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Janus kinase 3 inhibition with CP-690,550 prevents allograft vasculopathy

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Summary

Janus kinase 3 (JAK3) mediates signal transduction from cytokine receptors using the common γ chain. The rationally designed inhibitor of JAK3, CP-690,550, prevents acute allograft rejection in rodents and in nonhuman primates. Here we investigated the ability of CP-690,550, to prevent allograft vasculopathy in a rodent model of aorta transplantation. Aortas from AxC Irish ($RT1^a$) or Lewis ($RT1^l$) rats were heterotopically transplanted into the infrarenal aorta of Lewis recipients and harvested at 28 or 56 days. Treated recipients received CP-690,550 by osmotic pumps (mean drug exposure of 110 ± 38 ng/ml). Significant intimal hyperplasia was demonstrated in untreated allografts when compared with isografts at 28 days (2.08 ± 0.85% vs. 0.43 ± 0.2% luminal obliteration, respectively, $P = 0.001$) and 56 days (5.3 ± 2.4% vs. 0.38 ± 0.3%, $P = 0.002$). Treatment caused a 51% reduction in intimal hyperplasia at day 56. CP-690,550-treated animals also had a significant reduction of donor-specific IgG production and of the gene expression for suppressor of cytokine signaling-3 and with unchanged levels of expression of RANTES, IP-10 and transforming growth factor-β1. These results are the first to show that JAK3 blockade by CP-690,550 effectively prevents allograft vasculopathy in this rat model of aorta transplantation.

Introduction

Following vascularized organ transplantation, neointimal proliferation and alteration in vascular tone lead to accelerated transplant arteriosclerosis [1–3]. Allograft vasculopathy, also referred to as accelerated transplant arteriosclerosis, chronic allograft vasculopathy, cardiac allograft vasculopathy, transplant coronary disease and transplant vasculopathy, affects 30–40% of all transplants and ultimately results in allograft failure [2]. The 10-year survival rate following cardiac transplantation is approximately 50%, and although improvements have been made in preventing and treating acute rejection, the long-term success of cardiac transplantation continues to be limited by the development of allograft vasculopathy [2].

The pathophysiology of allograft vasculopathy has been attributed to both non antigen-specific factors of *innate* immunity (e.g. provoked by ischemia/reperfusion injury, infection, diabetes mellitus and hyperlipidemia) and antigen-specific factors of *adaptive* immunity (e.g. allo- and graft tissue-specific T- and B-cell responses) [4]. Injury from these nonimmunologic and immunologic factors occurs at the allograft endothelium [2,5]. Injured and activated allograft endothelium secretes inflammatory cytokines which promote vascular smooth muscle cell proliferation [5]. Because of its diffuse pattern, accelerated transplant arteriosclerosis is usually not amenable to surgical or percutaneous therapies used to treat atherosclerotic coronary artery disease. The only definitive therapy for allograft vasculopathy is retransplantation,

therefore motivating its prevention and improved treatment. Most current immunosuppressive drugs, although effective at preventing acute allograft rejection, do not prevent accelerated transplant arteriosclerosis.

We have recently shown that specifically targeting the cytoplasmic signaling protein Janus kinase 3 (JAK3) can effectively prevent acute allograft rejection [6–8]. JAK3 is a protein tyrosine kinase that mediates signals from cell surface type I cytokine (IL-2, -4, -7, -9, -15 and -21) receptors bearing the common gamma chain (γc) to the nuclei (reviewed in Refs [9,10]). Treatment with the specific JAK3 inhibitor CP-690,550 significantly prolonged organ allograft survival in rodent [7,8] and nonhuman primates [6,7].

Because of the critical roles of type I cytokine receptors in both *innate* and *adaptive* immune cell activation, function and development [11–13], we postulated that CP-690,550 can prevent the development of allograft vasculopathy. In this study, we investigated the ability of CP-690,550 to prevent allograft vasculopathy in a well-described rat heterotopic aorta transplantation model.

Materials and methods

Animals

Male, MHC class I-mismatched AxC Irish (ACI; $RT1^a$) and Lewis (LEW; $RT1^l$) rats (250–300 g) were obtained from Charles River Laboratories (Wilmington, MA, USA) and housed in cages with food and water *ad libitum*. Animal use was approved by the Stanford University Administrative Panel of Laboratory Animal Care, and complied with the Principles of Laboratory Animal Care formulated by the Institute of Laboratory Animal Resources, and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institutes of Health. LEW animals (n = 5–7 per group) were used as recipients of either isografts (LEW donors) or allografts (ACI donors). Recipients were observed for either 28 or 56 days post-transplant and complications (e.g. infection and wound dehiscence) were recorded.

Aorta transplantations

All surgical procedures were conducted under inhalation anesthesia using isoflurane (Iso-Thesia™; VetusAnimal Health, Burns Veterinary Supply Inc., Rockville, NY, USA). Aorta transplants were performed as described for a well-established rat model of allograft vasculopathy that has been used by other investigators to evaluate experimental therapeutic agents [14–18]. In brief, a 2-cm-long segment of thoracic aorta was procured from donor animals. The graft was flushed with cold saline and immersed in cold (4 °C) storage solution (Viaspan®; DuPont Pharma, Wilmington, DE, USA). Using an operating microscope (Leica Stereo zoom 4; Nightingale, Cape Floral, FL, USA), the graft was implanted below the renal arteries in an end-to-end fashion using a continuous 8–0 polypropylene suture (Pronova; Ethicon, Inc., Somerville, NJ, USA). The native aorta of the recipient was ligated at both ends and left *in situ*. Cold ischemia time did not exceed 15 min. Warm ischemia time ranged from 30 to 35 min.

Immunosuppression

Because of the limited oral bioavailability of CP-690,550 in rodents, a formulation of CP-690,550 was dissolved in polyethylene glycol (Sigma, St Louis, MO, USA) at 25 mg/ml and administered continuously via a subcutaneous osmotic pump (Alzet, Cupertino, CA, USA). Osmotic pumps were implanted 2 days prior to aorta transplantation so that therapeutic levels of drug would be present at the time of the procedure. Because of the limited capacities of the pumps available, animals that were followed for 56 days had to undergo pump replacement at day 26. To verify adequate exposure to CP-690,550, blood levels of this compound were measured 7 days after pump implantation and at necropsy using high-performance liquid chromatographic/liquid chromatographic/mass spectrometric methods as reported in detail elsewhere [19,20].

Graft removal and processing

Aortic grafts were harvested from euthanized recipients and immediately flushed with saline. A 0.5-cm medial segment was excised, placed in 10% buffered formalin solution and processed and embedded in paraffin for histopathological studies. Five-micrometer sections were stained with hematoxylin and eosin (H&E) and trichrome blue. One part of unfixed graft remnant was transferred into RNAlater (Qiagen, Valencia, CA, USA) immediately upon collection and stored at 4 °C overnight. RNAlater was then removed and samples were frozen at −80 °C until RNA extraction was performed. The remaining third of the harvested graft was embedded in optimal cutting temperature compound (Sakura, Torrance, CA, USA) and snap frozen in liquid nitrogen.

Histomorphometric analysis

Photomicrographs of each H&E stained section were captured with a high-resolution digital video camera (Technical Instrument Company, South San Francisco, CA, USA) mounted on a Leica microscope at 25× magnifica-

tion (Microsystems, Inc., Bannockburn, IL, USA. Morphometric analysis of each graft cross-section was performed using Scion Image Beta 4.0.2 (Scion Corporation, Frederick, MD, USA). Lumen, internal elastic lamina (IEL) and external elastic lamina were manually circumscribed by a single operator. Perimeters and areas of the circumscribed zones were calculated by the software. Intimal hyperplasia was expressed as an intimal area, defined as IEL area − luminal area and then expressed as a percentage of obstruction of the lumen using the following formula: standardized luminal obstruction percentage = (intimal area × 100 × 4π)/squared IEL perimeter. We have recently reported elsewhere in detail on the rationale for using this formula in this model as it compensates for shape heterogeneity of paraffin-embedded arterial cross-sections and treating each arterial cross section as if it were perfectly round [21].

Alloantibody production

Donor-specific IgG titers were measured in the recipient serum obtained at euthanasia. Undiluted serum was incubated with fresh donor splenocytes as target cells which were then washed and then incubated with a secondary FITC-conjugated F(ab′)2 goat anti-rat IgG (Caltag Laboratories, San Francisco, CA, USA). As a control for non-specific binding, splenocytes were incubated with autologous serum. Samples were analyzed on a FACSCalibur flow cytometer (BD, San Jose, CA, USA). Results were expressed as mean fluorescence intensity (MFI) and were compared within groups.

Intragraft gene expression

RNA was extracted from grafts using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Following DNAse treatment, purity and concentration of total RNA were determined on a spectrophotometer (NanoDrop, Montchanin, DE, USA).

Two-step quantitative TaqMan® real-time (RT)-PCR was performed to verify modulation of gene expression for the following set of five genes: growth factors [transforming growth factor beta 3 (TGF-β3) and TGF-β1], chemoattractant cytokine (RANTES and IP-10) and the negative regulator of the JAK/signal transducer and activator of transcription (STAT) pathway, suppressor of cytokine signaling-3 (SOCS-3). Primers and probes were either custom designed or purchased from Applied Biosystems (ABI, Foster City, CA, USA). The RT-PCR procedure was described in detail elsewhere [22]. Data were normalized to the ubiquitin 2e housekeeping gene from the same cDNA preparation to account for efficiency of RT-PCR reaction per sample, and subsequently normalized to appropriate native aortas. In our hands, ubiquitin 2e was shown to have the most stable and consistent expression among a group of several housekeeping genes (data not shown).

Statistical analysis

Data are presented as mean ± standard deviation. Differences between groups, at 28 and 56 days, were assessed using parametric tests, assuming normal distribution of the data. Unpaired *t*-tests were used for comparison between two groups (e.g. syngeneic versus allogeneic untreated). Comparison between three or more groups was performed using the one-way ANOVA method. SPSS GRADUATE Pack 11.5 and SIGMASTAT 2.03 software (both from SPSS Inc., Chicago, IL, USA) were used and a *P*-value of ≤0.05 was regarded as significant.

Results

Treatment with CP-690,550 significantly prevents the development of intimal hyperplasia in aortic allografts

When compared with isografts, untreated allografts had complete or near complete concentric intimal hyperplasia at both 28 and 56 days (Fig. 1). The lesions consisted of mononuclear cells admixed in a loose edematous myxoid stroma. On day 28, intimal hyperplasia with subsequent luminal reduction was demonstrated in untreated allografts when compared with isografts (2.08 ± 0.85% vs. 0.43 ± 0.2%, respectively, $P = 0.001$) (Fig. 2a). On day 56, luminal obliteration further increased in untreated allografts and was again significantly higher than that measured in corresponding isografts (5.3 ± 2.4% vs. 0.38 ± 0.3%, $P = 0.002$). CP-690,550 treatment had no effect on the development of intimal hyperplasia at day 28 as in either group, the observed luminal obliteration was not different from that seen in untreated allografts ($P = 0.187$). The cellular composition was similar in the different treatment groups at day 28 compared with the untreated allograft control (Fig. 1). At day 56, treatment with CP-690,550 with a mean steady-state blood concentration of 110 ± 38 ng/ml resulted in a significant prevention of luminal obliteration (2.61 ± 0.54%, $P = 0.015$ versus untreated allografts).

Treatment with CP-690,550 significantly prevents alloantibody production in aortic allograft recipients

No significant difference was found between groups with respect to donor-specific IgG production on day 28 (Fig. 2c). On day 56, however, donor-specific IgG production was significantly reduced in treated animals when compared with untreated controls (MFI: 743.66 ± 320.07 units vs. 2227.67 ± 471.93 units, $P < 0.001$).

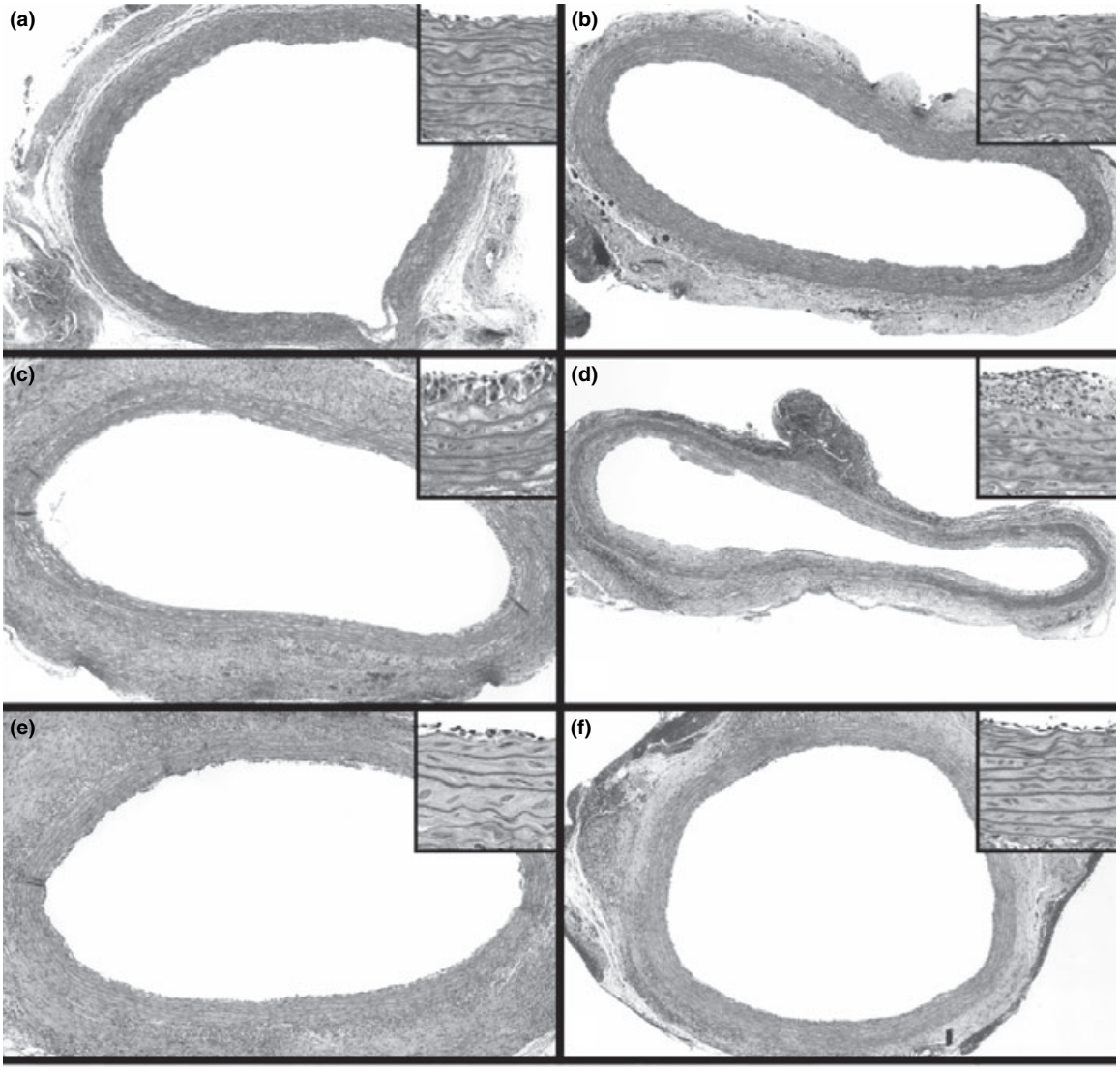

Figure 1 Histopathological findings in aortic transplants. (a) *Day 28 isograft* showing normal elastic artery [trichrome (TC) × 40]. Insert: high power magnification showing normal intimal and medial layers (TC × 400). (b) *Day 56 isograft* showing normal artery. (c) *Day 28 untreated allograft* showing intimal thickening by cellular proliferation (TC × 40). Insert: mononuclear cells are shown within the expanded intimal layer (TC × 400). (d) *Day 56 untreated allograft* showing further expansion of the chronic allograft vasculopathy (CAV) lesion with circumferential intimal thickening (TC × 40). Insert: the endothelial cell layer remains intact (TC × 400). (e) *Day 28 allograft treated with CP-690,550* showing an intact elastic artery (TC × 40). Insert: the sparse intimal layer contains endothelial cells and mononuclear cells (TC × 400). (f) *Day 56 allograft treated with CP-690,550* showing prevention of the neointimal lesion of CAV compared with (d) (TC × 40; insert × 400).

Effects of CP-690,550 on intragraft gene expression in rat aortic allograft recipients

To gain further insight into the molecular mechanisms by which CP-690,550 abrogated allograft vasculopathy, we measured the levels of expression of genes that have been previously described to be involved in fibrosis (TGF-β1 and TGF-β3), chemotaxis (RANTES) and inflammation (IP-10 and SOCS-3) [23–26].

Transforming growth factor-β1 expression was a significantly induced in untreated allografts at both timepoints (Fig. 3), whereas the expression for TGFβ-3 was not (data not shown). Similarly, at 28 and 56 days, there was a significant expression of RANTES (11.46 ± 3.29- and 13.13 ± 6.93-fold, respectively), IP-10 (226.79 ± 96.59- and 301.81 ± 96.26-fold) and SOCS-3 (29.82 ± 3.39 and 34.53 ± 5.58-fold). Compared with untreated allografts, CP-690,550 significantly reduced the expression of SOCS-3

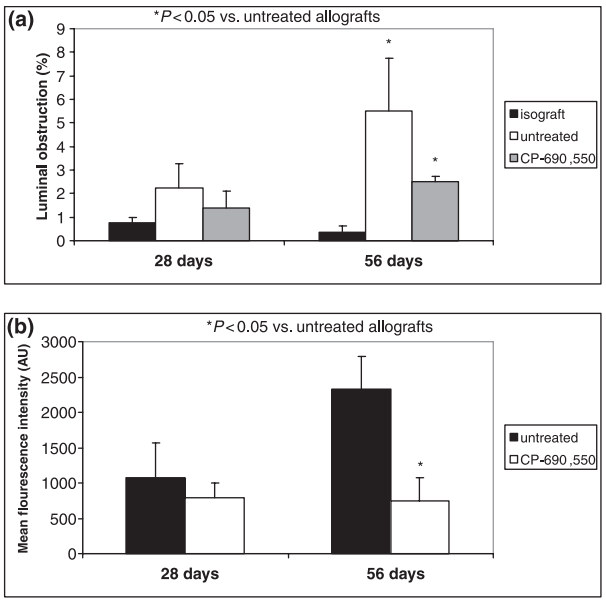

Figure 2 Chronic allograft vasculopathy parameters following aorta transplantation in rats. (a) Significant luminal obstruction develops at 28 and 56 days in untreated allografts and is significantly reduced in treated animals at day 56. (b) Treatment with CP-690,550 significantly reduces donor-specific IgG production in transplanted animals as indicated by a significant reduction of the mean fluorescence intensity on FACS analysis using donor thymocytes as targets. Data presented represent average ± standard deviation of at least five animals per group.

at 28 and 56 days (13.44 ± 6.47 and 10.79 ± 3.88, respectively, both $P < 0.05$ versus untreated allografts). The expression of RANTES at 28 and 56 days (7.26 ± 3.93 and 7.3 ± 5.89, respectively) was also reduced in CP-690,550-treated animals, although that reduction did not reach significance. Similarly, IP-10 gene expression levels were not significantly reduced upon CP-690,550 exposure at both time-points considered.

Treatment with CP-690,550 is well tolerated by rat aortic allograft recipients

Untreated recipients of aortic allografts maintained body weights similar to those measured in isografts at both 28 (349 ± 18 vs. 354 ± 11 g, respectively, $P = 0.921$) and 56 days (347 ± 13 vs. 351 ± 22 g, respectively, $P = 0.928$). Throughout the study, there were no significant differences in body weights between untreated and treated (341 ± 27 g, $P = 0.837$) animals. Similar findings were seen at completion of study at day 56 (data not shown). No infection was documented in any group during follow-up. Whereas no incisional hernias were seen in isograft animals (0/11), some were seen in untreated allograft animals (1/12, 8.3%), as well as in animals treated with CP-690,550 (1/12, 8.3%).

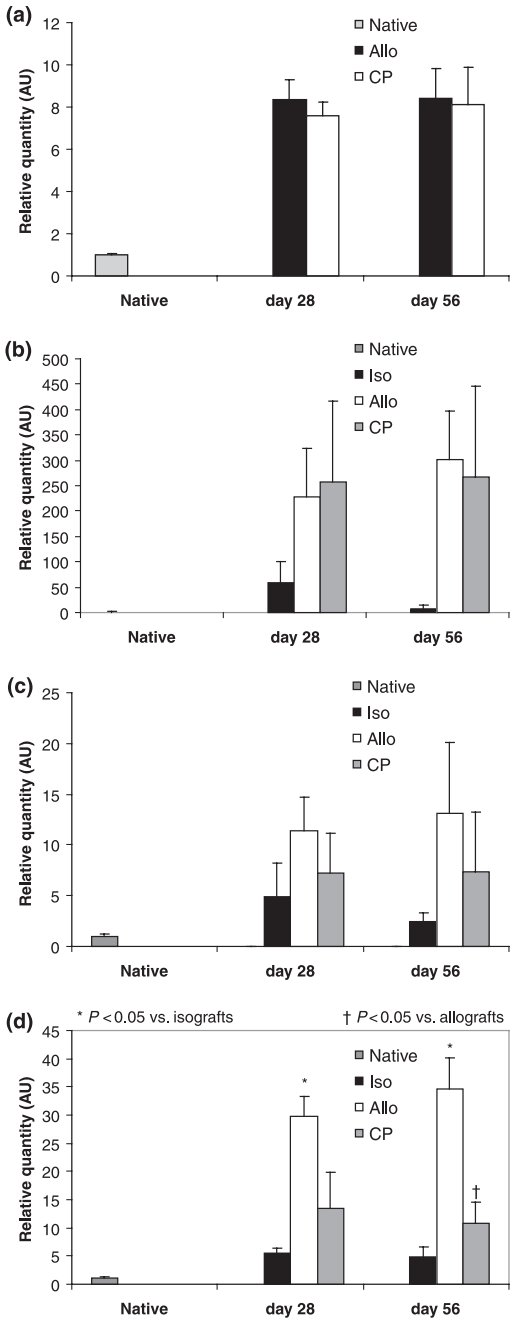

Figure 3 Effects of CP-690,550 on intragraft gene expression measured with TaqMan real-time PCR in rat aortas. Aorta allotransplantation results in significant overexpression of transforming grwth factor-β1 (a), IP-10 (b), RANTES (c) which is not significantly affected by treatment with CP-690,550. (d) Expression of suppressor of cytokine signaling-3 (SOCS-3) gene is significantly increased in aorta allografts. By day 56, treatment with CP-690,550 results in a significant reduction of SOCS-3 expression. Data presented represent average ± standard deviation of at least five animals per group. Iso, isograft; Allo, allograft; CP, CP-690,550.

Discussion

In our previous studies, CP-690,550 was found to be extremely potent at preventing acute allograft rejection in a variety of models developed both in rodents [7,8] and in cynomolgus monkeys [6,7]. Moreover, CP-690,550 afforded a significant prevention of the development of bronchiolitis obliterans, a form of chronic rejection in lung transplantation, in a rat heterotopic tracheal transplant model (M. Lau and D.C. Borie, unpublished data).

The rationale for our hypothesis that the JAK3 inhibitor CP-690,550 could prevent the development of allograft vasculopathy was that the inhibition of JAK3 blunts signaling by key cytokine receptors involved in both *innate* and *adaptive* immunological responses involved in the development of accelerated transplant arteriosclerosis [4]. More specifically, inhibition of JAK3 leads to the interruption of signals from both the IL-2 and IL-15 receptors. It is well recognized that IL-2 and T cells participate in the formation of transplant vasculopathy [27] and we have demonstrated in previous *in vitro* and *in vivo* studies that CP-690,550 potently inhibits T-cell activation and function as well as signals mediated by the IL-2 receptor [7,28].

IL-15 mediates natural killer (NK) cell development, activation, proliferation and function [29]. The HMG-CoA reductase inhibitor pravastatin has been shown to inhibit cardiac transplant vasculopathy at 1- and 10-year follow-up [30,31]. The mechanisms behind the efficacy of HMG-CoA reductase inhibitors in preventing allograft vasculopathy and chronic rejection in both clinical and experimental transplantation have yet to be determined, although NK cell inhibition has been implicated [30,32]. HMG-CoA reductase inhibitors have been shown to inhibit NK cell cytotoxicity *in vitro* and *in vivo* [32,33]. Recently, more definitive evidence from Uehara *et al.* [34] has shown that NK cells play a pivotal role in the evolution of allograft vasculopathy. These investigators showed through a series of heterotopic cardiac transplants in mice utilizing parenteral donors and F1 hybrid recipients that NK cells contributed to the formation of cardiac allograft vasculopathy and that this process is dependent on recipient-derived IFN-γ. Furthermore, using antibody-mediated depletion of NK cells and T cells, these investigators showed that the partial depletion of NK or T cells alone did not prevent cardiac allograft vasculopathy, whereas the depletion of both cell types resulted in complete prevention of cardiac allograft vasculopathy.

As signaling from the IL-15 receptor was expected to be inhibited by CP-690,550, it was also anticipated that this agent would inhibit NK cell development, activation, function and proliferation. Although not evaluated in the current study, there is evidence from our previous studies to argue that CP-690,550 inhibits NK cells *in vitro* and *in vivo*. We showed that CP-690,550 inhibited IL-15-induced CD69 expression in cynomolgus and human peripheral blood NK cells *in vitro* with an IC_{50} of 48 and 63 nM, respectively [35]. Furthermore, in our *in vivo* studies in a renal transplant model utilizing cynomolgus monkeys, we found that treatment with CP-690,550 resulted in a dramatic contraction of the NK cell population to 10% of pretreatment levels [28]. IFN-γ production by T cells in these animals was also significantly depressed; however, it remains to be determined if NK cell production of IFN-γ is also abrogated by CP-690,550. It is also plausible that CP-690,550 also inhibits NK cell IFN-γ production as the activation of NK cells by a variety of stimuli results in both increased CD69 expression and IFN-γ production [36,37].

Sensitization to allo-specific antigens may significantly affect graft survival and patients with anti-HLA antibodies are at increased risk of allograft vasculopathy [38–40]. Mice lacking both IL-4 and IL-21R exhibit a significant dysgammaglobulinemia and severely impaired IgG response [41] and we have shown that CP-690,550 potently inhibits IL-4-driven proliferation of the DND39 B-cell line [7]. Thus, it is reasonable to surmise that the pharmacological inhibition of JAK3 may contribute to the prevention of donor-specific antibody production and, therefore, chronic rejection.

Because our previous studies demonstrated that CP-690,550 had profound anti-T-cell activity, we expected to find significant decreases in the expression of RANTES and IP-10 [7,28]. A likely explanation for the contrary insignificant decreases in RANTES and IP-10 that we observed is that the measurement of these gene expressions occurred primarily in aortic allograft tissue where alternative signals for the upregulation of these genes may not be affected by JAK3. Certainly, we have observed that in acute allograft rejection in a heterotopic murine cardiac transplant model, there were up to 100 times lower RANTES and IP-10 expression in blood (where immune cells reside in greater numbers) when compared with heart allograft tissue [7].

Unlike our results observed in a rat model of transplant associated obliterative bronchiolitis (M. Lau, unpublished data), we did not observe a significant upregulation of TGF-β3, a pro-healing isoform of the cytokine [42]. Although more investigation is needed to delineate the role of JAK3 in TGFβ regulation, these results in two rat transplant models utilizing different allograft tissue suggest that CP-690,550 may have tissue-specific effects.

Suppressor of cytokine signaling-3 is a negative regulator of the JAK/STAT pathway and is activated upon IL-2 receptor activation [43,44]. Thus, we expected to find that in untreated allografts, SOCS-3 expression was increased because of presumed IL-2 receptor activation due to the

uninhibited alloimmune response and that treatment with CP-690,550 was accompanied by a significant reduction of the SOCS-3 gene expression because of abrogated IL-2 receptor signaling. These findings suggest that SOCS-3 levels may be used as a surrogate marker for CP-690,550 activity.

In conclusion, JAK3 inhibition by CP-690,550 results in the prevention of allograft vasculopathy in a rodent model of aortic transplantation. The activity of CP-690,550 on a variety of immune cells and recent insight into the pathogenesis of allograft vasculopathy suggest that inhibiting both the adaptive immune system *and* the innate immune system is necessary to the prevention of this process.

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