RESEARCH ARTICLE

Targeting Janus tyrosine kinase 3 (JAK3) with an inhibitor induces secretion of TGF- β by CD4⁺ T cells

Marina Cetkovic-Cvrlje^{1,2}, Marin Olson¹ and Ketaki Ghate^{1,2}

Regulatory T cells (Tregs) are critical for the peripheral maintenance of the autoreactive T cells in autoimmune disorders such as type 1 diabetes (T1D). Pharmacological inhibition of Janus tyrosine kinase 3 (JAK3) has been proposed as a basis for new treatment modalities against autoimmunity and allogeneic responses. Targeting JAK3 with an inhibitor has previously been shown to exhibit protective action against the development of T1D in non-obese diabetic (NOD) mice. As the mechanism of such preventative action has been unknown, we hypothesized that JAK3 inhibition induces generation of Tregs. Here, we show that the JAK3 inhibitor 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P131) suppresses proliferation of short-term cultured NOD CD4⁺ T cells through induction of apoptosis, while promoting survival of a particular population of long-term cultured cells. It was found that the surviving cells were not of the CD4⁺CD25⁺FoxP3⁺ phenotype. They secreted decreased amounts of IL-10, IL-4 and interferon (IFN)- γ compared to the cells not exposed to the optimal concentrations of JAK3 inhibitor. However, an elevated transforming growth factor (TGF)- β secretion was detected in their supernatants. *In vivo* treatment of prediabetic NOD mice with WHI-P131 did not affect the frequency and number of splenic and pancreatic lymph node CD4⁺FoxP3⁺ Tregs, while generating an elevated numbers of CD4⁺FoxP3⁺ TGF- β -secreting T cells. In conclusion, our data suggest an induction of TGF- β -secreting CD4⁺ T cells as the underlying mechanism for antidiabetogenic effects obtained by the treatment with a JAK3 inhibitor. To our knowledge, this is the first report of the JAK3 inhibitor activity in the context of the murine Tregs.

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INTRODUCTION

Regulatory CD4⁺ T cells (Tregs) have been shown to actively suppress the function of conventional T cells, and play a crucial role in controlling undesired immune responses to self-antigens. Their protective role has been described in a non-obese diabetic (NOD) mouse model of autoimmune type 1 diabetes (T1D),^{1–4} as well as in human T1D.⁵ There are two major types of Tregs, 'natural' Tregs (nTregs),⁶ and 'induced or adaptive' Tregs (iTregs).^{7,8} The extracellular expression of a high-affinity IL-2 receptor α -chain (CD25) and the nuclear expression of FoxP3 in CD4⁺ T cells are considered as the nTreg markers. There is no particular molecule that marks iTreg population. However, iTregs can be divided into at least two different groups based on the type of cytokines that they secrete: IL-10-secreting iTregs and transforming growth factor (TGF)- β -secreting Th3-type Tregs.^{4,7,8}

Janus tyrosine kinase 3 (JAK3), a member of JAK family, is cytoplasmic protein tyrosine kinase which plays a pivotal role in the initiation of cytokine-triggered signaling events by activating the cytoplasmic latent forms of signal transducer and activator of transcription (STAT) factor 5 *via* tyrosine phosphorylation on the specific tyrosine residues.⁹ JAK3 specifically associates with the γ c subunit of the IL-2 receptor, and is activated by the cytokines IL-2, IL-4, IL-7,

IL-9, IL-15 and IL-21 that use the γ c receptor.^{10,11} It is known that JAK3 is abundantly expressed in lymphoid cells and plays an important role in normal lymphocyte proliferation, differentiation and function, as evidenced by qualitative and quantitative deficiencies in the T-cell compartment of the immune system of JAK3-deficient mice^{12,13} and the development of severe combined immunodeficiency in JAK3-deficient patients.¹⁴ Therefore, targeting JAK3 has been proposed as a new modality for immunosuppression of T cell-mediated pathologies, such as transplant rejection and autoimmunity.^{15–18}

The structure-based design of specific inhibitors of JAK3 has been reported previously.¹⁹ It has been shown that the lead compound, 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline (WHI-P131) suppresses mitogen- and antigen-induced responses *in vitro*.^{20,21} *In vivo* treatment with WHI-P131 attenuated the severity of T cell-dependent graft-versus-host disease post murine allogeneic bone marrow transplantation,^{20,22,23} and prevented rejection of mouse allogeneic islet cell transplants.²⁴ Furthermore, WHI-P131 treatment prevented development of T1D in NOD mice.²⁵ When cytokine profile was analyzed in splenocytes of WHI-P131-treated NOD mice, a clear skewing from pathogenic Th1- towards protective Th2-type immune response was not found. Thus, the efforts to characterize the mechanisms of protective action of JAK3 inhibitors in T1D

¹Department of Biological Sciences, St. Cloud State University, St. Cloud, MN, USA and ²Laboratory for Immunology, St. Cloud State University, St. Cloud, MN, USA
Correspondence: Dr M Cetkovic-Cvrlje, Laboratory for Immunology, Department of Biological Sciences, St. Cloud State University, 720 Fourth Avenue South, St. Cloud, MN 56301, USA.

E-mail: m.cetkoviccvrlje@stcloudstate.edu

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development of NOD mice provided inconclusive results in the context of Th1/Th2 paradigm.²⁵

Recent studies have indicated that some conventional immunosuppressive drugs exhibit beneficial effects on Treg induction and function, whereas others may be detrimental.²⁶ For example, rapamycin can induce generation of mouse,²⁷ as well as human^{28,29} CD4⁺CD25⁺FoxP3⁺ Tregs under specific *in vitro* condition. Moreover, rapamycin has been found to expand Tregs *in vitro*, allowing this rare cell type to become available for clinical applications.^{27–29} In contrast, *in vitro* and *in vivo* studies with cyclosporine A showed inhibition of FoxP3 expression,³⁰ compromised thymic generation, and reduction in peripheral Tregs.³¹ The action of both cyclosporine A and rapamycin is associated with cytokine IL-2. Differential effects of those drugs on Tregs may be explained by differences in signal transduction pathways. Whereas cyclosporin inhibits phosphatase calcineurin, thereby suppressing the IL-2 production,³² rapamycin inhibits IL-2 signaling by binding the mammalian target of rapamycin, with a consequent suppression of a cell cycle.^{33,34} The JAK3 inhibitors inhibit IL-2 signaling as well, but through the JAK3/STAT5 pathway.^{10,11} While JAK3 signaling is fairly well understood, the influence of JAK3 inhibitors on the induction of Tregs *in vitro* and *in vivo* has not been studied yet.

Considering the beneficial effects of the JAK3 inhibitor WHI-P131 in the prevention of T1D, and its unclear mechanism of protection, along with its potential influence on the generation of Tregs, we hypothesized that the induction of Tregs is the mechanism of protective WHI-P131 action. Therefore, the aim of this study was to evaluate the effect of JAK3 inhibition by WHI-P131 on the murine Tregs *in vitro* and *in vivo*.

MATERIALS AND METHODS

Mice

NOD mice, originally bought from The Jackson Laboratory (Bar Harbor, ME, USA), were bred in our Animal Care Facility at St. Cloud State University (SCSU). Mice were fed *ad libitum* with rodent diet Harlan Teklad 18% Global Protein Diet 2018. We have shown that hyperglycemia can occur in our colony of NOD females as early as 8 weeks of age.³⁵ Therefore, all mice used in our experiments were 5- to 7-week-old prediabetic females that showed no signs of hyperglycemia. All procedures that included manipulation of live animals (euthanasia, detection of glycemia and drug injection regimens) were approved by the SCSU Institutional Animal Care and Use Committee.

Cell isolation, stimulation and culturing

Spleens were removed from NOD females and a single-cell suspension was prepared.³⁶ Lymphocytes were isolated from pancreata and pancreatic lymph nodes, as described previously.^{24,36} CD4⁺ cells were isolated by magnetic separation using anti-CD4-coated magnetic microbeads and MS and LS columns (Mytenyi Biotec; Auburn, CA, USA). Isolated CD4⁺ T cells were cultured in a concentration of 1–3×10⁶/ml in RPMI 1640 (supplemented with 10% fetal calf serum, Hyclone; Logan, UT, USA) and stimulated with plate-bound anti-CD3 (10 μ g/ml) and anti-CD28 (2 μ g/ml) antibodies (BD Biosciences; San Jose, CA, USA). JAK3 kinase inhibitor WHI-P131 (Calbiochem; San Diego, CA, USA) was added to the cell cultures in the concentrations of 6, 3 and 1.5 μ g/ml (18, 9 and 4.5 μ M, respectively). A control drug, rapamycin (Sigma; St. Louis, MO, USA), known to induce generation of CD4⁺CD25⁺FoxP3⁺ Tregs *in vitro*,²⁷ was added in the concentrations of 1000, 100 and 10 nM. Briefly, CD4⁺ T cells were stimulated and cultured with addition of drugs for 7 days. The cells were then washed, recounted and restimulated with addition of drugs. Three rounds of

stimulation of 7 days each were performed, as described previously.^{27,37} IL-2 (2 μ g/ml; BD Biosciences) was added starting from the second round of cell stimulation.^{27,37} At the end of the third week of culturing, the cells were washed, stimulated without the drug addition and analyzed appropriately.

Detection of cell proliferation

Proliferation was measured by using Cell Proliferation ELISA (BrdU) Immunoassay kit from Roche (Indianapolis, IN, USA). Isolated CD4⁺ T cells were stimulated as described above and cultured for 72 h with addition of WHI-P131. Cells were then labeled with BrdU according to manufacturer's instruction, and the optical density was determined within 30 min using a microplate reader at 450 nm.

Detection of cell viability during the long-term culturing

During the 3 weeks of culturing, at the end of each week, CD4⁺ T cells were collected, washed, counted and cell viability determined by trypan blue exclusion test. Based on the total number of viable cells, they were again put in the culture and restimulated by anti-CD3, anti-CD28 and IL-2 with addition of WHI-P131 or rapamycin.

Detection of apoptosis

Apoptosis was measured in CD4⁺ T cells cultured for 72 h (short-term) by using Cell Death Detection ELISA kit (Roche) according to the manufacturer's instruction. This kit provides relative quantification of histone-complexed DNA fragments of the cells that have undergone apoptosis. The optical density was determined using a microplate reader at 450 nm. Apoptosis was also measured by flow cytometry in long-term (3 weeks) vs. short-term (48 h) cultured CD4⁺ T cells by using the FITC Annexin V Apoptosis Detection kit (BD Biosciences) according to the manufacturer's instruction. Briefly, 1×10⁵ cells were stained by FITC-Annexin V and PI, incubated for 15 min and analyzed by FACSCalibur (BD Biosciences). Double-positive (Annexin V⁺PI⁺) cells were considered as apoptotic.

Immunophenotyping of cells by flow cytometry

Monoclonal antibodies against mouse CD3 and CD8 markers were purchased from BD Biosciences. Mouse Regulatory T Cell Staining kit (eBiosciences; San Diego, CA, USA), containing anti-CD4-FITC, -CD25-APC and -FoxP3-PE antibodies, was used for a characterization of CD4⁺ T cells either *in vitro* cultured for 3 weeks with addition of WHI-P131 or rapamycin, or *ex vivo* isolated from NOD mice treated *in vivo* by these drugs. Cells were stained according to the manufacturer's instruction and analyzed by BD FACSCalibur (BD Biosciences).

Detection of cytokines

ELISA tests (Quantikine mouse IL-2, IL-4, IL-10, interferon (IFN)- γ and human TGF- β 1; R&D Systems; Minneapolis, MN, USA) were used for quantifying cytokine concentrations in CD4⁺ cell supernatants obtained post long-term culturing with addition of WHI-P131 and rapamycin *in vitro*. The same cytokines were detected in the supernatants of the isolated CD4⁺ T cells obtained post *in vivo* treatment of NOD mice with WHI-P131 and rapamycin. The supernatants were collected and stored at –80 °C until the measurement. The optical density was determined using a microplate reader at 450 nm.

In vivo treatment of mice by WHI-P131 and rapamycin

In the first experiment, 4- to 6-week-old prediabetic NOD females ($n=7$) were injected intraperitoneally with 75 mg/kg of WHI-P131

(dissolved in 5% dimethyl sulfoxide in phosphate-buffered saline), daily (twice/day) for 10 days. Control mice ($n=6$) received vehicle control. After the treatment, mice were killed, and CD4⁺ T cells were magnetically isolated (as described above), and stimulated with anti-CD3 plus anti-CD28 antibodies for 48 h. Cell supernatants were collected for detection of cytokines (IL-10, IL-4, IL-2, IFN- γ and TGF- β 1). In the second experiment, 4- to 6-week-old NOD females ($n=6$) were treated with 1 mg/kg/day of rapamycin (dissolved in phosphate-buffered saline), while the controls received vehicle control for a duration of 10 days. Afterwards, CD4⁺ T cells were isolated, stimulated and cultured for 48 h. The supernatants were collected and cytokines were analyzed by ELISA.

RESULTS

WHI-P131 suppresses the proliferation of short-term cultured isolated CD4⁺ T cells

It has been previously shown that the JAK3 inhibitor WHI-P131 exhibits a potent dose-dependent suppression of proliferative responses of non-diabetic C57BL/6 and NOD mouse T cells induced by both mitogens^{20,25} and an autoantigen, GAD65.²⁵ However, these studies did not confirm the effects of the JAK3 inhibitor on a particular subpopulation of T cells, such as CD4⁺ T cells. To define the effect of WHI-P131 on isolated CD4⁺ T-cell population, CD4⁺ cells were isolated by positive magnetic beads separation from the spleens of NOD mice. Isolated cells were activated with plate-bound anti-CD3 and soluble anti-CD28 Ab with or without the addition of different concentrations (1.5, 3 and 6 μ g/ml) of WHI-P131, and proliferation was measured by BrdU ELISA post 72 h of culturing. The chosen concentrations of WHI-P131 were selected based on our previously obtained results showing that doses of 5–100 μ g/ml of WHI-P131 completely suppressed proliferation of a heterogeneous population of T cells.²⁰ Here, we show that the additions of 1.5, 3 and 6 μ g/ml of WHI-P131 induced a dose-dependent, statistically significant reduction in proliferation of CD4⁺ T cells compared to vehicle-exposed controls (Figure 1a).

WHI-P131 induces apoptotic cell death in short-term cultured isolated CD4⁺ T cells

It has been demonstrated that WHI-P131 induces apoptotic cell death of tumor cell lines *in vitro*.^{19,21} Therefore, we asked whether an apoptotic cell death is the mechanism of WHI-P131-induced suppression of CD4⁺ cell proliferation. Cells were activated by the combination of anti-CD3 and anti-CD28 antibodies, and cultured with the additions of 1.5, 3 and 6 μ g/ml of WHI-P131 for 72 h. Apoptotic cell death was detected by ELISA. Figure 1b shows that the addition of WHI-P131 causes a dose-dependent increase in apoptotic cell death in cultured CD4⁺ T cells. Therefore, an induction of cell death is the mechanism of suppressed proliferation of CD4⁺ cells cultured for a short period of time with the addition of WHI-P131.

WHI-P131 allows survival of isolated CD4⁺ T cells during long-term culturing

Rapamycin selectively expands murine CD4⁺CD25⁺FoxP3⁺nTregs²⁷ during long-term culturing consisting of three rounds of stimulations of naive CD4⁺ T cells. In contrast, the proapoptotic effect of rapamycin has been shown in the short-term cultures of both murine²⁷ and human CD4⁺ T cells.²⁹ To define the effects of long-term exposure of T cells on the JAK3 inhibitor, naive CD4⁺ T cells, isolated from NOD spleens, were activated with a plate-bound anti-CD3 and soluble anti-CD28 antibodies for 3 consecutive weeks in the presence or absence of

Figure 1 Proliferation (a) and apoptotic cell death (b) of CD4⁺ T cells exposed to WHI-P131 during short-term (72 h) culture period. Isolated CD4⁺ T cells were cultured with addition of WHI-P131 in the concentrations of 1.5, 3 and 6 μ g/ml (P1.5, P3 and P6, respectively). Cell proliferation was detected by BrdU ELISA kit (a); cell apoptosis was detected by cell death detection ELISA kit (b). Data presented as average O.D. $\pm$ s.e.m. of 5–6 samples/group. Statistical analysis performed by Student's *t*-test. * $P < 0.05$ compared to control. WHI-P131, 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazolin.

different concentrations of WHI-P131. The viability was evaluated by trypan blue exclusion test post each week of culturing. Rapamycin (10, 100 and 1000 nM) was used as a control drug-treatment. After the first week of culturing, there was a significant reduction in the percentages of viable cells observed in all of the WHI-P131-treated groups compared to the control cells (Figure 2). The highest concentration of the drug (6 μ g/ml) induced significantly more pronounced reduction in cell viability ($17.1\% \pm 1.4\%$) compared to the one observed in the cells exposed to 1.5 μ g/ml of the drug ($42.3\% \pm 1.7\%$). Rapamycin, as well, affected the viability of cells post first week of culturing (Figure 2). However, the dose-dependent response was not observed. After the second round of stimulation, there was a trend of reduced viability, which did not reach a significant difference in T-cell cultures exposed to 6, 3 and 1.5 μ g/ml of WHI-P131 compared to control cultures (Figure 2). In contrast, the numbers of viable cells obtained by the end of the third week of activation with the additions of 6, 3 and 1.5 μ g/ml of WHI-P131 were comparable to the control cells (Figure 2). The addition of rapamycin (all three concentrations) during the second, as well as the third week of stimulation, did not reduce the viability of cultured cells compared to controls (Figure 2).

As shown previously and here (Figure 1b), WHI-P131 induces apoptotic cell death in the primary cells²⁰ and cell lines¹⁹ cultured for a short period of time. Our cell viability data, observed in cultures exposed to WHI-P131 after the first and second week of stimulation, are in agreement with apoptosis induction. In contrast, an increased viability of drug-exposed isolated CD4⁺ T cells, observed post long-term culturing, suggests potential resistance of surviving cells to proapoptotic effect of WHI-P131, or the outgrowth of a pre-existing res-

Figure 2 Viability of CD4⁺ T cells, presented as a percentage of the initial cell number, exposed to WHI-P131 during long-term (3 weeks) culture period. Isolated CD4⁺ T cells were stimulated and cultured (three rounds of stimulation with anti-CD3 and anti-CD28 antibodies, 7 days each) with addition of WHI-P131 in the concentrations of 1.5, 3 and 6 μ g/ml (P1.5, P3 and P6, respectively), or rapamycin in the concentrations of 10, 100 and 1000 nM (R10, R100 and R1000, respectively). Viability was measured using trypan blue exclusion test after the first (clear bars), second (striped bars) and third (dotted bars) week of culturing. Data presented as average percentage $\pm$ s.e.m. of five experiments with 4–6 samples/group. Statistical analysis performed by Student's *t*-test. **P* < 0.05 compared to control. WHI-P131, 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazolin.

istant population. Therefore, we wanted to compare the apoptotic cell death in short- vs. long-term cultures of isolated CD4⁺ T cells. The same experimental design, consisting of three rounds of stimulation of NOD CD4⁺ T cells with exposure to WHI-P131 in each round was used, as described above. The short-term culture of isolated CD4⁺ cells was set up 48 h before the end of the third round of stimulation of 3-week-cultured cells, in order to analyze the apoptotic cell death of short- vs. long-term cultured cells simultaneously. Cells were double-stained with Annexin V/PI and analyzed by flow cytometry. Figure 3 shows that addition of all concentrations of WHI-P131 during the 48-h culture period significantly increased the percentage of double-positive (Annexin V⁺/PI⁺) apoptotic cells compared to controls. Addition of WHI-P131 in concentrations of 6 and 3 μ g/ml significantly elevated the percentages of apoptotic cells to 49% $\pm$ 7% and 52% $\pm$ 4%, respectively, compared to 30% $\pm$ 2% in 1.5 μ g/ml-treated group. In contrast, a significant reduction in the cell death percentages was observed in the cultures exposed to WHI-P131 during the 3 weeks vs. 48 h of culturing (Figure 3). The next experiment was set up in order to test whether all surviving long-term cultured T cells have been initially resistant to JAK3 inhibition. The CD4⁺ T cells were stimulated during the 3 weeks with anti-CD3 plus anti-CD28 antibodies without the exposure to WHI-P131. After the three rounds of culturing, cells were exposed to WHI-P131 for 48 h. While 8.3% $\pm$ 0.7% of control untreated cells were apoptotic, 47.2% $\pm$ 11.3%, 41.0% $\pm$ 8.7% and 25.1% $\pm$ 5.9% became apoptotic post exposure to 6, 3 and 1.5 μ g/ml of WHI-P131, respectively, confirming that long-term culturing *per se* does not generate T cells resistant to JAK3 inhibition.

Similar results were obtained by the addition of rapamycin in short- vs. long-term cultures of CD4⁺ cells. Thus, rapamycin (all tested concentrations) induced a significant increase in the percentage of cell death in short-term cultures of CD4⁺ T cells compared to controls (Figure 3). In contrast, flow cytometric analysis of Annexin V/PI double-positive cells, performed at the end of the third week, revealed a

Figure 3 Flow cytometric analysis of apoptotic cell death post short- (48 h) and long-term (3 weeks) culturing of CD4⁺ T cells with addition of WHI-P131. Apoptotic cell death was detected using Annexin-V/PI double staining of CD4⁺ T cells stimulated as described in Figure 2, and cultured with addition of WHI-P131 in the concentrations of 1.5, 3 and 6 μ g/ml (P1.5, P3 and P6, respectively) or rapamycin in the concentrations of 10, 100 and 1000 nM (R10, R100 and R1000, respectively) for 48 h (striped bars) or 3 weeks (dotted bars). Data are presented as an average percentage of double-positive (Annexin-V⁺/PI⁺) cells $\pm$ s.e.m. of five samples/group. Statistical analysis performed by Student's *t*-test. **P* < 0.05 compared to control; ***P* < 0.05 compared to the same drug concentration at different culturing periods; ****P* < 0.05 compared to the optimal rapamycin drug concentration (R100). WHI-P131, 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazolin.

significant reduction in the percentages of apoptotic cells in the cultures exposed to 1000, 100 and 10 nM of rapamycin, respectively, compared to the percentages of apoptotic cells observed post short-term exposure to the same concentrations of the drug. In conclusion, these data are in line with the viability data obtained post first (short-term) and third week of culturing (long-term) with the addition of WHI-P131. In addition, presented results suggest that a long-term exposure to the JAK3 inhibitor allows a particular fraction of cultured CD4⁺ T cells to become more resistant to proapoptotic action of this drug, or the outgrowth of a preexisting resistant population.

WHI-P131 does not promote growth of CD4⁺CD25⁺FoxP3⁺ cells during long-term culturing

After the three rounds of stimulation with anti-CD3 and anti-CD28 antibodies, CD4⁺ T cells were rested with the addition of IL-2 and restimulated with the same antibodies overnight. The next day, a flow cytometric analysis was performed in order to quantify the expression of surface markers CD4 and CD25, and intracellular marker FoxP3. The majority of control cells (82% $\pm$ 2%), generated over the 3 weeks of culturing, were CD4⁺CD25⁺ double-positive cells. The addition of WHI-P131 induced marked reduction (58% $\pm$ 7%) of CD4⁺CD25⁺ cells. In contrast, rapamycin addition in the concentrations of 10 and 1000 nM did not affect, whereas 100 nM significantly increased the percentage of CD4⁺CD25⁺ cells (91% $\pm$ 3%). An entire population of CD4⁺CD25⁺ cells represents the activated conventional T cells, as well as Tregs.⁸ In order to detect the CD25⁺ nTregs, we asked how many of the CD25⁺ cells express FoxP3, a hallmark marker of nTregs.^{7,8} There was 3.6% $\pm$ 0.8% of CD25⁺FoxP3⁺ T cells present in the control treatment (Figure 4a and c). The WHI-P131 addition in doses of 1.5, 3 and 6 μ g/ml, prominently reduced the populations of CD25⁺FoxP3⁺ cells in a dose-dependent manner to 2.0% $\pm$ 0.3%, 1.2% $\pm$ 0.2% and 0.3% $\pm$ 0.2%, respectively (Figure 4a and d). In contrast, addition of

Figure 4 Detection of CD4⁺CD25⁺FoxP3⁺ cells in cultures of CD4⁺ T cells exposed to WHI-P131 over the 3 weeks. (a) Percentage of CD25⁺FoxP3⁺ T cells. (b) Percentage of CD4⁺FoxP3⁺ T cells. CD4⁺ T cells were stimulated, cultured for 3 weeks with addition of WHI-P131 in the concentrations of 1.5, 3 and 6 μ g/ml (P1.5, P3 and P6, respectively) or rapamycin in the concentrations of 10, 100 and 1000 nM (R10, R100 and R1000, respectively) and stained for flow cytometric analysis with anti-CD4-FITC, anti-FoxP3-PE and anti-CD25-APC antibodies. Data are presented as average $\pm$ s.e.m. of six samples/group. Statistical analysis performed by Student's *t*-test. **P* < 0.05 compared to control. The representative dot plots of: CD25⁺FoxP3⁺ cells of the control (c), WHI-P131- (d) and rapamycin (Rapa)-treated (e) groups; CD4⁺FoxP3⁺ cells of the control (f), WHI-P131- (g) and Rapa-treated (h) groups; the percentages of double-positive cells are shown in the upper right quadrants. WHI-P131, 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazolin.

rapamycin in the concentrations of 10, 100 and 1000 nM significantly increased the percentages of CD25⁺FoxP3⁺ cells to 10.8% $\pm$ 3.3%, 15.2% $\pm$ 2.5% and 8.1% $\pm$ 1.4%, respectively (Figure 4a and e). Similarly, the trend of changes in CD4⁺FoxP3⁺ cell population exposed to WHI-P131 and rapamycin followed those observed in CD25⁺FoxP3⁺ cells. As previously described,²⁷ the exposure of CD4⁺ cells to 100 nM rapamycin most prominently increased the percentage of FoxP3⁺ cells to 19.2% $\pm$ 4.4% compared to 4.9% $\pm$ 1.1% in a control group (Figure 4b, f and h). In contrast to the rapamycin, addition of WHI-P131 did not induce FoxP3 expression in long-term cultures of CD4⁺ T cells: only 1.7% $\pm$ 0.2%, 1.5% $\pm$ 0.5% and 0.4% $\pm$ 0.3% of CD4⁺FoxP3⁺ cells were detected post exposure to 1.5, 3 and 6 μ g/ml of WHI-P131, respectively (Figure 4b and g).

WHI-P131 does not induce generation of IL-10-, but TGF- β -secreting T cells during long-term culturing

Next, we asked what types of cytokines are released post three rounds of stimulation of isolated CD4⁺ T cells cultured with the addition of

WHI-P131. It has been demonstrated that the addition of dexamethasone and vitamin D promotes growth of IL-10-secreting Tr-1-type Tregs during long-term culturing with three rounds of stimulations of naive CD4⁺ T cells.³⁷ An elevated secretion of IL-10 has been observed previously in a heterogeneous, concanavalin A-activated splenic T-cell population, isolated from NOD mice post *in vivo* treatment with WHI-P131.²⁵ Therefore, we hypothesized that WHI-P131, while not inducing FoxP3⁺ nTregs, can allow generation of IL-10-secreting Tr-1 cells during long-term culturing. Results shown on Figure 5a do not confirm our hypothesis. The secretion of IL-10 in the long-term cultures of stimulated CD4⁺ T cells was significantly decreased if the cells were treated with 6 and 3 μ g/ml of WHI-P131 (Figure 5a). The same supernatants were tested for the amount of IL-2, IL-4, IFN- γ and TGF- β . While IL-2 secretion was not detected at all (data not shown), the IFN- γ (Figure 5c) and IL-4 levels (Figure 5b) were significantly reduced in the supernatants of cells exposed to WHI-P131. These data suggest that the WHI-P131 addition to cultures of CD4⁺ cells does not induce generation of Tr-1 or Th1/Th2-type T

Figure 5 Cytokine profile of CD4⁺ T cells exposed to WHI-P131 during long-term culturing. Cytokines IL-10 (a), IL-4 (b), IFN- γ (c) and TGF- β (d) were detected by ELISA in the supernatants of CD4⁺ T cells stimulated and cultured for 3 weeks with addition of WHI-P131 in the concentrations of 1.5, 3 and 6 μ g/ml (P1.5, P3 and P6, respectively) or rapamycin in the concentrations of 10, 100 and 1000 nM (R10, R100 and R1000, respectively). Data are presented as average (pg/ml) $\pm$ s.e.m. of 5–6 samples/group. Statistical analysis performed by Student's *t*-test. **P* < 0.05 compared to control. IFN, interferon; TGF, transforming growth factor; WHI-P131, 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazolin.

cells. Surprisingly, a significantly elevated level of TGF- β was found in the supernatants of CD4⁺ cells exposed to WHI-P131 in the concentrations of 3 and 1.5 μ g/ml (Figure 5d), suggesting a Th3-type Treg phenotype induction during the long-term culturing under described *in vitro* conditions. The highest concentration of 6 μ g/ml of WHI-P131 did not increase TGF- β secretion, as this drug concentration induced significantly higher level of apoptotic cell death after the long-term culturing, compared to control (Figure 3). Addition of rapamycin did not affect either IL-10 (Figure 5a) or TGF- β (Figure 5d) levels, while reducing IFN- γ (Figure 5c) and IL-4 (Figure 5b) secretion of CD4⁺ cells, compared to controls.

***In vivo* treatment with WHI-P131 does not induce FoxP3 expression but elevates TGF- β secretion in CD4⁺ T cells**

The next set of experiments was performed in order to define the effects of *in vivo* treatment with WHI-P131 on nTregs (FoxP3 expression), as well as cytokine profiles of *ex vivo*-extracted CD4⁺ T cells. NOD females (*n* = 7) were treated with 75 mg/kg/day of WHI-P131, a dose expected to be effective *in vivo*.²⁵ A control group of mice received a vehicle control treatment (*n* = 6). Mice were killed post 10 days of treatment, spleens were removed and viability of splenocytes was determined by trypan blue exclusion test. It was found that WHI-P131 treatment *in vivo* does not affect viability of splenocytes, as 96% $\pm$ 5% were alive, compared to 89% $\pm$ 3% in controls. These results confirm previously published data regarding the non-toxic effects of this JAK3 inhibitor.^{25,38} The splenocytes obtained from WHI-P131-treated and control mice were analyzed for CD3, CD8, CD4 and FoxP3 expression by flow cytometry. There were no differences in the percentages of the CD3⁺, CD4⁺, CD8⁺ and CD4⁺FoxP3⁺ T cells between the drug-treated and control mice (Figure 6a). Interestingly, the absolute splenocyte number obtained post *in vivo* WHI-P131 treatment was significantly (*P* < 0.05) higher ($103 \times 10^6 \pm 6.2 \times 10^6$)

compared to the controls ($84.5 \times 10^6 \pm 8.6 \times 10^6$). Thus, when the splenocytes' percentages were adjusted per total cell counts, a significant increase in CD4⁺ T cells was observed in the spleens of WHI-P131-treated mice (Figure 6b). Cytokine secretion was determined following 48-h stimulation with anti-CD3 plus anti-CD28 antibodies, without addition of the drug, in CD4⁺ cells isolated from treated and control mice. It was found that *in vivo* WHI-P131 treatment does not affect IL-10 (Figure 7a), while significantly reducing the IL-4 (Figure 7b), IFN- γ (Figure 7c) and IL-2 (Figure 7d) production by CD4⁺ T cells. Interestingly, the only elevated cytokine found in this experiment was TGF- β (Figure 7e).

WHI-P131 treatment does not affect the frequency and total numbers of nTregs in the secondary lymphatic organ such as spleen. However, it should be considered that its effect could be observed in lymph nodes located in a vicinity of the pancreas. Therefore, an additional set of experiments was performed in order to answer whether *in vivo* treatment with WHI-P131 (75 mg/kg/day during a 10-day period) affects Treg population in the pancreatic lymph nodes (PLNs) of NOD females. Figure 6c shows that there were no differences observed in the percentages of CD3⁺, CD4⁺, CD8⁺ and CD4⁺FoxP3⁺ T cells in the PLNs of drug-treated and control mice. The average of the absolute cell numbers obtained from the PLNs of six mice administered with WHI-P131 was higher ($0.94 \times 10^6 \pm 0.23 \times 10^6$), but not significantly different, from the average PLN cell number of the six controls ($0.68 \times 10^6 \pm 0.11 \times 10^6$). Similar to the spleen results, the absolute number of PLN CD4⁺ T cells was significantly higher in mice treated with WHI-P131 than in controls, while the numbers of all other studied T-cell sub-populations, including the CD4⁺FoxP3⁺ cells, remained unaffected (Figure 6d).

The following three experiments were performed, with PLN and pancreatic infiltrate (PL) cells being pooled in each experiment, from the seven control and seven drug-treated mice. Results showed again

Figure 6 Flow cytometric analysis of CD3⁺, CD4⁺, CD8⁺ and CD4⁺FoxP3⁺ T cells in the spleens (**a, b**) and PLNs (**c, d**) of NOD females treated *in vivo* by WHI-P131. The percentages (**a, c**) and total cell numbers (**b, d**) were obtained from the NOD spleens and PLNs post 10-day treatment with WHI-P131 ($n=6$) and vehicle ($n=6$). Data are presented as average percentage (**a, c**) and total cell number ($\times 10^6$) (**b, d**) $\pm$ s.e.m. of WHI-P131-treated (gray bars) and control group (white bars). Statistical analysis performed by Student's *t*-test. * $P < 0.05$ compared to control. NOD, non-obese diabetic; PLNs, pancreatic lymph nodes; WHI-P131, 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazolin.

that WHI-P131 treatment did not affect the frequency of CD4⁺FoxP3⁺ PL cells, compared to controls (data not shown). In contrast, cytokine analysis showed that the PLs, isolated from the WHI-P131-treated mice, produced significantly ($P < 0.05$) higher amount of TGF- β (1810 ± 203 pg/ml) compared to controls (732 ± 125 pg/ml), along with decreased amount of IFN- γ (1860 ± 410 pg/ml vs. 4130 ± 502 pg/ml) and IL-2 (1200 ± 540 pg/ml vs. 3290 ± 478 pg/ml). Whereas a trend of increased production of IL-10 and lower IL-4 was observed in mice treated with WHI-P131, significant changes were not obtained (data not shown). Overall, the results observed in the PLNs and PLs agree with those obtained in the spleens, pointing to the increased number of CD4⁺FoxP3⁺ T cells and elevated production of TGF- β in NOD mice post *in vivo* exposure to the JAK3 kinase inhibitor. In addition, *in vivo* data are in agreement with those obtained *in vitro*, suggesting the WHI-P131-induced generation of CD4⁺FoxP3⁺ T cells that secrete TGF- β .

The next *in vivo* experiment included rapamycin administration (1 mg/kg/day) of NOD females for 10 days (six treated and six control mice), in order to compare the cytokine levels and immunophenotyping results obtained by WHI-P131 and rapamycin treatment. Flow cytometric analysis showed that there was a significant increase ($P < 0.05$) in CD4⁺CD25⁺ cells in rapamycin-treated ($6.3\% \pm 0.3\%$) vs. control mice ($5.0\% \pm 0.2\%$). FoxP3 expression was elevated as well. Thus, $5.9\% \pm 0.2\%$ of cells were CD4⁺FoxP3⁺ in rapamycin-treated, compared to $4.6\% \pm 0.4\%$ in control mice ($P < 0.05$). Figure 8 shows that rapamycin treatment significantly increased IL-10 (Figure 8a) and

decreased IL-4 (Figure 8b) secretion of CD4⁺ T cells. The levels of IFN- γ (Figure 8c), IL-2 (Figure 8d) and TGF- β (Figure 8e) were not affected by *in vivo* rapamycin treatment. In general, these data are in line with results obtained *in vitro*, suggesting different mechanisms of action of WHI-P131 and rapamycin on the Tregs.

DISCUSSION

Here, we reintroduced the question about the mechanism of protective antidiabetogenic WHI-P131 action in NOD mice, asking whether targeting JAK3 with this inhibitor would induce generation of Tregs. First, the effects of the JAK3 inhibition were studied *in vitro*. We showed that WHI-P131 acts directly on CD4⁺ T cells, suppressing the proliferation by inducing apoptotic cell death during short-term culturing of these cells. This observation is important, as previous studies explored the antiproliferative and apoptotic effects of WHI-P131 in the context of lymphoblastic and myeloblastic leukemia cells,^{19,21} and a heterogeneous population of murine splenic cells,^{15,20,24} not CD4⁺ T cells in particular. In contrast to short-term culturing, long-term exposure of CD4⁺ cells to WHI-P131 provided different experimental data. Thus, the viability of cells, significantly reduced during the first week of exposure to WHI-P131, reached the control values post 3 weeks of culturing. In line with viability data, the flow cytometric analysis revealed a significant reduction in apoptotic cell death of CD4⁺ cells exposed to WHI-P131 over the period of 3 weeks, compared to short-term exposure. Similar results were obtained in rapamycin-treated groups, which are in accordance with

Figure 7 Cytokine secretion of CD4⁺ T cells isolated from NOD mice treated with WHI-P131 (P131). Cytokines IL-10 (a), IL-4 (b), IFN- γ (c), IL-2 (d) and TGF- β (e) were detected by ELISA in the supernatants of CD4⁺ T cells isolated from the spleens of 5-week-old NOD females treated for 10 days with 75 mg/kg of WHI-P131 ($n=7$) or vehicle ($n=6$). The cells were stimulated and cultured for 48 h. Data are presented as average (pg/ml) $\pm$ s.e.m. of WHI-P131-treated and control group. Statistical analysis performed by Student's *t*-test. * $P<0.05$ compared to control. IFN, interferon; NOD, non-obese diabetic; TGF, transforming growth factor; WHI-P131, 4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazolin.

previously published data, showing that short-term exposure of CD4⁺ cells to rapamycin induces apoptotic cell death and reduction in viability, while long-term stimulation increases viability and generates a several-fold increase in the cell number.^{27–29} The final number of viable cells, obtained post 3 weeks of stimulation, was comparable in WHI-P131- and rapamycin-exposed groups.

It has been shown that rapamycin promotes differentiation of CD4⁺CD25⁺FoxP3⁺ Tregs during long-term stimulation of CD4⁺ cells *in vitro*.^{27–29} In contrast to rapamycin, confirmed in our case as well, WHI-P131 exhibited a different effect on induction of nTregs *in vitro*. Thus, the population of nTregs, characterized as CD4⁺FoxP3⁺ and CD25⁺FoxP3⁺ cells, was significantly reduced in WHI-P131-treated CD4⁺ cells, compared to rapamycin-treated and control cultures. It is now clear that IL-2/IL-2 receptor signaling is fundamental for the peripheral activity of nTregs. The IL-2-mediated proliferation and survival of activated T cells require a coordinated action between multiple signaling pathways downstream of the IL-2 receptor. The initial events upon IL-2 binding result in recruitment of the JAK3/STAT5 pathway, as well as activation of PI3K kinase and Ras-mitogen-activated protein kinase. An important observation on signaling in Tregs is the finding that IL-2 primarily activates the JAK/STAT

pathway in these cells.^{33,39} Thus, engagement of the IL-2 receptor on Tregs resulted in the activation of the JAK/STAT signaling pathway, but failed to activate downstream targets of the PI3K signaling pathway, such as AKT or p70-S6 kinase.³³ Furthermore, it has been shown that IL-2-mediated upregulation of FoxP3 in purified CD4⁺CD25⁺ T cells involves the binding of STAT3/5 proteins to a highly conserved STAT binding site located in the first intron of the FoxP3 gene.³⁹ Our data clearly show that the JAK3 inhibitor WHI-P131 inhibits differentiation of CD4⁺ T cells towards the nTreg CD4⁺CD25⁺FoxP3⁺ phenotype, confirming the essential role of the JAK3/STAT5 signaling pathway for the induction of CD4⁺FoxP3⁺ T cells *in vitro*. Furthermore, these results are in line with studies performed in JAK3-deficient mice.⁴⁰ Mayack and Berg⁴⁰ showed that activated JAK3^{−/−} CD4⁺ T cells, while able to suppress the proliferation of effector cells, do not express FoxP3.

Here, we provide experimental evidence that CD4⁺ T cells generated by *in vitro* exposure to WHI-P131 secrete an elevated level of TGF- β . An absence of IL-2 secretion, accompanied by the decreased levels of IFN- γ , IL-4 and IL-10 suggests that these cells are neither conventional Th1-/Th2- nor IL-10-secreting Tr-1-type T cells. In line with our data, it has been described that CD4⁺ cells isolated from

Figure 8 Cytokine secretion of CD4⁺ T cells isolated from NOD mice treated with rapamycin (Rapa). Cytokines IL-10 (a), IL-4 (b), IFN- γ (c), IL-2 (d) and TGF- β (e) were detected by ELISA in the supernatants of CD4⁺ T cells isolated from the spleens of 5-week-old NOD females treated for 10 days with 1.0 mg/kg of rapamycin ($n=6$) or vehicle ($n=6$). The cells were stimulated and cultured for 48 h. Data are presented as average (pg/ml) $\pm$ s.e.m. of rapamycin-treated and control group. Statistical analysis performed by Student's *t*-test. * $P<0.05$ compared to control. IFN, interferon; NOD, non-obese diabetic; TGF, transforming growth factor.

JAK3-deficient mice secrete an elevated level of TGF- β .⁴⁰ However, increased IL-10 secretion was found as well. Interestingly, decreased IFN- γ secretion, like in our case, was observed in phytohemagglutinin-stimulated blood leukocytes of kidney transplant patients treated by the JAK3 inhibitor CP-690,550.⁴¹ Unfortunately, the TGF- β secretion in these patients was not measured. Numerous studies have identified roles for TGF- β in preventing T1D in NOD mice by blocking the cytotoxic effects of diabetogenic effector lymphocytes.⁴² Recent studies have demonstrated that regulatory T cells protect from T1D in the context of TGF- β .^{43,44} Furthermore, the protective treatments of NOD mice attributed their beneficial actions to upregulation of TGF- β -producing Tregs as well.^{45,46} These TGF- β -producing Tregs, called by some as Th3-type Tregs, are believed to develop in the presence of TGF- β .⁸ While some reports have defined them as FoxP3⁺ adaptive Tregs,⁷ a substantial number of studies have demonstrated that Th3 cells express FoxP3.^{47,48} However, it should be noted that research in the Th3 field has focused on mucosal tissue in the intestine, as these cells are found to be important in intestinal diseases and in maintaining tolerance to intestinal antigens.^{47,48}

It has been shown that WHI-P131 in the doses of 50 and 100 mg/kg was effective in the prevention of T1D in NOD mice.^{15,25} Moreover, there were no drug-related toxic lesions found during histopathological

examinations in any of the organs of mice treated with 100 mg/kg/day.²⁵ In accordance with these studies, we chose a dosage of 75 mg/kg of WHI-P131 for our *in vivo* experiments. As previously described,²⁵ we again observed that WHI-P131 treatment does not change the frequency of CD3⁺, CD4⁺ and CD8⁺ splenocytes. The same results were observed in the PLNs, the secondary lymphoid organs directly involved in immunopathogenesis of the autoimmune attack. However, an analysis of the absolute numbers of these T-cell sub-populations pointed out towards the elevated numbers of CD4⁺ cells in the spleens, as well as in the PLNs of mice treated with WHI-P131, suggesting that JAK3 inhibitor increases the CD4⁺ T-cell numbers *in vivo*. Furthermore, the frequency, as well as the absolute numbers of CD4⁺FoxP3⁺ T cells, in the spleens and PLNs of NOD mice was not affected by WHI-P131 treatment. A recent study investigated the effect of JAK3 kinase inhibitor CP-690,550 on Treg number and function in human kidney transplant recipients enrolled in a phase 1 dose-escalation safety trial.⁴¹ A marked reduction in the CD4⁺CD25^{bright} population was observed in patients treated with CP-690,550 over the 2 weeks, compared to baseline value. However, this study lacked a placebo control group and analyzed a very small sample size.⁴¹ Moreover, as those patients had already been immunosuppressed from the beginning of the study, more clinical research should be done before the final conclusion can be

drawn regarding the influence of JAK3 inhibition on human CD4⁺FoxP3⁺ Tregs. In contrast to the results obtained by *in vivo* treatment with the WHI-P131, rapamycin administration exhibited significant increase in splenic CD4⁺CD25⁺FoxP3⁺ T cells, confirming the previously published results.⁴⁵ TGF- β was the only cytokine significantly increased in the spleens, PLNs, as well as in the pancreata of NOD mice *in vivo* treated by WHI-P131. All other studied cytokines were either significantly decreased, such as IL-2, IFN- γ and IL-4, or not significantly different than the controls, such as IL-10. The cytokine profiles, obtained from stimulated CD4⁺ cells isolated from spleens of rapamycin-treated mice, while in accordance with elevated IL-10 and unchanged TGF- β levels described by Battaglia *et al.*,⁴⁵ were different from those observed post WHI-P131 treatment. Thus, we clearly show here a disparate action of rapamycin vs. WHI-P131 on the murine Tregs *in vitro* and *in vivo*.

Conventional immunosuppressive drugs have various direct and indirect effects on Tregs.^{26,49} The majority of reports have reflected on the effects of these drugs on the CD4⁺FoxP3⁺ Tregs.^{26,49} Rapamycin's beneficial effects on the induction of FoxP3⁺ Tregs *in vitro*^{27–29} and *in vivo*^{45,50} have been well documented and discussed throughout this paper. It has been shown that corticosteroids induce FoxP3 expression *in vitro*,⁵¹ and expand nTreg population post short-term administration *in vivo*.⁵² Mycophenolate mofetil neither altered the expression of FoxP3, nor affected Treg function *in vitro*.⁵³ In contrast, calcineurin inhibitors, such as cyclosporine A and tacrolimus, exhibited negative effects on CD4⁺FoxP3⁺ Tregs both *in vitro* and *in vivo*.^{30,31} Here, we show that the JAK3 inhibitor does suppress generation of CD4⁺FoxP3⁺ cells *in vitro*, while not depleting them *in vivo*. To our knowledge, this is the first report exploring a direct *in vitro* and *in vivo* effect of JAK3 inhibition on the murine nTregs. Further studies would need to address the functional capability of these CD4⁺FoxP3⁺ cells post-treatment with WHI-P131. Interestingly, our results are in line with studies performed with IL-2 receptor blocking antibodies. It has been shown that these antibodies, which directly interfere with receptor signaling by inhibiting IL-2 binding and subsequent phosphorylation of the IL-2 receptor β - and γ -chains, inhibit FOXP3 mRNA induction,³⁰ and expansion of CD4⁺CD25⁺ cells *in vitro*,⁵⁴ while not depleting the peripheral Treg numbers after the *in vivo* treatment.⁵⁵

In summary, we have shown that JAK3 inhibition by WHI-P131 promotes TGF- β secretion by CD4⁺ T cells *in vitro* and *in vivo*. Induction of these cells might be the underlying mechanism of anti-diabetogenic action of WHI-P131 in NOD mice. Our results confirm that JAK3 signaling is a prerequisite for the induction of FoxP3⁺ Tregs *in vitro*. Despite clear *in vitro* effects, the negative impact of JAK3 inhibition on the FoxP3⁺ Tregs was not observed *in vivo*. Overall, these data provide additional evidence that JAK3 inhibition is a suitable mechanism for the modulation of autoimmune responses.

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1 Jaeckel E, von Boehmer H, Manns MP. Antigen-specific FoxP3-transduced T cells can control established type 1 diabetes. *Diabetes* 2005; **54**: 306–310.

- 2 Tang Q, Bluestone JA. Regulatory T-cell physiology and application to treat autoimmunity. *Immunol Rev* 2006; **212**: 217–237.
- 3 Tang Q, Henriksen KJ, Mingyong B, Finger EB, Szot G, Ye J *et al*. *In vitro*-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med* 2004; **199**: 1455–1465.
- 4 You S, Thieblemont N, Alyanakian MA, Bach JF, Chatenoud L. Transforming growth factor-beta and T-cell-mediated immunoregulation in the control of autoimmune diabetes. *Immunol Rev* 2006; **212**: 185–202.
- 5 Riley RL, June CH, Blazar BR. Human T regulatory cell therapy: take a billion or so and call me in the morning. *J Immunol* 2009; **30**: 656–665.
- 6 Sakaguchi S, Ono M, Setoguchi R, Yagi H, Shohai H, Fehervari Z *et al*. Foxp3⁺CD25⁺CD4⁺ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* 2006; **212**: 8–27.
- 7 Curotto de Lafaille MA, Lafaille JJ. Natural and adaptive Foxp3⁺ regulatory T cells: more of the same or a division of labor? *Immunity* 2009; **30**: 626–635.
- 8 Workman CJ, Workman-Szymczak AL, Collison LW, Pillai MR, Vignali DA. The development and function of regulatory T cells. *Cell Mol Life Sci* 2009; **66**: 2603–2622.
- 9 Ihle JN, Kerr IR. Jaks and Stats in signaling by the cytokine receptor superfamily. *Trends Genet* 1995; **11**: 69–74.
- 10 Leonard WJ, O'Shea JJ. Jaks and Stats: biological implications. *Annu Rev Immunol* 1998; **16**: 293–322.
- 11 Shuai K, Liu B. Regulation of JAK-STAT signaling in the immune system. *Nat Rev Immunol* 2003; **3**: 900–911.
- 12 Nosaka T, van Deursen JM, Tripp RA, Thierfelder WE, Witthuhn BA, McMickle AP *et al*. Defective lymphoid development in mice lacking Jak3. *Science* 1995; **270**: 800–802.
- 13 Park SY, Saijo K, Takahashi T, Osawa M, Arase H, Hirayama N *et al*. Developmental defects of lymphoid cells in Jak3 kinase-deficient mice. *Immunity* 1995; **3**: 771–782.
- 14 O'Shea JJ, Husa M, Li D, Hofmann SR, Watford W, Roberts JL *et al*. Jak3 and the pathogenesis of severe combined immunodeficiency. *Mol Immunol* 2004; **41**: 727–737.
- 15 Cetkovic-Cvrlje M, Tibbles HE. Therapeutic potential of Janus kinase 3 (JAK3) inhibitors. *Curr Pharm Des* 2004; **10**: 176–84.
- 16 O'Shea J, Pesu M, Borie DC, Changelian PS. A new modality for immunosuppression: targeting the JAK/STAT pathway. *Nat Rev Drug Discov* 2004; **3**: 555–564.
- 17 Wilson LJ. Recent patents in the discovery of small molecule inhibitors of JAK3. *Expert Opin Ther Pat* 2010; **20**: 609–623.
- 18 Xiong Z, Ma A, Chen H. JAK3 inhibitors in organ transplantation and autoimmune disease. *Recent Pat Inflamm Allergy Drug Discov* 2010; **4**: 75–81.
- 19 Sudbeck EA, Liu XP, Narla RK, Mahajan S, Ghosh S, Mao C *et al*. Structure-based design of specific inhibitors of Janus kinase 3 as apoptosis-inducing anti-leukemic agents. *Clin Cancer Res* 1999; **5**: 1569–1582.
- 20 Cetkovic-Cvrlje M, Roers BA, Waurzyniak B, Liu XP, Uckun FM. Targeting Janus kinase 3 to attenuate the severity of acute graft-versus-host disease across the major histocompatibility barrier in mice. *Blood* 2001; **98**: 1607–1613.
- 21 Cetkovic-Cvrlje M, Uckun FM. Targeting Janus kinase 3 in the treatment of leukemia and inflammatory diseases. *Arch Immunol Ther Exp* 2004; **52**: 69–82.
- 22 Cetkovic-Cvrlje M, Roers BA, Schonhoff D, Waurzyniak B, Liu XP, Uckun FM. Treatment of post-bone marrow transplant acute graft-versus-host disease with a rationally designed JAK3 inhibitor. *Leuk Lymphoma* 2002; **43**: 1447–1553.
- 23 Cetkovic-Cvrlje M, Uckun FM. Dual targeting of Bruton's tyrosine kinase and Janus kinase 3 with rationally designed inhibitors prevents graft-versus-host disease (GVHD) in a murine allogeneic bone marrow transplantation model. *Br J Haematol* 2004; **126**: 821–827.
- 24 Cetkovic-Cvrlje M, Dragt AL, Uckun FM. Prevention of islet allograft rejection in diabetic mice by targeting Janus kinase 3 with [4-(4'-hydroxyphenyl)-amino-6,7-dimethoxyquinazoline] (JANEX-1). *Drug Res* 2003; **53**: 648–654.
- 25 Cetkovic-Cvrlje M, Dragt AL, Vassilev A, Liu XP, Uckun FM. Targeting JAK3 with JANEX-1 for prevention of autoimmune type 1 diabetes in NOD mice. *Clin Immunol* 2003; **106**: 213–225.
- 26 Demirkiran A, Hendriks TK, Baan CC, van der Laan LJW. Impact of immunosuppressive drugs on CD4⁺CD25⁺FOXP3⁺ regulatory T cells: does *in vitro* evidence translate to the clinical setting? *Transplantation* 2008; **85**: 783–789.
- 27 Battaglia M, Stabellini A, Roncarolo MG. Rapamycin selectively expands CD4⁺CD25⁺FoxP3⁺ regulatory T cells. *Blood* 2005; **105**: 4743–4748.
- 28 Battaglia M, Stabellini A, Migliaiaccia B, Horejs-Hoeck J, Kaupper T, Roncarolo MG. Rapamycin promotes expansion of functional CD4⁺CD25⁺FOXP3⁺ regulatory T cells of both healthy subjects and type 1 diabetic patients. *J Immunol* 2006; **177**: 8338–8347.
- 29 Strauss L, Whiteside TL, Knights A, Bergmann C, Knuth A, Zippelius A. Selective survival of naturally occurring human CD4⁺CD25⁺FoxP3⁺ regulatory T cells cultured with rapamycin. *J Immunol* 2007; **178**: 320–329.
- 30 Baan CC, van der Mast BJ, Klepper M, Mol WM, Peeters AM, Korevaar SS *et al*. Differential effect of calcineurin inhibitors, anti-CD25 antibodies and rapamycin on the induction of FOXP3 in human T cells. *Transplantation* 2005; **80**: 110–117.
- 31 Coenen JJ, Koenen HJ, van Rijssen E. Rapamycin, not cyclosporine, permits thymic generation and peripheral preservation of CD4⁺CD25⁺FoxP3⁺ regulatory T cells. *Bone Marrow Transplant* 2007; **39**: 537–545.
- 32 Hamawy MM. Molecular actions of calcineurin inhibitors. *Drug News Perspect* 2003; **16**: 277–282.

- 33 Bensinger SJ, Walsh PT, Zhang J, Carroll M, Parsons R, Rathmell JC *et al*. Distinct IL-2 receptor signaling pattern in CD4⁺CD25⁺ regulatory T cells. *J Immunol* 2004; **172**: 5287–5296.
- 34 Malek TR, Bayer AL. Tolerance, not immunity, crucially depends on IL-2. *Nat Rev Immunol* 2004; **4**: 665–674.
- 35 Olson M, Johnson S, Cetkovic-Cvrlje M. Incidence of type 1 diabetes in the colony of NOD/LtJ mice at St. Cloud State University. *J Mm Acad Science* 2008; **71**: 24.
- 36 Cetkovic-Cvrlje M, Gerling IC, Muir A, Atkinson MA, Elliott JF, Leiter EH. Retardation or acceleration of diabetes in NOD/Lt mice mediated by intrathymic administration of candidate beta cell antigens. *Diabetes* 1997; **46**: 1975–1982.
- 37 Vieira PL, Christensen JR, Minaee S, O'Neil EJ, Barrat FJ, Boonstra A *et al*. IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4⁺CD25⁺ regulatory T cells. *J Immunol* 2004; **172**: 5986–5993.
- 38 Uckun FM, Ek O, Liu X-P, Chen C-L. *In vivo* toxicity and pharmacokinetic features of the Janus kinase 3 inhibitor WHI-P131 [4-(4'-hydroxyphenyl)-amino-6, 7-dimethoxyquinazoline]. *Clin Cancer Res* 1999; **5**: 2954–2962.
- 39 Zorn E, Nelson EA, Mohseni M, Porcheray F, Kim H, Litsa D *et al*. IL-2 regulates FOXP3 expression in human CD4⁺CD25⁺ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells *in vivo*. *Blood* 2006; **108**: 1571–1579.
- 40 Mayack SR, Berg LJ. Cutting edge: an alternative pathway of CD4⁺ T cell differentiation is induced following activation in the absence of gamma-chain-dependent cytokine signals. *J Immunol* 2006; **176**: 2059–2063.
- 41 van Gurp EA, Schoordijk-Verschoor W, Klepper M, Korevaar SS, Chan G, Weimar W *et al*. The effect of the JAK inhibitor CP-690, 550 on peripheral immune parameters in stable kidney allograft patients. *Transplantation* 2009; **87**: 79–86.
- 42 Rabinovitch A, Suarez-Pinzon WL. Roles of cytokines in the pathogenesis and therapy of type 1 diabetes. *Cell Biochem Biophys* 2007; **48**: 159–163.
- 43 Richer MJ, Straka N, Fang D, Shanina I, Horwitz MS. Regulatory T-cells protect from type 1 diabetes after induction by coxsackievirus infection in the context of transforming growth factor-beta. *Diabetes* 2008; **57**: 1302–1311.
- 44 Tonkin DR, Haskins K. Regulatory T cells enter the pancreas during suppression of type 1 diabetes and inhibit effector T cells and macrophages in a TGF-beta-dependent manner. *Eur J Immunol* 2009; **39**: 1313–1322.
- 45 Battaglia M, Stabilini A, Draghici E, Migliavacca B, Gregori S, Bonifacio E *et al*. Induction of tolerance in type 1 diabetes *via* both CD4⁺CD25⁺ T regulatory cells and T regulatory type 1 cells. *Diabetes* 2006; **55**: 1571–1580.
- 46 Tian B, Hao J, Zhang Y, Tian L, Yi H, O'Brien TD *et al*. Upregulating CD4⁺CD25⁺FOXP3⁺ regulatory T cells in pancreatic lymph nodes in diabetic NOD mice by adjuvant immunotherapy. *Transplantation* 2009; **87**: 198–206.
- 47 Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N *et al*. Conversion of peripheral CD4⁺CD25⁺ naive T cells to CD4⁺CD25⁺ regulatory T cells by TGF-beta induction of transcription factor FoxP3. *J Exp Med* 2003; **198**: 1875–1886.
- 48 Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell population by foreign antigen. *Nat Immunol* 2005; **6**: 1219–1227.
- 49 Wekerle T. T regulatory cells—what relationship with immunosuppressive agents? *Transplant Proc* 2008; **40**: 13–16.
- 50 Monti P, Scirpoli M, Maffi P, Piemonti L, Secchi A, Bonifacio E *et al*. Rapamycin monotherapy in patients with type 1 diabetes modifies CD4⁺CD25⁺FOXP3⁺ regulatory T cells. *Diabetes* 2008; **57**: 2341–2347.
- 51 Karagiannis C, Akdis M, Holopainen P, Woolley NJ, Hense G, Rückert B *et al*. Glucocorticoids upregulate FOXP3 expression and regulatory T cells in asthma. *J Allergy Clin Immunol* 2004; **114**: 1425–1433.
- 52 Chen X, Oppenheim JJ, Winkler-Pickett RT, Ortaldo JR, Howard OM. Glucocorticoid amplifies IL-2-dependent expansion of functional FoxP3⁺CD4⁺CD25⁺ T regulatory cells *in vivo* and enhances their capacity to suppress EAE. *Eur J Immunol* 2006; **36**: 2139–2149.
- 53 Zeiser R, Nguyen VH, Beilhack A, Buess M, Schulz S, Baker J *et al*. Inhibition of CD4⁺CD25⁺ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood* 2006; **108**: 390–399.
- 54 Lopez M, Clarkson MR, Albin M, Sayegh MH, Najafian N. A novel mechanism of action for anti-thymocyte globulin: induction of CD4⁺CD25⁺Foxp3⁺ regulatory T cells. *J Am Soc Nephrol* 2006; **17**: 2844–2853.
- 55 Kohm AP, McMahon JS, Podojil JR, Begolka WS, DeGutes M, Kasproicz DJ *et al*. Cutting edge: anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of CD4⁺CD25⁺ T regulatory cells. *J Immunol* 2006; **176**: 3301–3305.