Peroxisome Proliferator–Activated Receptor-γ Agonists Prevent In Vivo Remodeling of Human Artery Induced by Alloreactive T Cells

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Background—Ligands activating the transcription factor peroxisome proliferator–activated receptor-γ (PPARγ) have antiinflammatory effects. Vascular rejection induced by allogeneic T cells can be responsible for acute and chronic graft loss. Studies in rodents suggest that PPARγ agonists may inhibit graft vascular rejection, but human T-cell responses to allogeneic vascular cells differ from those in rodents, and the effects of PPARγ in human transplantation are unknown.

Methods and Results—We tested the effects of PPARγ agonists on human vascular graft rejection using a model in which human artery is interposed into the abdominal aorta of immunodeficient mice, followed by adoptive transfer of allogeneic (to the artery donor) human peripheral blood mononuclear cells. Interferon-γ–dependent rejection ensues within 4 weeks, characterized by intimal thickening, T-cell infiltrates, and vascular cell activation, a response resembling clinical intimal arteritis. The PPARγ agonists 15-deoxy-prostaglandin-J_2, ciglitazone, and pioglitazone reduced intimal expansion, intimal infiltration of $CD45RO^+$ memory T cells, and plasma levels of inflammatory cytokines. The PPARγ antagonist GW9662 reversed the protective effects of PPARγ agonists, confirming the involvement of PPARγ-mediated pathways. In vitro, pioglitazone inhibited both alloantigen-induced proliferation and superantigen-induced transendothelial migration of memory T cells, indicating the potential mechanisms of PPARγ effects.

Conclusion—Our results suggest that PPARγ agonists inhibit allogeneic human memory T cell responses and may be useful for the treatment of vascular graft rejection. **(*Circulation*. 2011;124:196-205.)**

Key Words: arteriosclerosis ■ mice ■ PPARγ ■ T-lymphocytes ■ vascular diseases

Inflammation of the arterial wall by T lymphocytes is characteristic of many arteriopathies, including atherosclerosis, Takayasu arteritis, periarteritis nodosa, giant-cell arteritis, and Kawasaki disease.[1,2] T cells may also infiltrate the arterial wall in certain forms of acute (intimal arteritis) or chronic (transplant vasculopathy) vascular rejection of allografts and is a major cause of allograft loss.[3] These 2 forms of vascular rejection, which may represent aggressive and more indolent forms, respectively, of the same process, are resistant to conventional immunosuppression.[3]

Clinical Perspective on p 205

Recent studies in rodents suggest that PPARγ agonists may be good candidates for the treatment of both acute and chronic phases of allograft rejection.[4,5] PPARγ is a member of a nuclear receptor family that, on binding an agonist, increases glucose uptake, stimulates lipogenesis,[6] and has antiinflammatory effects.[4,5] The most potent natural PPARγ agonist is a metabolite of prostaglandin D_2, 5-deoxy-prostaglandin-J_2 (PGJ2). In addition, multiple PPARγ ligands have been synthesized with both agonistic and antagonistic properties. The notable agonists are ciglitazone, a prototypical compound for the thiazolidinedione class of drugs, and its 2 analogs, rosiglitazone and pioglitazone, which are Food and Drug Administration–approved drugs for type 2 diabetes mellitus.[7,8] The irreversible antagonistic ligand GW9662 makes it possible to distinguish PPARγ-dependent and -independent effects of PPARγ agonists.[9]

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Although rodent transplantation models have been used to study the pathogenesis of acute and chronic forms of allograft vascular rejection, these models are limited in their applicability to human transplantation. For example, rejected aortic interposition grafts in rats or mice develop lesions with intimal expansion, but the vascular cells within the expanded intima are host derived and accumulate only after the allogeneic graft cells have been completely destroyed.[10] This type of injury is not seen in the grafts of immunosuppressed patients, in which the vast majority of stromal cells within the neointima are of graft origin.[11] Although other types of rodent models may avoid this pitfall, rodent transplantation differs in several significant ways from human transplantation. Human recipients have a strong T-cell memory response to alloantigens that is typically missing in rodents.[12] Moreover, human endothelial cells are able to activate resting alloreactive CD4 memory T cells to become effector cells,[13] whereas rodent endothelial cells do not.[14] This latter response depends on the expression of major histocompatibility complex class II (MHC II; human leukocyte antigen [HLA-DR]) molecules by human endothelial cells.[15] Although HLA-DR is observed on human coronary artery endothelium in situ,[16] it appears to depend on low levels of interferon-γ (IFNγ) for maintenance of expression.[17] In contrast, rodent endothelial cells do not typically express MHC II molecules.[18] Of particular relevance to this project, a PPARγ-responsive element is present in the murine inducible nitric oxide synthase promoter that is not present in the human gene.[19] Such differences underlie the importance of investigating PPARγ agonists in human allogeneic vascular rejection.

To address the limitations of conventional mouse transplantation models, we have used a humanized mouse model in which human artery segments are interposed into the aortas of immunodeficient C.B-17 severe combined immunodeficiency (SCID)/beige mice, after which human peripheral blood mononuclear cells (PBMCs) allogeneic to the artery donor are adoptively transferred into the same animal.[20] Within 1 week, human memory T cells are found in the circulation of these mice. The engrafted T cells produce some IFNγ, which sustains HLA-DR expression on the human endothelial cells lining the arterial graft. These mice reproducibly develop expansive intimal lesions containing human smooth muscle–like cells, as well as human T-cell intimal and adventitial infiltrates confined to the interposed human artery segment. Luminal endothelial cells show evidence of injury, but the media is largely spared.[20] The T cells likely respond to the graft endothelial cells in the absence of professional antigen-presenting cells (passenger leukocytes) necessary to initiate rejection in rat models.[12] The infiltrating T cells produce more IFNγ, which acts as a mitogen for human vascular smooth muscle cells.[21] Over a time course of 4 to 6 weeks, intimal expansion outpaces outward vessel remodeling, and the lumen of the artery is progressively compromised.[21] This process much more closely resembles the lesions observed in human allograft arteries than that observed in rodent arterial interposition models. Here, we report that PPARγ agonists reduce activation of alloreactive human T cells in this model, resulting in reduced HLA-DR expression on vascular cells, reduced T-cell infiltration in the vessel intima, and reduced intimal expansion. These effects may occur as a result of inhibition of T-cell reactivity to alloantigens and reduced migration through the endothelium.

Methods

Animals

CB.17 SCID/beige mice[20] (Taconic, Germantown, NY, or Harlan, Indianapolis, IN) were used at 6 to 12 weeks of age. Animals were housed in microisolator cages and given sterilized water and mouse chow. All experimental protocols were approved by the Institutional Animal Care and Use Committee of Yale University. From our pilot data on the effects of pioglitazone on neointimal formation, we estimated that 8 animals per group would be required to detect an effect size of 2 in neointimal reduction ($\alpha=0.05$; power=80%).

Arterial Engraftment and Adoptive Human Cell Transfer

Human epigastric, coronary, or internal mammary artery implantations in mice were performed as described previously.[20,22] Alloreactive PBMCs were obtained from adult healthy volunteers with leukapheresis under a protocol approved by the Yale Human Investigation Committee, and 3×10^8 PBMCs in 1 mL PBS were injected into mice intraperitoneally. The level of human lymphocyte engraftment was assessed at 2 and 4 weeks after PBMC injection by fluorescence-activated cell sorter analysis of mouse peripheral blood.

Treatment of Mice With Peroxisome Proliferator–Activated Receptor-γ Agonists and/or Antagonist

The treatment was started at least 10 days postoperatively and 2 days before injection of PBMCs and continued daily for 28 days, when the experiment was terminated and the human arterial graft was removed for analysis. The drugs were injected intraperitoneally with the following doses: PGJ2, 50 $\mu g \cdot kg^{-1} \cdot d^{-1}$ in 60 μL (1 mg/mL PGJ2 in 95% ethanol diluted 1:49 in PBS); ciglitazone, 2 $mg \cdot kg^{-1} \cdot d^{-1}$ in 60 μL (dimethyl sulfoxide:PBS, 1:4); pioglitazone, 10 mg/kg in 30 μL dimethyl sulfoxide; and GW9662, 300 $\mu g \cdot kg^{-1} \cdot d^{-1}$ or 2 $mg \cdot kg^{-1} \cdot d^{-1}$ (in pioglitazone-treated mice) in 60 μL (dimethyl sulfoxide:PBS, 1:1). The effects of diluents without any drug were tested in pilot experiments; no effects were observed.

Evaluation of Arterial Intima

Arterial grafts were removed 28 days after injection of alloreactive PBMCs, frozen in optimal-cutting-temperature medium (Tissue Tek, Sakura Finetek, Torrance, CA), and stored at −80°C. The vessels were sectioned (5 μm). Thickness of arterial intima, immunohistochemistry for CD45RO (memory T cells), and HLA-DR expression were assessed as described previously.[21] More detailed analysis of graft cells was done by double staining with immunofluorescent antibodies for HLA-DR, CD3 (T-cell marker), α-smooth muscle actin (SMA; smooth muscle marker), CD31 (endothelial marker), and $CD56^+$ (NK cells). Details on the reagents are given in the online-only Data Supplement.

Analysis of Human Immune Cells and Human Cytokines in Mouse Blood

Approximately 500 μL mouse blood was obtained through retro-orbital bleeding. Blood was centrifuged at 400*g*, and plasma was collected and stored at −80°C until the evaluation of human cytokine expression with Luminex-based Milliplex MAP multicytokine kit and kits for osteopontin and transforming growth factor-β1 according to the manufacturer's instructions (Millipore, Billerica, MA). From our pilot experimental results and previously published data,[4] we hypothesized that T cell–derived cytokines such as IFNγ and interleukin-10, tumor necrosis factor-α, and soluble CD25 would be reduced after pioglitazone treatment. Hence, the Bonferroni correction for multiple comparisons was not performed for those cytokines. For comparisons of additional cytokines, an α of 0.0036

was used to determine statistical significance (Bonferroni correction for 14 comparisons).

Subsets of human memory CD45RO T cells (CD4 and CD8) and subsets of CD4 cells were quantified (regulatory T cells and T helpers Th1, Th2, Th17). The online-only Data Supplement provides additional details.

T-Lymphocyte Transmigration Assay

Transendothelial migration assays were performed with tumor necrosis factor-α–pretreated, CIITA-transduced, superantigen-overlaid human coronary artery endothelial cell monolayers and either $CD4^+CD45RO^+CCR7^-$ $CD62L^-$ effector memory or $CD8CD45RO^+$ total memory T cells enriched from leukopheresis-isolated and cryopreserved PBMCs in flow chambers as previously described.[23] The purified T-cell subsets were pretreated overnight with vehicle or pioglitazone before the transmigration assays. The online-only Data Supplement provides a detailed description. The percentages of transmigrated $CD4^+$ T cells were calculated for 100 to 200 $V\beta2TCR^+$ and 100 to 200 $V\beta2TCR^-$ cells per sample by analyzing 5 to 10 groups of 20 cells each (1 cell at a time, covering the entire area of flow), calculating the percentage for each group, and calculating the mean and SD for the groups.

In Vitro T-Lymphocyte Proliferation to Allogeneic Endothelial Cells

Total memory T lymphocytes and CD4 or CD8 memory T lymphocyte subsets were cocultured with IFNγ-pretreated human umbilical vein endothelial cells. T-cell proliferation was compared between untreated and pioglitazone-treated cells by [^{3}H] thymidine incorporation or carboxyfluorescein succinimidyl ester dye dilution as described in the Methods section in the online-only Data Supplement. The mean of sample replicates (n=6) was calculated in each experiment and is shown as percent of decrease of vehicle control considered to be 100%.

Statistical Analysis

The data were analyzed by use of a 2-tailed paired *t* test in which each pair of mice received human artery from the same donor and alloreactive PBMCs from another donor and in which the mice differed only in pharmacological treatment. The data are expressed as mean±SD. A difference with a value of $P<0.05$ was considered statistically significant. Similarly, in vitro data were analyzed by use of a 2-tailed paired *t* test in which the pioglitazone- and vehicle-treated groups were compared. Sample size estimation was performed for a Wilcoxon rank-sum test with the use of a 2-sided α of 0.05.

Comparisons of levels of cytokines assayed by Luminex-based Milliplex MAP multicytokine kit for which a priori hypotheses were not defined were carried out with a Bonferroni adjustment, and an α of 0.0036 (0.05/14) was considered to be statistically significant.

Results

Effects of Peroxisome Proliferator–Activated Receptor-γ Agonists on Intimal Thickening

The SCID/beige mice that were engrafted with human artery and received adoptive transfer of human alloreactive PBMCs developed a neointima in the human arterial graft, whereas the mouse vasculature remained unchanged, as previously described.[20] In pilot experiments, we established an optimal daily dose of PGJ2 that reduces the neointima as 50 $\mu g \cdot kg^{-1} \cdot d^{-1}$. A lower dose of 15 $\mu g \cdot kg^{-1} \cdot d^{-1}$ and a higher dose of 100 $\mu g \cdot kg^{-1} \cdot d^{-1}$ were not inhibitory. A similar narrow therapeutic window was previously described for PGJ2 in a mouse model of multiple sclerosis.[24] The reason for narrow therapeutic window is not entirely clear. The effects of PGJ2 are complex and involve intracellular targets, as well as membrane DP1 and DP2 receptors (receptors for prostaglandin D2).[25] Treatment of the mice with 50 $\mu g \cdot kg^{-1} \cdot d^{-1}$ PGJ2 consistently abolished neointima formation in a PPARγ-dependent manner (see below) (0.1 ± 0.09 mm^2 in treated mice versus 0.55 ± 0.4 mm^2 in untreated mice; n=8 pairs; $P=0.012$; Figure 1A). Ciglitazone (2 mg/kg IP)[26] also reduced but did not completely inhibit neointima formation (0.50 ± 0.25 for untreated mice versus 0.26 ± 0.20 mm^2 for the ciglitazone-treated group: n=4 pairs; $P=0.026$; Figure 1B), whereas pioglitazone (10 mg/ kg IP)[4,27] completely blocked neointima formation; a 91% reduction was observed after treatment compared with untreated mice (0.86 ± 0.56 versus 0.08 ± 0.09 mm^2; n=8; $P=0.006$; Figure 1C).

To determine whether the effects of these PPARγ agonists are mediated via PPARγ-dependent mechanisms,[28] we compared mice treated with a relevant PPARγ agonist alone and those treated with an agonist combined with the PPARγ antagonist GW9662.[9] The effects of all 3 agonists were reversed by GW9662. The neointimal thickness in mice treated with PGJ2 alone was 0.07 ± 0.1 mm^2, whereas the neointima in mice treated with both PGJ2 and GW9662 (300 $\mu g \cdot kg^{-1} \cdot d^{-1}$ IP) was 0.36 ± 0.35 mm^2 (n=5 pairs; $P=0.067$; Figure 1D). The inhibitory effect of GW9662 on intima formation in ciglitazone-treated animals (0.46 ± 0.5 versus 0.9 ± 0.6 mm^2; n=4 pairs; $P=0.006$; Figure 1E) and pioglitazone-treated animals (0.17 ± 0.1 versus 0.85 ± 0.24 mm^2; n=4 pairs; $P=0.017$; GW9662, 2 $mg \cdot kg^{-1} \cdot d^{-1}$ IP; Figure 1F) was also significant.

Effects of Peroxisome Proliferator–Activated Receptor-γ Agonists on Cells Within the Human Arterial Grafts

In our model of T cell–mediated vascular rejection, the majority of the cells within the neointima are infiltrating T cells. The number of infiltrating CD45RO memory T cells in the neointima was reduced after PGJ2 treatment compared with untreated mice (40 ± 42 versus 190 ± 117 cells per intima; n=6 pairs; $P=0.034$; Figure 2A, left, and 2D), which was reversed by GW9662 cotreatment, but it did not reach statistical significance (27 ± 49 versus 106 ± 88; n=4 pairs; $P=0.07$; Figure 2A, right, and 2D). The effect of ciglitazone on CD45RO cell infiltration was not significant (89 ± 51 versus 72 ± 54; n=4; $P=0.65$; Figure 2B, left), and cotreatment with GW9662 did not alter the infiltration (88 ± 37 versus 120 ± 74; n=4 pairs; $P=0.507$; Figure 2B, right). Pioglitazone treatment reduced CD45RO cell infiltration (120 ± 73 versus 6 ± 9 cells; n=8 pairs; $P=0.002$; Figure 2C, left, and 2E). The reversal effect of GW 9662 on pioglitazone-induced inhibition of T-cell infiltration was notable, although it did not reach statistical significance (2 ± 3 versus 74 ± 83; n=4 pairs; $P=0.18$; Figure 2C, right, and 2E).

The HLA-DR–positive cells were identified by immunohistochemistry only in arterial grafts retrieved from mice that received allogeneic PBMCs, whereas they were absent in grafts of mice that did not receive PBMCs. The HLA-DR–positive cells were primarily localized close to the arterial lumen. The same localization was found for α-actin$^+$ smooth muscle–like cells or at the interface of lumen and neointima for $CD31^+$ endothelial cells. Treatment of mice with PGJ2 or

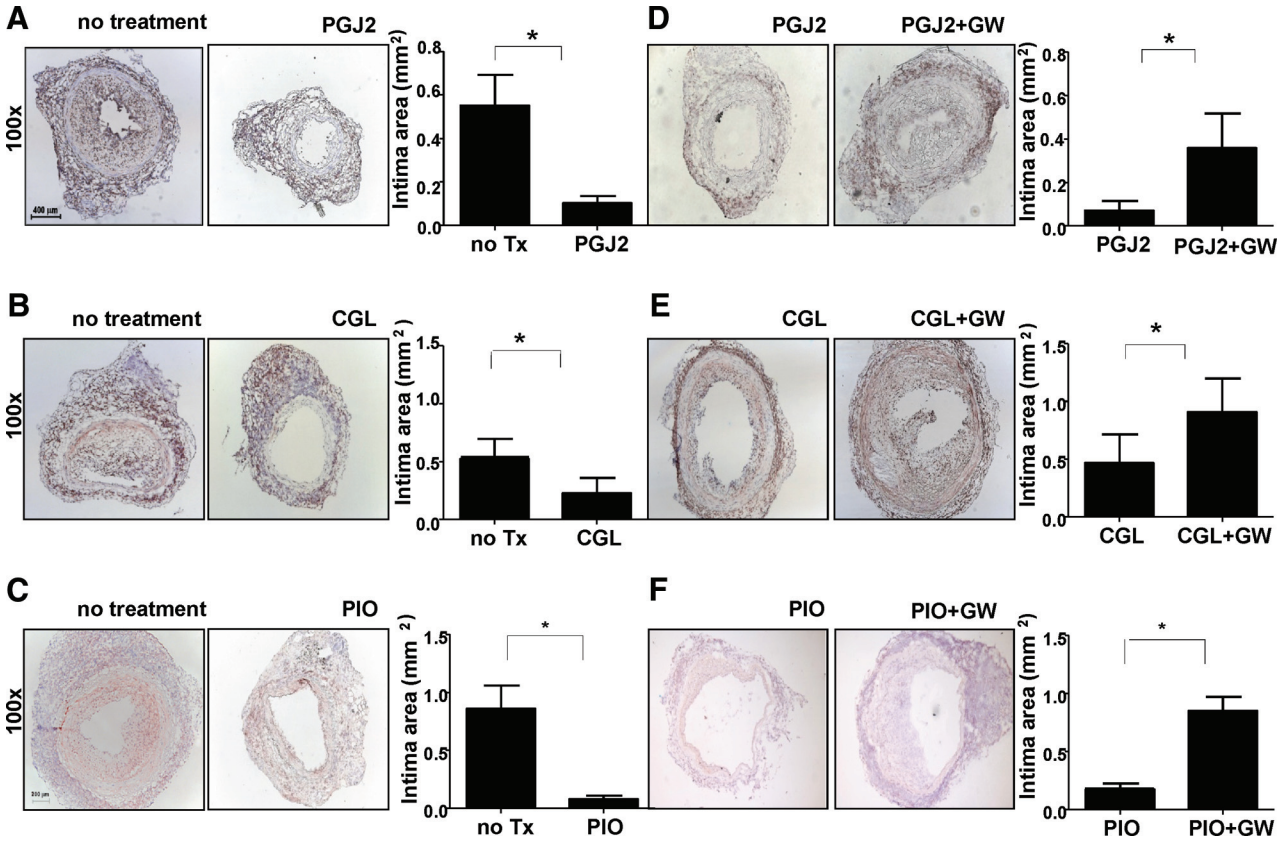

Figure 1. Neointima formation in human arterial allografts in severe combined immunodeficiency (SCID)/beige mice treated with peroxisome proliferator–activated receptor-γ (PPARγ) agonists alone or together with the PPARγ antagonist GW9662. Treatment with the PPARγ agonist PGJ2 (**A**) (scale bar=400 μm), ciglitazone (CGL; **B**), or pioglitazone (PIO; **C**) abolished the formation of arterial neointima induced by alloreactive T cells compared with untreated control group. Cotreatment with GW9662 reversed the inhibitory effects of PGJ2 (**D**), ciglitazone (**E**), or pioglitazone (**F**) (magnification ×100).

ciglitazone did not significantly affect the expression of HLA-DR (65±38 cells in untreated mice versus 39±11 cells in PGJ2 treated mice, n=6 pairs, P=0.185, Figure 3A and 3D; ciglitazone treatment, 69±32 cells versus 42±12 cells, n=4, P=0.104, Figure 3B). Pioglitazone treatment significantly reduced the number of HLA-DR–positive cells (78±36 cells in untreated mice versus 20±23 cells in pioglitazone-treated mice; n=8 pairs; P=0.005), and this

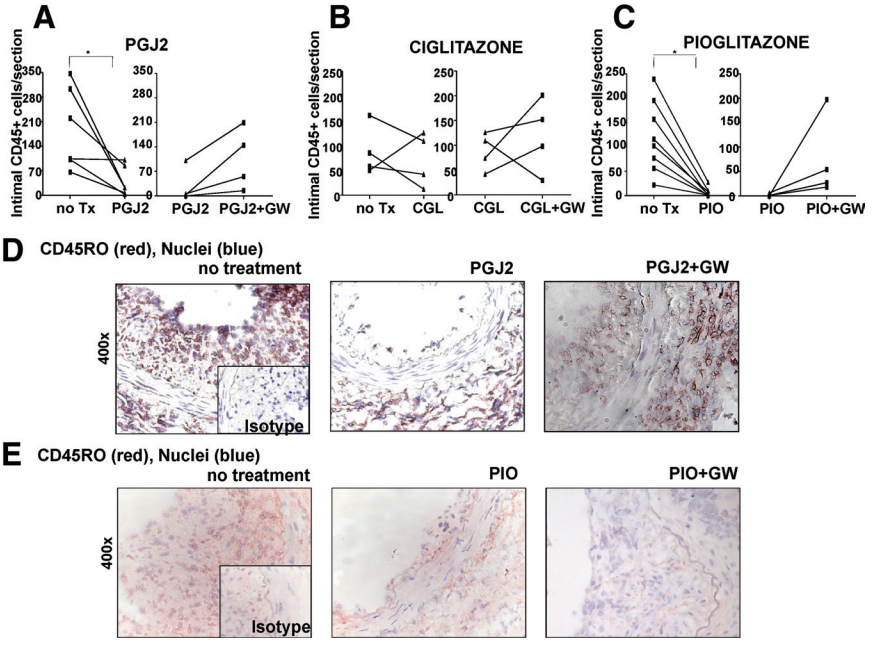

Figure 2. Effects of treatment with peroxisome proliferator–activated receptor-γ (PPARγ) agonists on infiltration of human CD45RO$^+$ cells into the neointima arterial graft. Infiltration of CD45RO$^+$ cells was reduced by PGJ2 (**A**) and pioglitazone (PIO; **C**) but not ciglitazone (**B**). Cotreatment with the PPARγ antagonist GW9662 partially reversed the protective effects of PGJ2 (**A**) and pioglitazone (**C**). The effects of PGJ2 and pioglitazone are shown in transverse sections of representative samples of human arterial grafts (**D** and **E**, respectively) that were stained with CD45RO (red) or relevant isotype controls (inset, left). The nuclei were counterstained with hematoxylin (blue) and quantified (magnification ×400).

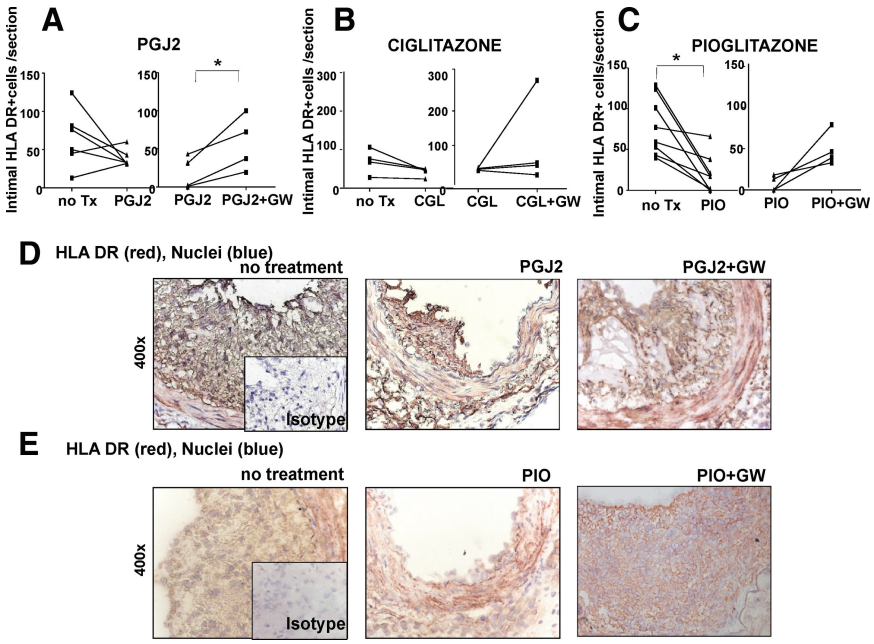

Figure 3. Effects of treatment with peroxisome proliferator–activated receptor-γ (PPARγ) agonists on the expression of HLA-DR in cells of human arterial graft. Expression of HLA-DR was not affected by PGJ2 (**A**) or ciglitazone (**B**) but was reduced by pioglitazone (PIO; **C**). Cotreatment with the PPARγ antagonist GW9662 reversed the effects of PGJ2 (**A**) and pioglitazone (**C**). Transverse sections of representative samples of human arterial grafts show the effects of PGJ2 (**D**) and pioglitazone (**E**) that were stained with HLA-DR (red) or relevant isotype control (inset, left). The nuclei were counterstained with hematoxylin (blue) and quantified (magnification ×400).

effect was reversed by GW9662 cotreatment (8±9 versus 49±20 cells; n=4 pairs; $P=0.05$; Figure 3C and 3E).

The evidence of T-cell infiltration of the neointima in untreated mice and inhibition of the infiltration was reproduced in grafts stained with immunofluorescent anti-CD3 antibody (Figure 4A and Figure I in the online-only Data Supplement). In the majority of untreated specimens, $CD3^+$ cells were not positive for HLA-DR staining (Figure 4A and Figure I in the online-only Data Supplement).

We then performed double staining of human arterial grafts with fluorochrome-labeled antibodies for HLA-DR and SMA (smooth muscle marker; Figure 4B) or CD31 staining (endothelial cell marker; n=4 pairs; Figure 4C). A remarkable quantity of HLA-DR–positive cells in the subendothelial layer of neointima of human arteries costained with SMA, suggesting that these are smooth muscle–like cells (Figure 4B). Pioglitazone treatment abolished the HLA-DR expression on SMA-positive cells (Figure 4B). Expression of HLA-DR was also observed on $CD31^+$ endothelial cells at the interface of the lumen and intima. Pioglitazone appears to reduce the expression of HLA-DR on these cells (Figure 4C).

Some of the cells in the graft expressed CD11c, a marker often used to identify dendritic cells. In the arterial allograft of untreated mice, however, CD11c was expressed by a subset of $CD3^+$ T cells (Figure 4D) that also expressed CD8 (data not shown). $CD8^+CD11c^+$ lymphocytes in murine models of viral infections appear to be activated cytotoxic, IFNγ-producing cells. In humans, $CD8^+CD11c^+$ lymphocytes are elevated in patients with chronic graft-versus-host disease,[29] but their function in allograft rejection is unknown. Pioglitazone abolished the presence of all $CD3^+$ lymphocytes in the intima of the arterial grafts (Figure 4A and 4D). No $CD56^+$ NK cells were found in the neointima in any of the samples examined (data not shown).

Effects of Peroxisome Proliferator–Activated Receptor-γ Agonists on Human T Cells in the Peripheral Blood of SCID/Beige Mice

The number of human $CD45^+$ (panleukocytic marker) cells in mouse peripheral blood was not altered by any of the drug treatments as detected by flow cytometry 4 weeks after adoptive transfer of human PBMCs (16.4±7% as percent of total leukocytes in PGJ2-treated animals versus 20.4±16.6% in untreated mice, n=6, $P=0.556$; 18.7±2.8 in ciglitazone-treated mice versus 28±27.2% in untreated mice, n=4, $P=0.549$; and 27.5±19.9% in pioglitazone-treated mice versus 19.8±11.6%, n=7 pairs, $P=0.18$ in untreated mice; Figure 5A through 5C). In mice treated with pioglitazone, we also evaluated human T-cell subpopulations. We found that pioglitazone treatment led to a slight but statistically significant increase in $CD4^+$ cells (31.6±10.2% of human $CD45^+$ cells in untreated mice versus 42.7±14.5%; n=7 pairs; $P=0.01$; Figure 5D) and a decrease in $CD8^+$ cells (67.7±11.4% of human $CD45^+$ cells in untreated mice versus 56.7±14.7%; n=7 pairs; $P=0.008$). GW9662 appeared to partly reverse these effects of pioglitazone, but the change did not reach statistical significance (data not shown). We also compared the number of $CD4^+CD25^+Foxp3^+$ regulatory T cells and observed no differences between treated and untreated engrafted mice ($CD4^+CD25^+Foxp3^+$ cells, 4.1±5.04% of human $CD4^+$ cells in untreated versus 3.6±3.3% in pioglitazone-treated mice; n=5; $P=0.76$).

To assess whether pioglitazone selectively affects any specific subpopulation of lymphocytes, we evaluated $CD4^+$ $CD45RO^+$ subpopulations circulating in mice inoculated with human PBMCs but not bearing artery grafts using the following markers: CXCR3 and CCR5 for Th1 cells, CCR4 and CCR3 for Th2 cells, and CCR4 and CCR6 for Th17 cells.[30] Pioglitazone treatment did not cause any significant change in the ratio of T-helper subpopulations (Th1, 51.04±22.3% of double-positive population in the untreated

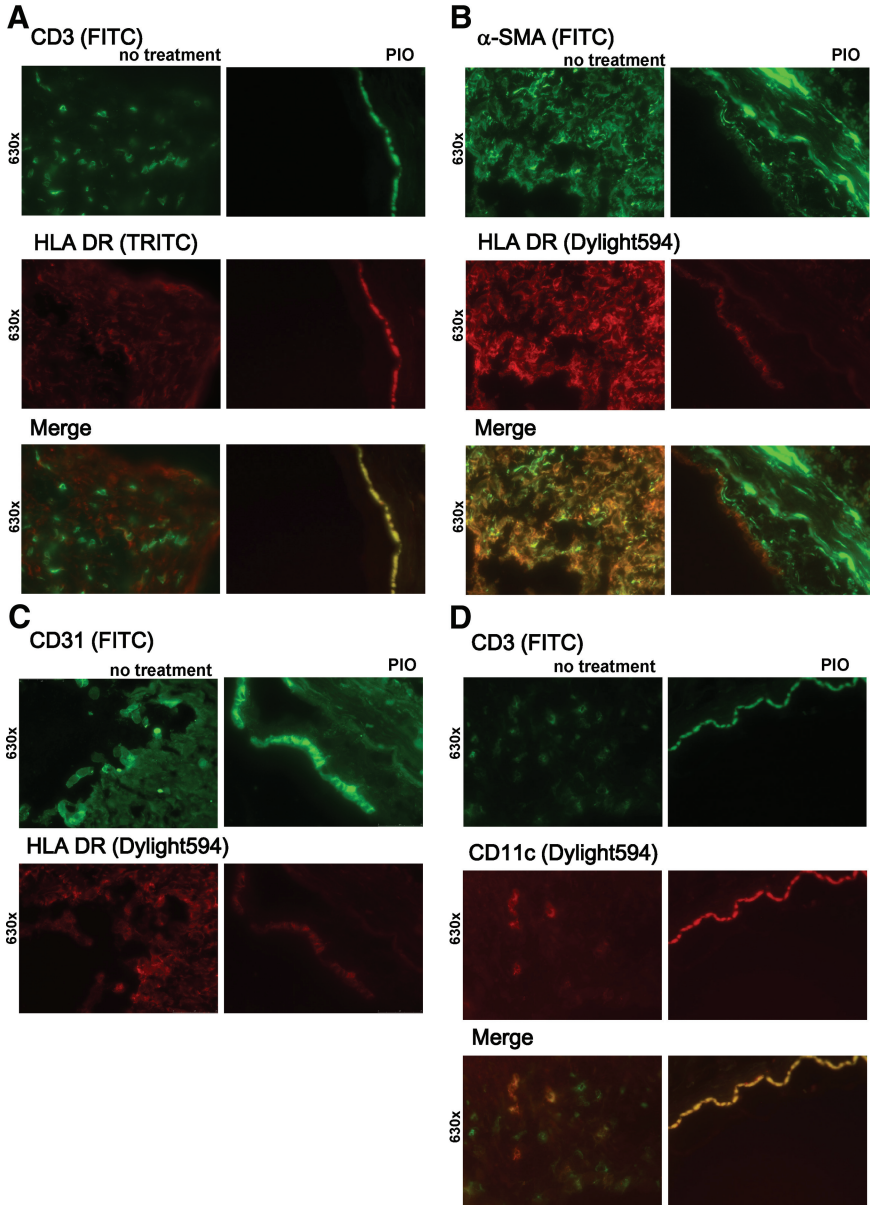

Figure 4. Effects of pioglitazone (PIO) on cells in human arterial grafts. **A**, Intima of human arteries of untreated mice was positive for CD3 and HLA-DR, but no colocalization was observed. Both markers were reduced after pioglitazone treatment. **B**, A remarkable number of α-actin–positive smooth muscle–like cells (α-SMA) are HLA-DR positive. Pioglitazone treatment reduced the numbers of α-actin–positive cells and abolished HLA-DR expression. **C**, HLA-DR expression was also present in areas of $CD31^+$ endothelial cells lining the arterial lumen, and pioglitazone treatment reduced the HLA-DR expression (magnification $\times$630). **D**, A subset of $CD3^+$ cells in the neointima show positivity for the CD11c marker. Pioglitazone treatment abolished the presence of all $CD3^+$ cells (Internal elastic lamina shows nonspecific staining in both the pioglitazone-treated and untreated groups; the internal elastic lamina is not visible in untreated samples because neointima occupies visual field).

versus 54.3±27.4% in the treated group, $P=0.5$; $Th2^+$, 55.05±34.3% in the untreated versus 43.7±30.2% in the treated group, $P=0.47$; $Th17^+$, 42.9±26.4% in the untreated versus 32.7±32.2% in the treated mice, $P=0.57$; all n=7 pairs).

Effects of Pioglitazone on Plasma Levels of Human Cytokines

To assess the effects of pioglitazone on soluble inflammatory mediators in vivo, we investigated the plasma of pioglitazone-treated engrafted mice or the untreated group. The effect of pioglitazone was assessed in both nonengrafted and engrafted mice. In engrafted mice, treatment with pioglitazone significantly decreased plasma levels of IFNγ ($P=0.02$), interleukin-10 ($P=0.005$), interleukin-2Rα ($P=0.03$), tumor necrosis factor-α ($P=0.03$), and macrophage inflammatory protein-1β ($P=0.0006$) and reduced osteopontin levels ($P=0.05$), whereas it caused elevation of plasma levels of transforming growth factor-β ($P=0.04$; Figure 6). In nonengrafted mice, pioglitazone had no effect on levels of human cytokines (Figure II in the online-only Data Supplement). None of the human cytokines examined were detected in the plasma of engrafted or nonengrafted mice not inoculated with PBMCs, excluding the possibility of cross-reactivity with inflammatory mediators of murine origin.

Effect of Pioglitazone on T-Lymphocyte Responses In Vitro

Human T cells transmigrate across endothelial cell monolayers under conditions of flow in response either to chemokines presented on the apical surface of the endothelial cell or to antigens presented by endothelial cells that are recognized by the T-cell receptor (TCR) for antigen; TCR signals inhibit chemokine-driven trans endothelial migration (TEM) and activate a transmigratory program that depends on distinct

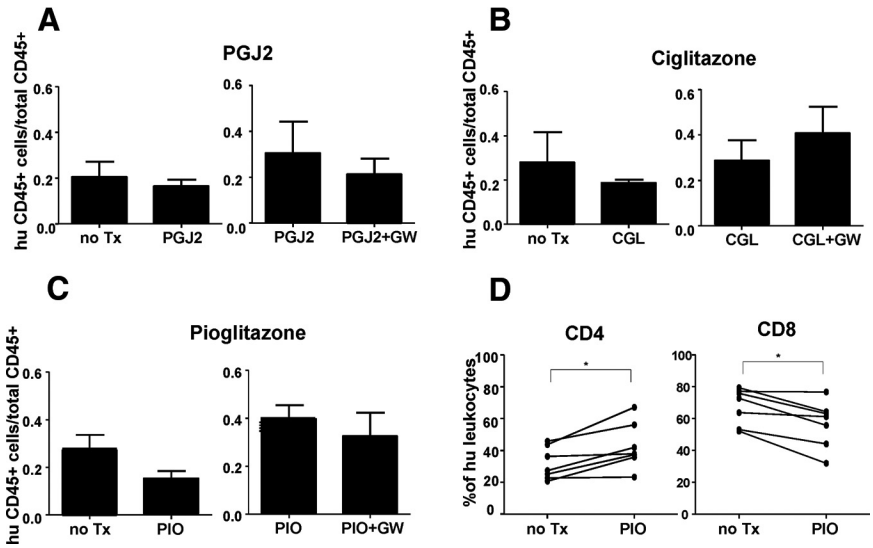

Figure 5. Effect of peroxisome proliferator–activated receptor-γ (PPARγ) agonists on human T cells circulating in mouse peripheral blood. The level of reconstitution was evaluated by the number of human $CD45^+$ cells (panleukocytic marker) in mouse peripheral blood. **A** through **C**, Treatment with PPARγ agonists or antagonist did not significantly affect the numbers of human $CD45^+$ cells. **D**, Pioglitazone (PIO) induced an increase in $CD4^+$ and a decrease in $CD8^+$ human T-cell numbers in peripheral blood of the engrafted mice.

endothelial cell junctional proteins and lymphocyte receptors.[23,31–33] Both rapid chemokine and TCR-dependent TEM are restricted to the effector memory $CD4^+$ or total (both central and effector) memory $CD8^+$ T-cell populations (Manes and Pober[23] and personal observations, T.D.M.). We investigated whether pioglitazone affected TCR- and chemokine-driven TEM using an in vitro flow assay with freshly purified human effector memory $CD4^+$ T cells ($CD4^+CD45RA^-$, $CCR7^{low}$, $CD62L^{low}$) and total memory $CD8^+$ T cells ($CD8^+CD45RA^-$). The endothelial cell monolayers were formed by human coronary artery endothelial cells that had been transduced with CIITA (to reinduce expression of MHC II), activated by tumor necrosis factor-α (to induce the expression of adhesion molecules and chemokines necessary to capture flowing T cells), and overlaid with the superantigen TSST-1 (to selectively engage the TCR of those T cells that used a Vβ2 segment to form their TCR, typically ≈5% to 20% in most individuals). Induction of MHC II is necessary to bind and present the superantigen to T cells. T cells that do not have the Vβ2TCR (referred to as $VB2^-$ cells in this report) respond to chemokines presented on the endothelial cell apical surface and transmigrate within 15 minutes, whereas the $V\beta2TCR^+$ cell response to chemokine is blocked and instead undergoes TCR-dependent TEM ≈30 minutes later.[23] Strikingly, pioglitazone treatment of T cells inhibited TCR-driven TEM but not chemokine-driven TEM (Figure 7).

In vitro proliferation of memory $CD45RO^+$ or $CD4^+CD45RO^+$ cells in response to human umbilical vein endothelial cells was reduced by pioglitazone in a dose-dependent manner. At

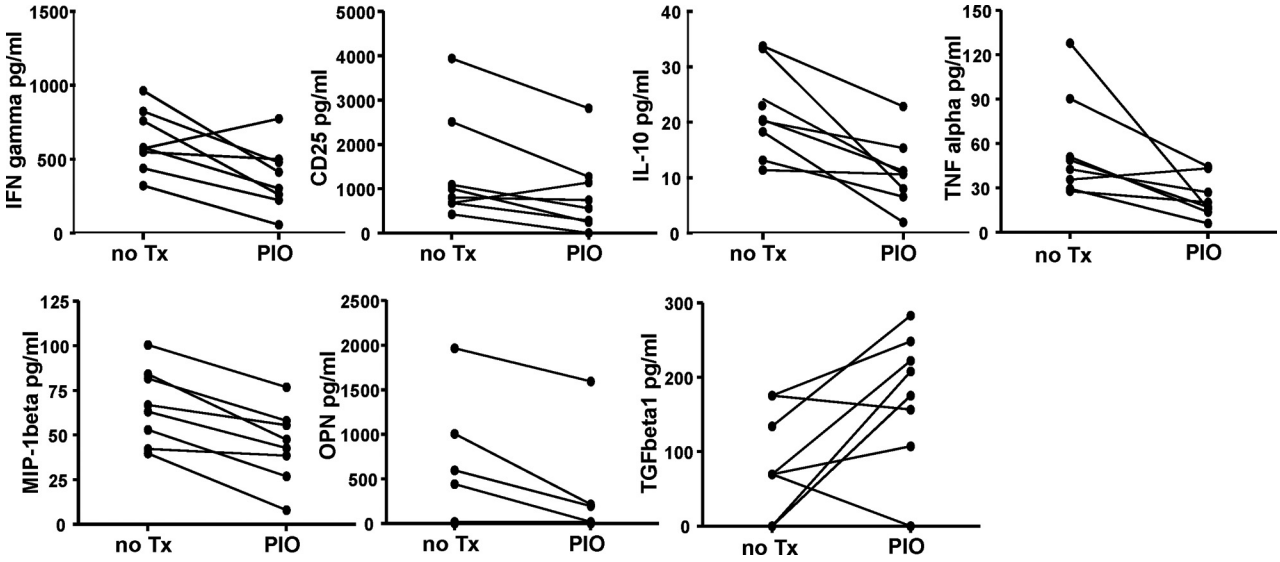

Figure 6. Effects of pioglitazone (PIO) on the levels of human cytokines in mouse plasma. Plasma levels of human cytokines in pioglitazone-treated mice showed a significant decrease in interferon-γ (IFNγ), interleukin (IL)-10, IL-2Rα, macrophage inflammatory protein-1β (MIP-1β), tumor necrosis factor-α (TNFα), and osteopontin (OPN) compared with untreated mice (no Tx), whereas the levels of transforming growth factor-β (TGF-β) were increased. No significant difference between the untreated and pioglitazone-treated groups was found for other investigated cytokines such as epidermal growth factor, granulocyte-macrophage colony stimulating factor, IL1-α, IL1-β, IL-1R antagonist, IL-2, IL-13, IL-17, or monocyte chemoattractant protein-1.

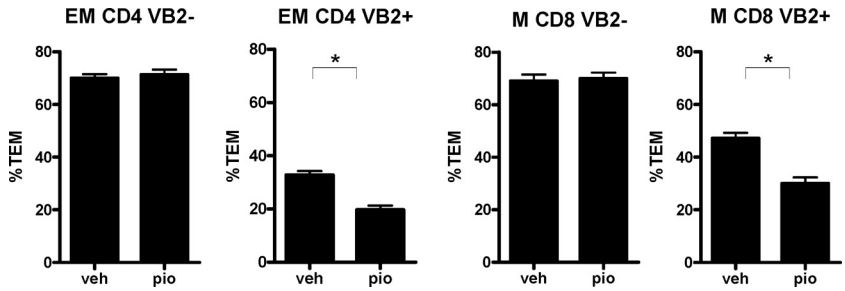

Figure 7. Pioglitazone inhibits transendothelial migration of T-cell receptor–driven T effector memory (EM) cells. The TEM assay assessed under conditions of venular shear stress effector memory and memory (M) CD8 T cells treated overnight with vehicle (veh) or 10 μmol/L pioglitazone (pio) on CIITA-transduced human coronary artery endothelial cells treated with tumor necrosis factor and overlaid with TSST-1 superantigen. Graphs show the percentage of transmigrating TCR-driven ($VB2^+$) and chemokine-driven ($VB2^-$) T cells (results of 3 independent experiments). *$P<0.001$.

the highest concentration (10 μmol/L), the inhibition was 38% compared with vehicle-treated controls (total memory T cells, 61.92±10.91% versus 100%, n=4, $P=0.006$; CD4 memory cells, 69.9±6% versus 100% vehicle control, n=3, $P=0.01$). Similar observations were made for CD4 or CD8 lymphocyte proliferation by carboxyfluorescein succinimidyl ester dye dilution (percent of CD4 cells, 1.65±0.4% versus 2.86±1.03%, n=2, $P=0.056$; percent of CD8 cells, 3.32±1.24% versus 5.3±2.5%, n=2, $P=0.045$). Analysis of the expression of CD25, CD69, HLA-DR, or CTLA4 on CD4 and CD8 memory subpopulations by fluorescence-activated cell sorter did not show any significant differences in the pioglitazone-treated group compared with vehicle controls at day 1 or 3 of coculture (data not shown). There was also no statistically significant difference in apoptosis of these T cells as assessed by annexin V and propidium iodide staining (data not shown).

Discussion

In this study, we show that in vivo remodeling of human arteries induced by alloreactive memory T lymphocytes can be prevented by treatment with PPARγ agonists, including endogenous eicosanoid, PGJ2, and 2 synthetic PPARγ agonists, namely ciglitazone (a prototypical compound for the thiazolidinedione class of drugs) and pioglitazone (an analog of ciglitazone that is currently 1 of 2 PPARγ agonists available for clinical use).[7,8] The effects of all 3 agonists were reversed by the antagonistic ligand GW9662.

In the humanized mouse model of vascular rejection, thickening of the arterial intima results from infiltration of memory CD4 and CD8 T lymphocytes into the subendothelial layer of the artery.[20] Memory T cells formed during previous encounters with microbial antigens may cross-react with allogeneic MHC/peptide complexes of the graft with high frequency,[34] which correlates with rejection rates.[35] A subpopulation of IFNγ-producing effector memory T cells recognize allogeneic endothelial cells and cause allograft injury.[13] Preexisting alloreactive memory T cells are resistant to induction of tolerance[36] and thus represent a significant therapeutic challenge. Our data suggest that PPARγ agonists may be a useful addition to the current therapeutic regimen for vascular graft rejection by targeting these alloreactive human memory T cells.

A key issue raised by our study is how PPARγ agonists regulate the human allogeneic immune response. The absence of T cells in the treated specimens prevented us from investigating alterations in T-cell subpopulations directly at this site. Pioglitazone caused a slight but statistically significant elevation in the number of $CD4^+$ cells and a reciprocal decrease in the number of $CD8^+$ T cells in the circulation of treated mice. These adoptively transferred human T cells express memory T-cell markers, and the circulating $CD4^+$ T cells can be further subdivided into different T-helper-cell subsets based on patterns of chemokine receptor expression. Pioglitazone did not appear to alter the ratio of these helper subsets of circulating $CD4^+$. In animals bearing arterial grafts, pioglitazone reduced the circulating levels of human cytokines. Although circulating cytokines were also detected in animals that lacked arterial grafts but received human PBMCs, pioglitazone treatment did not reduce cytokines in these animals. This observation suggests that the primary effect of pioglitazone was inhibition of the activation of T cells that were responding to alloantigen. Our in vitro experiments are consistent with this interpretation. Direct alloresponses and transmigration of TCR-driven but not chemokine-driven CD4 T cells across an endothelial cell monolayer were inhibited by pioglitazone. Another critical parameter of T-cell immunity is the regulatory T-lymphocyte subpopulation. These cells inhibit vascular graft disease,[37] and PPARγ and its agonists are important in their function.[38,39] We quantified the number of $CD4^+CD25^{high}Foxp3^+$ regulatory T cells. Pioglitazone did not affect the number of these cells in peripheral blood, and the low frequency of these cells in peripheral blood of mice made it impossible to evaluate these cells functionally.

In clinical studies, treatment of arterial inflammatory disorders with pioglitazone was demonstrated to reduce the progression of carotid intima-media thickening,[40] in-stent neointima formation,[41] and coronary artery disease.[42] The inhibitory effects of pioglitazone have been believed to be related mainly to improvement in metabolic parameters, particularly decreased levels of triglycerides and increased levels of high-density lipoprotein cholesterol.[43] Comparison of the effects of pioglitazone and glimepiride on carotid intima-media thickness in recent studies suggested that the reduction was independent of glycemic control in patients and was related to soluble inflammatory markers in human serum, including monocyte chemotactic factor-1, tissue plasminogen activator, high-sensitivity C-reactive protein, and matrix metalloproteinase-9.[44,45] Recent reports and our in

vivo experimental data raise the possibility that the protective effects of this agent may be related to prevention of human T-cell infiltration into the vascular wall.

Intimal expansion in graft arteries results in lumen loss that cannot be adequately compensated for by vascular remodeling. Vascular resistance in conduit arteries and the corresponding loss of organ perfusion increase linearly with the length of the stenotic region and inversely with the radius of the lumen to the fourth power. Unlike discrete atheromas in which luminal narrowing must be extensive (>70%) to be clinically significant, transplant recipients develop long, contiguous regions of stenosis that can impair organ perfusion with lesser degrees of luminal narrowing. Consequently, a small reduction in intimal expansion over the whole length of the vessel with only a correspondingly small increase in the lumen may significantly increase graft perfusion. The large reductions in intimal expansion observed in our study using PPARγ agonists, especially pioglitazone, could have a very significant impact on transplant recipients even if only partly replicated in the clinical setting.

Many effects induced by PPARγ agonists may be mediated via PPARγ-independent mechanisms.[46] To confirm that the protective effects of PPARγ agonists are due to activation of PPARγ, we used GW9662, the irreversible antagonist of PPARγ. The suppressive effects of PGJ2, ciglitazone, and pioglitazone on neointima formation were reversed by cotreatment with GW9662, suggesting the involvement of PPARγ-driven mechanisms.

Conclusions

The protective effects of PPARγ agonists in human graft vasculopathy are in accord with findings in rodent models[4,5] despite significant differences in the mechanism of human and mouse graft vasculopathy.[12–19] The present study represents an important link in the "bench to bedside" process[47] in which discoveries in conventional rodent models often cannot be directly extrapolated to human diseases. Our findings suggest that PPARγ agonists have the capacity to suppress memory alloreactive T cells responsible for vascular graft rejection via a PPARγ-dependent mechanism. Interestingly, in the present study, we show complete prevention of neointima formation by pioglitazone; in our previous study testing the effects of statins,[48] we reported a reduction in but not complete inhibition of T cell–driven arterial remodeling. The PPARγ agonists are approved for treatment of type 2 diabetes mellitus. The combination of antiinflammatory and antidiabetic effects may be particularly useful for the management of vascular graft rejection in patients who develop posttransplantation diabetes mellitus.[49] Lastly, T-cell infiltration drives the pathogenesis of other arterial inflammatory disorders, and the observations made in the present study may be relevant to the investigation of the effects of PPARγ agonists on other types of arteritis or T cell–driven inflammatory disorders. Benefits of these drugs compared with existing antiinflammatory therapies and their use in combination therapies need to be established.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Human allograft rejection differs from that which occurs in rodent models owing to differences in the immune properties of graft cells, especially of endothelial cells, and to the presence in humans of memory T cells that can respond to and reject an allograft. Consequently, therapies that work in rodents often fail in humans. Peroxisome proliferator–activated receptor-γ agonists, in addition to their role in lipid metabolism, can suppress inflammation and immunity. We show in this study that these agents can suppress the responses of human memory T cells to allogeneic blood vessels in vivo and to allogeneic endothelial cells in vitro. These data suggest the possibility that peroxisome proliferator–activated receptor-γ agonists could have benefit in clinical transplantation.