

# Combined Inhibition of c-Abl and PDGF Receptors for Prevention and Treatment of Murine Sclerodermatous Chronic Graft-versus-Host Disease

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**Chronic graft-versus-host disease (cGvHD) is a common complication of allogeneic bone marrow transplantation, and has a major effect on the long-term prognosis. The molecular mechanisms underlying cGvHD have been only partially revealed, and molecular targeted therapies have not yet been established for clinical use. We examined the effects of the combined inhibition of the Abelson kinase (c-Abl) and platelet-derived growth factor receptors (PDGFR) in experimental sclerodermatous cGvHD. Treatment using imatinib or nilotinib abolished the aberrant activation of c-Abl and PDGFR and protected against experimental cGvHD. Preventive therapy using imatinib or nilotinib inhibited the development of sclerodermatous cGvHD. Clinical features such as weight loss, alopecia, and skin ulcers, and histologic features with dermal thickening and accumulation of collagen were significantly reduced in mice that received imatinib or nilotinib therapy, but not in mice that received prednisone therapy. Of note, imatinib and nilotinib were also effective for treatment of experimental cGvHD that had already been clinically manifested. In**

**summary, the combined inhibition of c-Abl and PDGFR is effective for prevention and treatment of experimental sclerodermatous cGvHD. Considering the high morbidity associated with cGvHD, the lack of efficient molecular therapies for clinical use, and first positive signals from uncontrolled studies of imatinib, combined inhibition of c-Abl and PDGFR might be a promising future strategy for treatment of sclerodermatous cGvHD. (Am J Pathol 2012, 181:1672–1680; <http://dx.doi.org/10.1016/j.ajpath.2012.07.017>)**

Allogeneic stem cell transplantation is curative in a variety of hematologic, neoplastic, and genetic disorders.<sup>1</sup> The transplant not only replaces the bone marrow of the recipient after conditioning, but also exhibits therapeutic effects by initiating an immune response against malignant recipient cells, that is, the graft-versus-leukemia or graft-versus-tumor reaction. The immune response, however, is not specific for malignant cells. Donor T cells that recognize polymorphic

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histocompatibility antigens can also initiate immune responses against host tissues that manifest as acute or chronic graft-versus-host disease (cGvHD).<sup>2</sup> Inasmuch as both forms of GvHD are associated with high morbidity and mortality, it is emerging as an important limitation of allogeneic bone marrow transplantation.<sup>3</sup>

While good progress has been made in acute GvHD, the molecular mechanisms leading to tissue damage in cGvHD are still incompletely understood, and effective specific therapies for cGvHD for clinical use are not available. Previous studies of the etiology of cGvHD have suggested that either donor or host antigen presenting cells induce the pathogenic expansion of donor-derived T cells that subsequently attack target tissue through cytolytic attack, secretion of inflammatory and fibrotic cytokines, or B-cell activation and autoantibody-mediated damage.<sup>4–12</sup>

Skin is commonly involved in cGvHD. One form of cGvHD, so-called sclerodermatous cGvHD, is a wasting and fibrosing disease similar to scleroderma.<sup>13,14</sup> The histopathologic hallmark of scleroderma and cutaneous cGvHD is excessive accumulation of extracellular matrix (ECM) components.<sup>15</sup> The accumulation of ECM results from excessive release of collagens and other components of the ECM by aberrantly activated fibroblasts. Numerous lines of evidence suggest that profibrotic cytokines such as transforming growth factor- $\beta$  (TGF- $\beta$ ) and platelet-derived growth factor (PDGF) have a key role in the pathologic activation of fibroblasts.<sup>16</sup>

Imatinib and nilotinib are both small molecules that target specifically TGF- $\beta$  and PDGF signaling pathways by inhibiting the tyrosine kinase activity of c-Abl and PDGF receptors. Thus, imatinib and nilotinib simultaneously interfere with two major pathways for activation of fibroblasts. In preclinical trials, both imatinib and nilotinib have been used successfully in the treatment of fibrotic disorders such as scleroderma.<sup>17</sup> Because of pathophysiologic similarities between scleroderma and cGvHD, and on the basis of the first encouraging data in cGvHD in humans,<sup>18–20</sup> we evaluated imatinib and nilotinib as novel therapeutic approaches in an established model of murine sclerodermatous cGvHD. We found that c-Abl and PDGFR signaling are activated in human and murine cGvHD. Imatinib and nilotinib effectively improved experimental cGvHD. Preventive as well as therapeutic regimens controlled clinical signs of cGvHD such as weight loss, alopecia, and formation of skin ulcers, and also reduced dermal thickening, myofibroblast differentiation, and accumulation of collagens.

## Materials and Methods

### Human Samples

Human sample collection was approved by the local ethics boards and authorities in Erlangen and Kiel, Germany, and in Manchester, UK. All patients gave written informed consent according to the regulations of the local ethics committees. A clinical characterization of patients with cGvHD is given in Table 1. Nonfibrotic skin samples were obtained from 10 age- and sex-matched healthy individuals.

**Table 1.** Clinical Characteristics of Patients with Sclerodermatous cGvHD

Variable	Value
No. of patients	12
Sex	
Female	6 (50)
Male	6 (50)
Age, year	
Recipient	51.9 $\pm$ 11.2
Donor	47.2 $\pm$ 10.1
Time between HSCT and onset of cGVHD, day	7.8 $\pm$ 2.7
Biopsy site	
Thigh	8 (67)
Forearm	4 (33)
Therapy	
Cyclosporine A	4 (33)
Mycophenolate mofetil	8 (67)

Values are given as No. (%) or mean  $\pm$  SD. cGvhd, chronic graft-versus-host disease; HSCT, hematopoietic stem cell transplantation.

### Mice

The B10.D2 $\rightarrow$ BALB/c (H-2(d)) minor histocompatibility antigen-mismatched model, which reflects clinical and pathologic symptoms of human sclerodermatous cGvHD, was used in the present study.<sup>21,22</sup> BALB/c (H-2d) mice were purchased from Centre d'Élevage Janvier SA (Le Genest St. Isle, France), and B10.D2 (H-2d) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained in specific pathogen-free conditions, with sterile pellet food and water and a normal day-night cycle. All animal experiments were approved by the local ethics committee.

### Bone Marrow Transplantation

For isolation of unfractionated bone marrow cells, tibial and femoral bones were prepared under sterile conditions. Phosphate-buffered saline solution was used to flush bone marrow cells from bone marrow cavities. Subsequently, bone marrow cells were filtered through 70- $\mu$ m nylon meshes (BD Biosciences, Heidelberg, Germany), followed by erythrocyte hemolysis. The remaining bone marrow cells were kept on ice until transplantation. No further purification or *in vitro* expansion of a particular subset of cells was performed. Eight-week-old recipient mice, BALB/c (H-2d), underwent total body irradiation consisting of two 6-Gy doses separated by an interval of 3 hours. Sixteen hours after the second irradiation, all BALB/c (H-2d) recipients received bone marrow from either BALB/c (H-2d) in syngeneic transplantation (synBMT) or B10.D2 (H-2d) in allogeneic transplantation (alloBMT). For transplantation,  $2 \times 10^6$  splenocytes and  $1 \times 10^6$  bone marrow cells from donor mice were resuspended in 0.2 mL PBS and injected via tail veins.<sup>21,22</sup> In recipient mice, BALB/c (H-2d), preventive treatment was started at 12 days after bone marrow transplantation (BMT), and treatment of first

clinical signs at 21 days after BMT.<sup>23</sup> Samples were obtained at 21, 42, or 80 days after BMT.

### *Treatment with Imatinib, Nilotinib, and Prednisone*

Imatinib was administered in polyethylene glycol 300 (PEG 300) as suspension. Nilotinib was dissolved in 1-methyl-2-pyrrolidone at a concentration of 200 mg/mL. This stock solution was further diluted in PEG 300 before use. Imatinib was given at a final concentration of 150 mg/kg once a day, and nilotinib at 37.5 mg/kg twice a day, in a total volume of 0.1 mL PEG 300 via oral gavage. Prednisone was dissolved in 0.9% NaCl to a final concentration of 1 mg/kg, and was given in a total volume of 0.1 mL once daily via oral gavage. The given doses are pharmacologically relevant because they resulted in serum concentrations that can also be achieved in humans.<sup>17</sup> The other groups consisted of BALB/c→BALB/c mice undergoing synBMT and B10.D2→BALB/c mice undergoing allogBMT; both groups received sham treatment with PEG 300 via oral gavage. Animals were sacrificed at 21, 42, or 80 days after BMT via cervical dislocation, and skin samples were obtained from a precisely defined 1-cm<sup>2</sup> area on the upper back.

### *Immunohistochemistry*

Paraffin-embedded sections from the treatment and control groups were deparaffinized, and incubated with 3% H<sub>2</sub>O<sub>2</sub>, followed by serum blocking with 10% goat serum in 5% bovine serum albumin. Phosphorylated c-Abl, phosphorylated PDGFR- $\beta$ , and PDGF-BB were detected by incubating with polyclonal rabbit anti-mouse phospho-c-Abl, polyclonal rabbit anti-mouse phospho-PDGFR- $\beta$ , and polyclonal rabbit anti-mouse PDGF-BB antibodies (Abcam PLC, Cambridge, UK) at 4°C overnight. Isotype antibodies in the same concentration were used in control mice (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Myofibroblasts were identified by staining for  $\alpha$ -smooth muscle actin using monoclonal anti-actin  $\alpha$ -smooth muscle antibodies (Sigma-Aldrich Corp., Steinheim, Germany) as described previously.<sup>17</sup>

Antibodies labeled with horseradish peroxidase (Dako, Hamburg, Germany) were used as secondary antibodies. The expression of phospho c-Abl, phospho PDGFR- $\beta$ , PDGF-BB, and  $\alpha$ -smooth muscle actin was visualized using diaminobenzidine peroxidase substrate solution (Sigma-Aldrich).

Immunohistochemical staining of fibroblasts was evaluated semiquantitatively at  $\times 400$  magnification in three randomly chosen high-power fields per patient, with 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining.

### *Clinical Score of Cutaneous cGvHD*

Recipient mice were clinically monitored once daily from the day of transplantation to the indicated days after transplantation to determine the incidence and severity of

cutaneous cGvHD, and mobility, diarrhea, and weight loss. The following scoring system for cutaneous cGvHD was used: 0 = healthy appearance, 1 = skin lesions with alopecia  $< 1$  cm<sup>2</sup> in area, 2 = skin lesions with alopecia 1 to 2 cm<sup>2</sup> in area, and 3 = skin lesions with alopecia  $> 2$  cm<sup>2</sup> in area. Incidence was expressed as the percentage of mice that exhibited clinical manifestations as described previously.<sup>22,24</sup>

### *Histologic Analysis*

The 1-cm<sup>2</sup> murine skin samples were fixed in 4% formalin and embedded in paraffin. Two-micrometer sections were stained with H&E. Dermal thickness was analyzed using a Nikon Eclipse 80i microscope (Nikon Instruments Europe BV, Badhoevedorp, the Netherlands) at  $\times 200$  magnification by measuring the distance between the epidermal-dermal junction and the dermal-subcutaneous junction at sites of induration in four different areas, with two independent measurements per area in each animal.<sup>25</sup>

### *Quantification of Collagen Content in Lesional Skin*

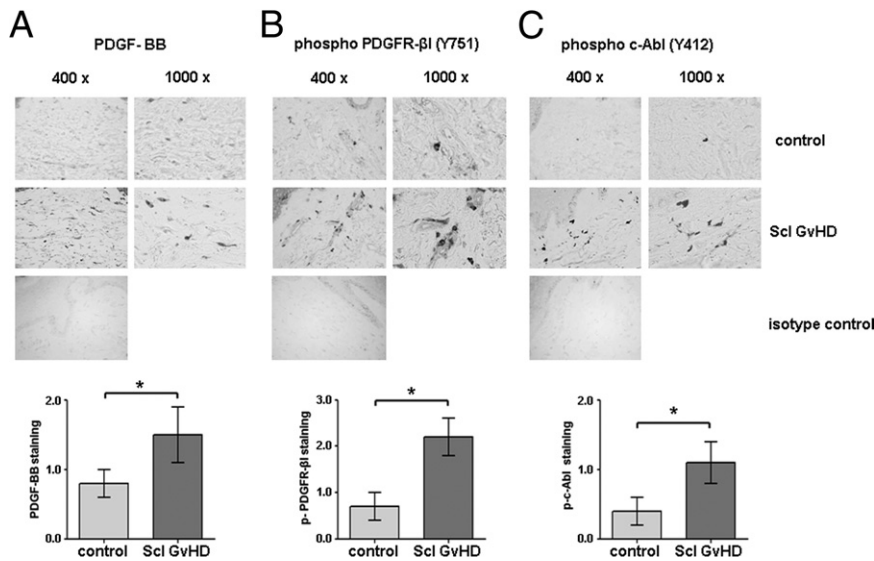
To analyze the collagen content in skin samples, a hydroxyproline assay was performed as previously described.<sup>26</sup> After digestion of 0- to 3-mm skin biopsy samples in 6 mol/L HCl for 3 hours at 120°C, 0.06 mol/L chloramine T was added, and samples were mixed and incubated for 20 minutes at room temperature. Perchloric acid at a concentration of 3.15 mol/L and 20% *p*-dimethylaminobenzaldehyde were added, and samples were incubated for additional 20 minutes at 60°C. Absorbance was determined at 557 nm using a Spectra MAX 190 microplate spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA).

### *Fluorescence in Situ Hybridization with Murine Y Chromosome Paint*

Tissue sections were deparaffinized in xylene and rehydrated in an ethanol series, followed by antigen retrieval in 10 mmol/L sodium citrate for 15 minutes at 96°C. Further pretreatment of the slides and hybridization using red-labeled Y chromosome probes were performed according to guidelines of the Mouse iDetect Chromosome Paint Probes (ID Labs, London, ON, Canada). Nuclei were visualized using DAPI.

### *Statistical Analysis*

Data are given as mean  $\pm$  SD. The results in the sham-treated synBMT mice were set to 100%, and all other results were normalized to the control group. The SD in the control group was calculated from the percentage of change from the mean in the individual samples. The Wilcoxon signed rank test for related samples and the Mann-Whitney *U*-test for nonrelated samples were used for statistical analyses.  $P < 0.05$  was considered statistically significant.



**Figure 1.** PDGF and c-Abl pathways are activated in human sclerodermatous cGvHD. PDGF-BB, phospho PDGFR- $\beta$  (Y751), and phospho c-Abl (Y412) were detected at immunohistochemistry in skin biopsy samples from patients with cGvHD ( $n = 12$ ) and control individuals ( $n = 10$ ). Representative tissue sections stained for PDGF-BB (A), phospho PDGFR- $\beta$  (B) and phospho c-Abl (C) are shown at  $\times 400$  and  $\times 1000$  magnification. Respective isotype controls are shown at  $\times 400$  magnification.  $*P < 0.05$ .

## Results

### *c-Abl and PDGF Pathways Are Activated in Human and Murine Sclerodermatous cGvHD*

We first analyzed whether the PDGF and c-Abl signaling cascades are activated in human sclerodermatous cGvHD. The levels of PDGF-BB, a homodimer of PDGF-B subunits with the highest potency to induce PDGFR- $\beta$  signal transduction, were increased in skin sections of patients with sclerodermatous cGvHD (Figure 1A). Consistent with the increased levels of PDGF-BB, PDGF signaling was activated with increased staining for Tyr751 phosphorylated active PDGFR- $\beta$  (Figure 1B). Similar to phosphorylation of PDGFR- $\beta$  at Tyr751, phosphorylation of c-Abl at Tyr412 serves as a

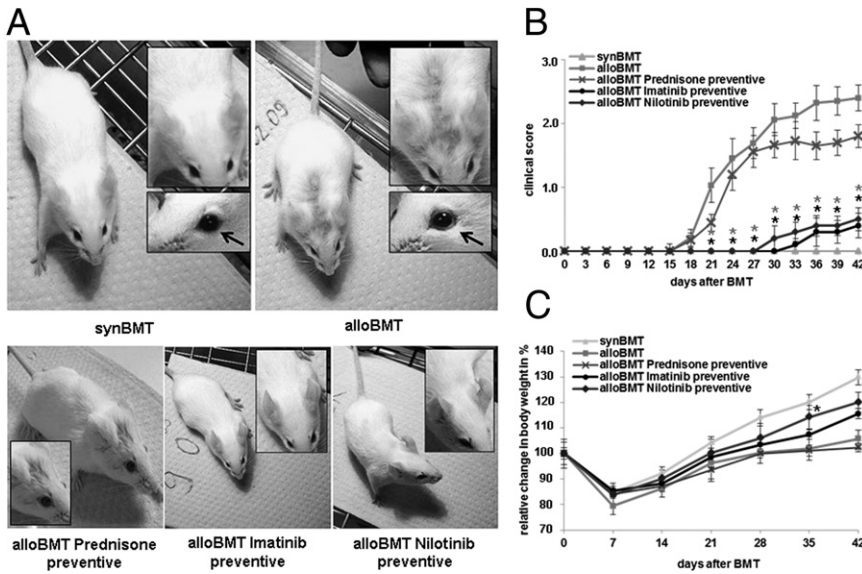
marker for activation of c-Abl.<sup>27–29</sup> Staining for Tyr412 phosphorylated c-Abl was significantly increased in patients with cGvHD as compared with control individuals (Figure 1C).

We next investigated whether PDGF and c-Abl signaling is also activated in murine sclerodermatous cGvHD. Findings in murine sclerodermatous cGvHD resembled those in human cGvHD, with increased levels of PDGF-BB (Figure 2A), phosphorylated PDGFR- $\beta$  (Figure 2B), and phosphorylated c-Abl (Figure 2C) in alloBMT mice compared with synBMT control mice.

Treatment with imatinib and nilotinib inhibited the activation of PDGF and c-Abl signaling in murine cGvHD. The intensity of the stainings for Tyr751 phosphorylated PDGFR- $\beta$  and Tyr412 phosphorylated c-Abl in alloBMT mice were reduced to the levels in synBMT control mice receiving treatment with imatinib and nilotinib. Levels of PDGF-BB were

**Figure 2.** Murine sclerodermatous cGvHD mimics the activation of PDGF and c-Abl pathways in human cGvHD. Representative tissue sections of the treatment groups and the control groups are shown at  $\times 400$  and  $\times 1000$  magnification ( $n = 8$  each). Isotype controls are shown at  $\times 400$  magnification. **A:** Expression of PDGF-BB is strongly increased at 42 days after alloBMT as compared with syngeneic controls. As expected, treatment with imatinib and nilotinib did not alter expression of PDGF-BB. **B:** Increased staining for phosphorylated PDGFR- $\beta$  in alloBMT mice compared with synBMT control mice at 42 days after alloBMT. Treatment with both imatinib and nilotinib prevented increased phosphorylation of PDGFR- $\beta$ . **C:** cAbl signaling is activated in murine cGvHD, with increased phosphorylation of c-Abl. Treatment with both imatinib and nilotinib prevented activation of c-Abl and reduced the levels of phosphorylation to that in synBMT control mice.  $*P < 0.05$ .





**Figure 3.** Preventive treatment with both imatinib and nilotinib was well tolerated and prevented the clinical features of sclerodermatous cGvHD. **A:** Representative pictures of a syngeneic control mouse (synBMT) in comparison with a sham-treated alloBMT mouse show skin lesions on the upper back and xerotic changes around the eyes (arrow). **B:** Treatment with either imatinib (alloBMT imatinib preventive) or nilotinib (alloBMT nilotinib preventive) initiated after BMT prevented xerosis, alopecia, and ulcer formation. Consistently, the clinical composite score for cutaneous cGvHD was significantly lower in mice that received preventive treatment with imatinib and nilotinib as compared with sham-treated mice and mice treated with prednisone (asterisks). **C:** Preventive treatment with imatinib and nilotinib, but not with prednisone, prevented weight loss after alloBMT and resulted in a significantly higher body weight (asterisks).

not affected by the treatment, which is consistent with the mode of action of imatinib and nilotinib as inhibitors of the kinase activity of PDGFR- $\beta$  and c-Abl (Figure 2, A–C).

### Prevention of Dermal Fibrosis in a Murine Model of Sclerodermatous cGvHD by Imatinib and Nilotinib

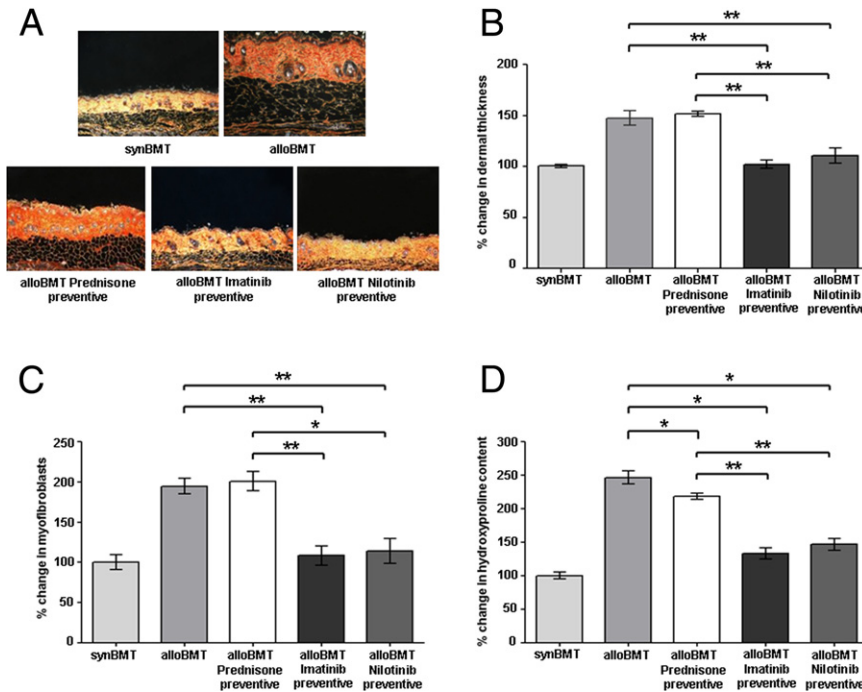
#### Imatinib and Nilotinib Prevent Clinical Features of Sclerodermatous cGvHD

BALB/c mice that received bone marrow cells from B10.D2 mice with a minor HLA incompatibility developed clinical features of sclerodermatous cGvHD including skin lesions and alopecia (Figure 3A), with reduced activity, diarrhea, and weight loss until 42 days after alloBMT. The manifestations of cutaneous cGvHD were quantified using a clinical scoring system based on progression of skin lesions and occurrence of alopecia. Twenty-one days after BMT, sham-treated alloBMT mice exhibited clinical features of sclerodermatous cGvHD, with a mean clinical score of  $1.1 \pm 0.3$  and skin lesions of about  $1 \text{ cm}^2$  (Figure 3B). Their clinical score increased rapidly to  $2.3 \pm 0.3$  between days 21 and 36 after transplantation, and reached its maximum value of  $2.4 \pm 0.2$ , with skin lesions  $>2 \text{ cm}^2$  in area, at 42 days after alloBMT. In contrast, the control animals did not show any evidence of skin lesions or alopecia, and the clinical score for synBMT controls remaining at 0 during the entire observation period (Figure 3B). Due to total body irradiation, mice in all groups lost about 15% to 20% of their initial body weight in the first week after BMT (Figure 3C). Sham-treated alloBMT mice gained almost no weight during the observation period, and reached a body weight of only  $106\% \pm 3\%$  at the end of the observation period. In contrast, synBMT controls gained weight quickly, and reached their initial body weight at day 21 after BMT. At the end of the observation period, the synBMT control mice were at  $130\% \pm 3\%$  of their baseline weight (Figure 3C).

Treatment with imatinib and nilotinib prevented alopecia and skin ulceration (Figure 3A). In this context, the nilotinib treatment groups exhibited first evidence of efficiency, with a clinical score of  $0.2 \pm 0.2$  at day 30 after BMT, and a final score of only  $0.5 \pm 0.2$  at day 42, whereas the imatinib treatment groups had a minor extent of skin lesions, with first notable clinical scores around  $0.1 \pm 0.1$  at day 33 after BMT, and a final score of only  $0.4 \pm 0.2$  at day 42 (Figure 3B). At 80 days of follow-up, the clinical score had not increased further, but stabilized at around 0.4. Compared with standard therapy using prednisone, treatment with imatinib and nilotinib was more effective in reducing the clinical signs of cGvHD (Figure 3B). Imatinib and nilotinib treatment groups had comparable changes in body weight. Mice in both treatment groups gained weight significantly faster and reached a greater body weight at 42 days after BMT, compared with sham-treated or prednisone-treated alloBMT mice (Figure 3C). Induction of mixed chimerism was excluded via fluorescence in situ hybridization analysis of bone marrow and fluorescence-activated cell sorter analysis of peripheral blood.

#### Imatinib and Nilotinib Prevented Dermal Fibrosis in Sclerodermatous cGvHD

Skin from sham-treated alloBMT mice showed dense accumulation of collagen bundles in the dermis (Figure 4A). Treatment with imatinib or nilotinib strongly reduced the accumulation of ECM within the dermis. Histologic analysis of skin tissue isolated 42 days after BMT revealed an increase in dermal thickness of  $48\% \pm 7\%$  in sham-treated alloBMT mice compared with synBMT control mice ( $P < 0.001$ ) (Figure 4B). Combined inhibition of c-Abl and PDGF pathways by either imatinib or nilotinib largely prevented these histologic changes, whereas treatment with prednisone had no effect. The dermal thickness of alloBMT mice treated with imatinib did not differ from that of synBMT controls, demonstrating that imatinib could completely prevent dermal thickening ( $P =$



**Figure 4.** Imatinib and nilotinib prevent the histologic changes of experimental sclerodermatous cGvHD. **A:** Treatment with imatinib and nilotinib, but not with prednisone, prevented fibrosis after alloBMT. Representative skin sections of synBMT control mice, sham-treated alloBMT mice, and alloBMT mice that received preventive treatment with imatinib, nilotinib, or prednisone stained with Sirius Red are shown at  $\times 100$  magnification. Preventive treatment with both imatinib and nilotinib reduced dermal thickening (**B**), prohibited the differentiation of resting fibroblasts into myofibroblasts (**C**), and decreased the hydroxyproline content of the skin (**D**), as compared with sham-treated alloBMT mice, whereas treatment with prednisone was ineffective. \* $P < 0.05$ , \*\* $P < 0.01$ .

0.004 compared with sham-treated alloBMT mice). In addition, nilotinib treatment in alloBMT mice significantly reduced dermal thickening by  $77\% \pm 7\%$  when compared with synBMT control mice ( $P = 0.007$ ) (Figure 4B).

Myofibroblasts, which can be identified by their expression of  $\alpha$ -smooth muscle actin, release large amounts of collagen and are key effectors in fibrotic diseases. The number of myofibroblasts in the skin of sham-treated alloBMT mice was increased by  $95\% \pm 9\%$  compared with skin of synBMT control mice ( $P = 0.001$ ) (Figure 4C). Imatinib reduced the number of myofibroblasts by  $84\% \pm 15\%$  in alloBMT mice compared with synBMT controls ( $P = 0.001$ ). Moreover, nilotinib significantly decreased the number of myofibroblasts by  $87\% \pm 17\%$  ( $P = 0.002$ ) (Figure 4C).

The hydroxyproline content was significantly increased by  $147\% \pm 10\%$  in skin samples of sham-treated alloBMT mice compared with synBMT controls ( $P = 0.008$ ) (Figure 4D). Both imatinib and nilotinib prevented collagen deposition, and reduced the hydroxyproline content by  $77\% \pm 8\%$  ( $P = 0.003$ ) and  $68\% \pm 9\%$  ( $P = 0.003$ ) compared with healthy syngeneic control mice (Figure 4D). In contrast to imatinib and nilotinib, prednisone had no beneficial effect.

#### *Imatinib and Nilotinib are Effective for the Treatment of Clinically Manifested Experimental cGvHD*

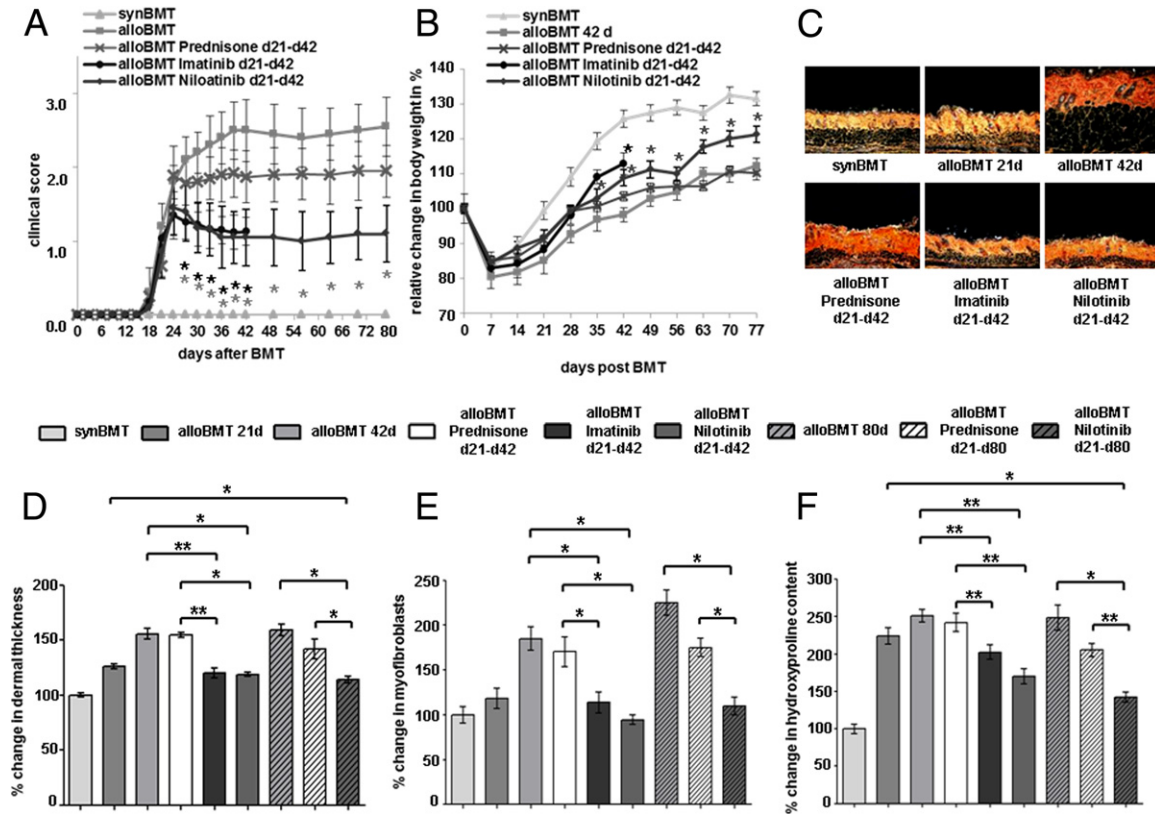
Although preventing cGvHD should be the central goal after alloBMT, therapeutic approaches may also be effective when initiated after the onset of clinical disease. We therefore evaluated the efficacy of imatinib and nilotinib for the treatment of cGvHD in a therapeutic regimen in which imatinib was administered after the occurrence of first clinical manifestations of experimental cGvHD at day 21 after alloBMT.

Treatment with imatinib and nilotinib stopped the progression of cutaneous cGvHD. In contrast to sham-treated mice, in which the clinical score increased progressively to  $2.5 \pm 0.4$  on day 42, the clinical score in imatinib-treated mice peaked at  $1.4 \pm 0.3$  on day 24, and declined slightly to  $1.1 \pm 0.3$  on day 42 after alloBMT (Figure 5A). Furthermore, sham-treated mice only slowly gained weight, whereas imatinib treated mice recovered much faster and reached a greater body weight at the end of the observation period (Figure 5B). Although treatment with prednisone had mild beneficial effects on clinical outcomes, imatinib and nilotinib were significantly more effective.

Prolonged treatment after alloBMT, initiated at first clinical signs of cGvHD on day 21 until day 80, induced regression of the histologic changes of sclerodermatous cGvHD. Treatment with imatinib or nilotinib from days 21 to 42 after alloBMT improved the histologic changes of cGvHD and reduced dermal thickness, myofibroblast counts, and hydroxyproline to levels in mice sacrificed at day 21 after BMT (Figure 5, C–F). To investigate whether the combined inhibition of PDGF and c-Abl signaling might also induce regression of experimental sclerodermatous cGvHD, mice were treated with nilotinib from days 21 to 80 after alloBMT. Prolonged treatment not only stopped progression of dermal thickening, but reduced dermal thickness below baseline levels in untreated mice sacrificed at 21 days after alloBMT ( $P = 0.018$ ) (Figure 5D). In line with these results, the hydroxyproline content was reduced to below baseline levels ( $P = 0.02$ ), confirming regression of fibrosis (Figure 5F). Prednisone therapy did not reverse histologic changes in murine cGvHD.

#### *Discussion*

The potent profibrotic cytokines PDGF and TGF- $\beta$  activate fibroblasts to release excessive amounts of ECM.



**Figure 5.** Therapeutic regimen of imatinib and nilotinib initiated after the onset of first clinical signs ameliorate clinical and histologic changes of sclerodermatous cGvHD. Treatment with imatinib at first clinical signs improves clinical features of cGvHD, as evidenced by a decreased composite score (A) and by increased recovery of body weight after alloBMT (B). Asterisks indicate statistically significant differences compared to sham-treated, allogeneically transplanted mice of the same time-point. Long-term inhibition of c-Abl and PDGFR, but not prednisone, induced regression of the histologic changes of cGvHD compared with pretreatment levels (C), with decreased dermal thickening (D), comparable myofibroblast counts (E), and reduced hydroxyproline content (F). alloBMT 21d, sham-treated mice with samples obtained at 21 days after alloBMT; alloBMT 42d, sham-treated mice with samples obtained at 42 days after alloBMT; alloBMT Prednisone/Imatinib/Nilotinib d21–d42, alloBMT mice treated with imatinib, nilotinib, or prednisone after onset of clinical disease and with samples obtained at 42 days after alloBMT; alloBMT 80d, sham-treated mice with samples obtained at 80 days after alloBMT; alloBMT Prednisone/Nilotinib d21–d80, alloBMT mice treated with nilotinib or prednisone after onset of clinical disease and with samples obtained at 80 days after alloBMT. \**P* < 0.05, \*\**P* < 0.01.

Imatinib and nilotinib are considered candidate therapies for fibrosis because they simultaneously target both pathways by inhibiting almost selectively the tyrosine kinase activity of PDGFRs and c-Abl.<sup>30–32</sup> In the present study, we provided a molecular basis for the use of these tyrosine kinase inhibitors in sclerodermatous cGvHD. We found that c-Abl is phosphorylated at Tyr412, and thereby is activated in both human and murine cGvHD. We also found that PDGF signaling is activated with increased expression of PDGF-BB and subsequent phosphorylation of PDGFR-β.

In addition to overexpression of their ligands, PDGFRs might also be activated by stimulatory autoantibodies, which have recently been identified in patients with cGvHD.<sup>10</sup> A recent phase I study indicated that these antibodies correlated with disease activity in only 4 of 7 patients.<sup>20</sup> The same study found that treatment with imatinib reduced the phosphorylation rates of PDGFR-α or PDGFR-β in only 1 of 4 patients, and those changes in the phosphorylation rate did not correlate with clinical response. Thus, clinical response to imatinib is not fully explained by inhibition of PDGFR activity. Together, these findings indicate that c-Abl is an important target for imatinib in sclerodermatous cGvHD. However, changes

in the phosphorylation of c-Abl were not assessed in that study.<sup>20</sup>

In contrast to sclerodermatous cGvHD, staining for phosphorylated PDGFR and c-Abl was virtually absent in synBMT control mice, which suggests that selective inhibition of PDGFR and c-Abl in cGvHD might not be hindered by toxicity. Furthermore, effective reduction of the phosphorylation of PDGFR and c-Abl by imatinib or nilotinib were not accompanied by clinical signs of toxicity in alloBMT mice. We also did not observe hematotoxicity in our mice, which is a major adverse effect in humans. Consistent with our observations, clinical trials of imatinib or nilotinib in chronic myeloid leukemia indicate that these inhibitors are well tolerated: only about 1% of patients discontinued therapy because of adverse effects.<sup>33–35</sup> In addition to the limited activation of PDGFR and c-Abl under physiologic conditions and the selectivity for those targets, the distinct mechanism of action compared with current regimens might contribute to the relatively low rate of severe adverse events. In contrast to current therapeutic regimens for cGvHD that use immunosuppressive or cytostatic agents, imatinib and nilotinib do not have significant inhibitory effects on the immune system,



and significantly increased rates of infections have not been reported in clinical trials.<sup>33</sup> Inasmuch as patients with cGvHD are already immunocompromised and infections are among the major causes of death in these patients, the absence of immunosuppressive effects of imatinib and nilotinib might be favorable when compared with conventional therapies.

We found that the combined inhibition of PDGF- and TGF $\beta$ -signaling cascades by imatinib and nilotinib effectively prevented experimental cGvHD. Of note, the dosages of imatinib and nilotinib used in the present study resulted in mean plasma concentrations that are pharmacologically relevant in humans.<sup>36,37</sup> When initiated directly after stable engraftment of the transplanted bone marrow, treatment with imatinib and nilotinib prevented the histologic changes of cutaneous cGvHD, with complete abrogation of dermal thickening and myofibroblast differentiation. Preventive treatment also improved the clinical features of cGvHD. In contrast to sham-treated mice, mice treated with imatinib and nilotinib did not develop skin ulcers and increased hair loss. Moreover, systemic features of cGvHD such as immobility, weight loss, and diarrhea were almost absent, indicating that the beneficial effects of imatinib and nilotinib are not restricted to cutaneous cGvHD.

Therapeutic approaches, however, should not be effective only in preventive regimens; they should also stop progression and reduce disease activity when given in therapeutic regimens only after clinically detectable cGvHD. This enables initiation of therapy as needed and averts unnecessary treatment. In this context, treatment with imatinib and nilotinib, when initiated after development of alopecia at 21 days after BMT, not only stopped progression of cGvHD but also induced regression of the histologic findings of sclerodermatous cGvHD. Imatinib- and nilotinib-treated mice regained weight faster, and progression to a higher composite score was prevented. With prolonged treatment, the dermal thickness and hydroxyproline content decreased to less than pretreatment levels. Thus, tyrosine kinase inhibitors that target PDGFR and c-Abl simultaneously are effective in both preventing and treating experimental cGvHD. When compared with the standard treatment using high-dose prednisone, treatment using imatinib and nilotinib resulted in superior effects. Our results in experimental sclerodermatous cGvHD are supported by three recent uncontrolled clinical studies that reported response rates of 40%, 50%, and 79%, respectively, in patients with established cGvHD unresponsive to other therapies.<sup>18–20</sup>

Because the efficacy of current therapies for cGvHD is limited, inhibitors of c-Abl and PDGFR such as imatinib and nilotinib might be a promising future strategy for the treatment of cGvHD. The broad efficacy and good tolerability in experimental cGvHD, as in the present study, and first positive signs from uncontrolled clinical trials favor further clinical testing of imatinib and nilotinib in cGvHD.

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