast cells also

express JAK3⁷⁵. Moreover, biochemical and genetic evidence were provided that JAK3 is a key regulator of IgE-mediated mast cell responses⁷⁴. Specifically, it was shown that JAK3 is activated upon IgE receptor cross-linking in mast cells. Furthermore, JAK3-deficient mast cells release reduced amounts of inflammatory mediators upon IgE receptor cross-linking as compared with wild type mast cells⁷⁴. *In vitro* treatment of mast cells with a potent and specific JAK3 inhibitor JANEX-2 (WHI-P154)⁷⁴ and JANEX-1⁷⁵, significantly abrogated degranulation and proinflammatory mediator release after IgE receptor/FcεRI cross-linking. *In vivo* administration of JANEX-1 at a non-toxic dose level prevented mast cell degranulation and the development of cutaneous as well as systemic anaphylaxis in mice⁷⁵. Treatment of ovalbumin-sensitized mice with another JAK3 kinase inhibitor, WHI-P⁹⁷, prevented the development of airway hyperresponsiveness to methacholine and inhibited eosinophil recruitment to the airway lumen after ovalbumin challenge in a dose-dependent fashion⁷³.

In summary, these results have confirmed that targeting JAK3 with a specific inhibitor such as JANEX-1 may provide the basis for new and effective treatment as well as prevention programs for mast cell-mediated allergic reactions.

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