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PPARγ antagonist attenuates mouse immune-mediated bone marrow failure by inhibition of T cell function

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ABSTRACT

Acquired aplastic anemia is an immune-mediated disease, in which T cells target hematopoietic cells; at presentation, the bone marrow is replaced by fat. It was reported that bone marrow adipocytes were negative regulators of hematopoietic microenvironment. To examine the role of adipocytes in bone marrow failure, we investigated peroxisomal proliferator-activated receptor gamma, a key transcription factor in adipogenesis, utilizing an antagonist of this factor called bisphenol-A-diglycidyl-ether. While bisphenol-A-diglycidyl-ether inhibited adipogenesis as expected, it also suppressed T cell infiltration of bone marrow, reduced plasma inflammatory cytokines, decreased expression of multiple inflammasome genes, and ameliorated marrow failure. *In vitro*, bisphenol-A-diglycidyl-ether suppressed activation and proliferation, and reduced phospholipase C gamma 1 and nuclear factor of activated T-cells 1 expression, as well as inhibiting calcium flux in T cells. The *in vivo* effect of bisphenol-A-diglycidyl-ether on T cells was confirmed in a second immune-mediated bone marrow failure model, using different strains and non-major histocompatibility antigen mismatched: bisphenol-A-diglycidyl-ether ameliorated marrow failure by inhibition of T cell infiltration of bone marrow. Our data indicate that peroxisomal proliferator-activated receptor gamma antagonists may attenuate murine immune-mediated bone marrow failure, at least in part, by suppression of T cell activation, which might hold implications in the application of peroxisomal proliferator-activated receptor gamma antagonists in immune-mediated pathophysiologies, both in the laboratory and in the clinic. Genetically "fatless" mice developed bone marrow failure with accumulation of marrow adipocytes in our model, even in the absence of body fat, suggesting different mechanisms of systematic and marrow adipogenesis and physiologic *versus* pathophysiologic fat accumulation.

Introduction

Aplastic anemia (AA) is the paradigmatic bone marrow (BM) failure syndrome in humans.[1,2] AA behaves as an immune-mediated disease in most patients: activated cytotoxic T cells and type I cytokines destroy hematopoietic stem and progenitor cells, resulting in pancytopenia and absence of hematopoietic precursors in the BM.[1,2] The BM of patients with AA is typically described as "empty", but in reality the hypocellular marrow space is occupied by fat, and specifically increased numbers of large adipocytes.[3] BM adipocytes in AA have been assumed to passively

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occupy marrow and to be metabolically inert under most physiological conditions.[4] Recently, evidence has been presented to support the notion that BM adipocytes might play a central function in regulating hematopoiesis.[4-6] Gene expression profiles suggest that mouse BM adipocytes possess a phenotype functionally distinct from extramedullary fat cells:[6] for example, inflammatory response genes, such as *Tnfa, Il6,* and *Tgf1*, are highly expressed, while expression of adipose-specific genes is low.[6] In one prominent report, pharmacological inhibition

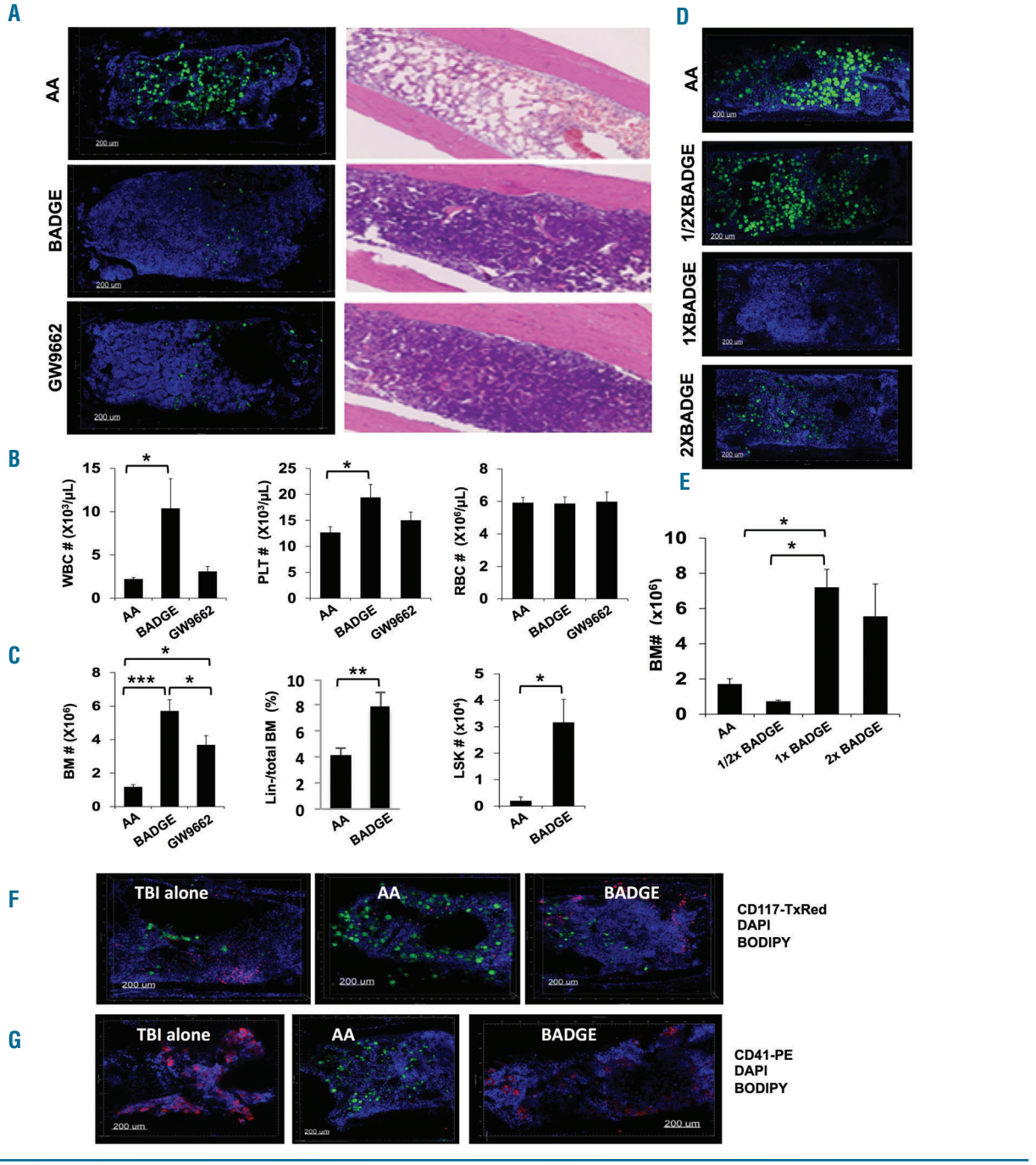

Figure 1. PPARγ antagonists ameliorate immune-mediated BM failure in AA mice. (A) Effects of PPARγ antagonists on BM adipocytes and cellularity in AA mice. In confocal imaging (left panels), adipocytes and nuclei in sternums were stained with BODIPY (493/503 nm, green) and DAPI (405 nm, blue). Right panels show H&E staining of mouse femurs in different groups. Representative images of AA (n=16), BADGE-treated (n=16), GW9662-treated (n=10) are shown. Blood counts (B) and BM total nucleated cell numbers of AA (n=16), BADGE-treated (n=16), and GW9662-treated (n=10), frequency of BM lineage negative cells (Lin^-) and absolute number of $Lin^-Sca1^+c\text{-}kit^+$ (LSK) cells (C) of AA (n=7), BADGE-treated mice (n=7). Dose response to BADGE on BM adipogenesis in confocal imaging (D) and on BM total nucleated cell numbers (E) in AA (n=6), low dose BADGE (15 mg/kg, n=6), standard dose BADGE (30 mg/kg, n=8), and high dose BADGE (60 mg/kg, n = 6) treated mice. All results shown are at 2 weeks. All error bars indicate SEM. **P*<0.05; ***P*<0.01; ****P*<0.001. BM hematopoietic stem and progenitor cells (HSPCs, CD117-Texas Red) (F), megakaryocytes (CD41-PE) (G), and adipocytes (BODIPY, green) in TBI only, AA control mice, and BADGE-treated mice were visualized by confocal imaging of sternums at 2 weeks in representative images. Original magnification, x100 (A left, D, F, and G); x40 (A right).

of peroxisomal proliferator-activated receptor gamma (PPARγ), a master regulator of adipogenesis, enhanced BM engraftment in mice after BM transplantation.[7]

PPARγ is a family of ligand-activated nuclear receptor transcription factors. There are two distinct isoforms, PPARγ1 and PPARγ2.[8-10] PPARγ2 is exclusively expressed in adipose tissue, while PPARγ1 is ubiquitously expressed at lower levels in other tissues, such as skeletal muscle, liver, breast, prostate, and colon.[8-12] PPARγ plays a critical role in lipid metabolism and also in immune function, cell growth, differentiation, and apoptosis.[8-15] In particular, cells of the immune system, such as normal macrophages, dendritic cells, eosinophils, T cells and B cells, also express PPARγ. Collectively, these findings prompted us to revisit the role of adipogenesis in BM failure and the possibility of PPARγ as a therapeutic target in AA.

In this study, we investigated the roles of bisphenol-A-diglycidyl-ether (BADGE, a PPARγ antagonist) in our AA mouse model. BADGE not only suppressed BM adipogenesis, but also inhibited T cell infiltration of BM and improved the marrow inflammatory environment, eventually ameliorating pancytopenia and BM destruction. *In vitro* BADGE suppressed T cell activation and proliferation, and reduced T-cell cytokine secretion. We also tested the antagonist in a second immune-mediated BM failure murine model, using different strains and non-major histocompatibility (non-MHC) mismatched. Unexpectedly, we observed the accumulation of BM adipocytes in genetically "fatless" mice in our marrow failure model.

Methods

Mice

Inbred C57BL/6 (B6, $H2^{b/b}$), DBA/1J (DBA/1, $H2^{q/q}$), FVB/NJ (FVB, $H2^{q/q}$) mice and congenic C.B10- $H2^{b/b}$/LilMcd (CB10, $H2^{b/b}$) mice

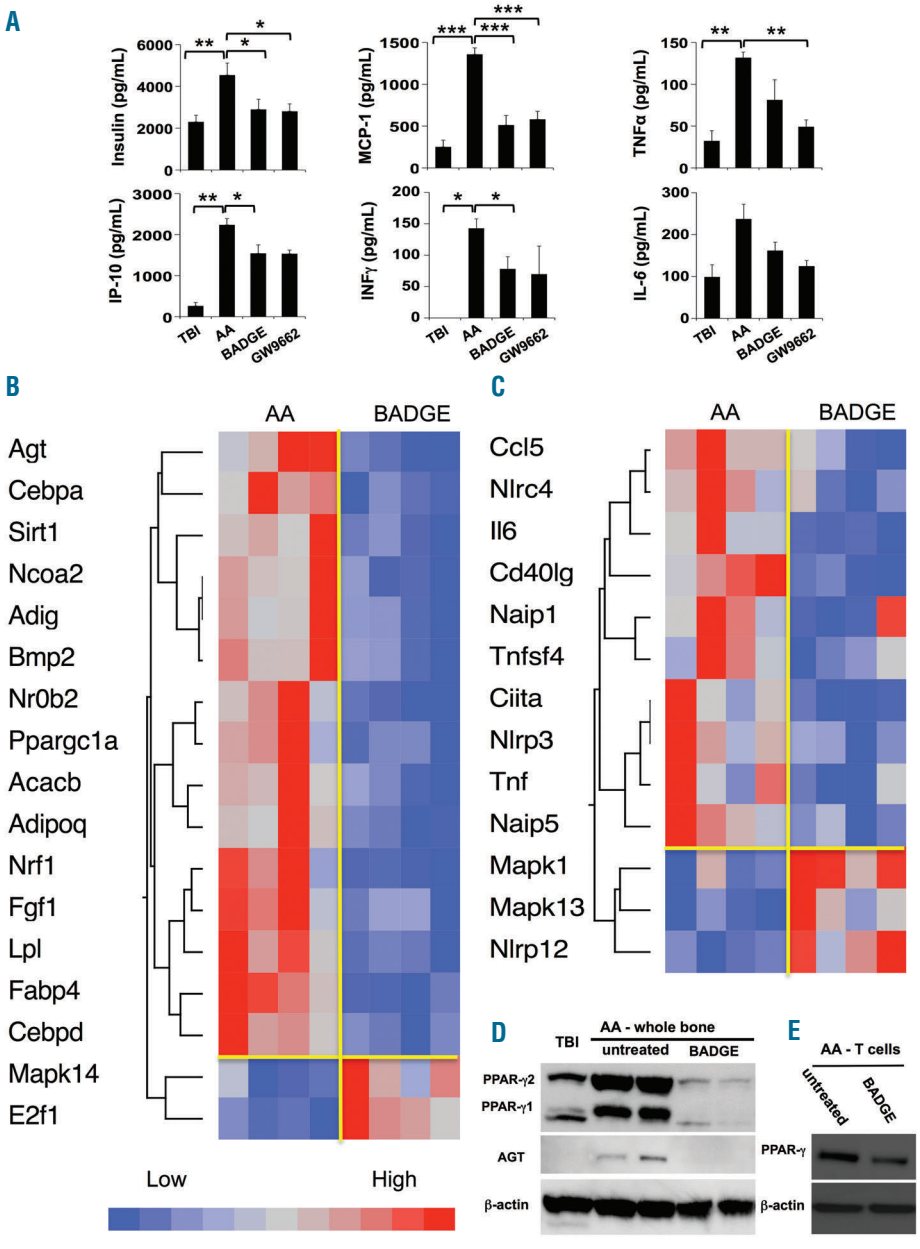

Figure 2. Alterations of inflammation and adipogenesis by PPARγ antagonists. (A) Inflammation, Th1 and adipogenesis related cytokines in AA mice treated with PPARγ antagonists. Plasma samples were collected at 2 weeks, and were divided for each of two different MILLIPLEX MAP Mouse Magnetic Bead Panels in order to measure cytokine concentrations. All error bars indicate SEM of TBI alone (n=5), AA (n=8), BADGE-treated (n=8), GW9662-treated (n=5) mice. *P<0.05; **P<0.01; ***P<0.001. Quantitative analysis of mRNA expression of adipogenesis- (B), and inflammasome- (C) related genes in whole femurs showing more than 2-fold changes in BADGE-treated AA mice (n=4), compared with untreated AA mice (n=4) at 2 weeks. Blue represents low expression, red indicates high expression. Each column of the heat map represents one individual mouse. (D) Immunoblot to validate protein levels of selected adipogenesis-related genes PPARγ and AGT obtained from PCR arrays in whole femurs. (E) Protein levels of PPARγ in T cells from BM of AA mice and BADGE-treated AA mice. β-actin was used as a loading control. Representative images of at least three independent experiments are shown.

were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). FVB.A-ZIP/F1 (A-ZIP/F) "fatless" mice and their wild-type littermates were kindly provided by Dr. Charles Vinson (NCI, NIH). All mice were bred and maintained at NIH animal facilities under standard care and nutrition. Female mice aged from 8 to 12 weeks were used in each experiment. All animal studies were approved by the National Heart, Lung, and Blood Institute's Animal Care and Use Committee.

AA mouse models

AA mice were generated through immune-mediated BM failure as previously described.[16,17] Briefly, recipient CB10 mice were exposed to 5 Gy of total body irradiation (TBI). Lymph node (LN) cells were obtained from B6 donors, and infused into recipients by tail vein injection at 5.0 x 10^6 cells per mouse. Wild-type FVB and A-ZIP/F1 "fatless" AA mice were created as was the CB10 AA model, except that recipient FVB or A-ZIP/F "fatless" mice were irradiated at 6.8 Gy and injected with LN cells from MHC H2-matched donor DBA/1 mice at 10 x 10^6 per mouse.

Treatment of mice

PPARγ antagonists, BADGE or GW9662 (Sigma, St. Louis, MO, USA), were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. The aliquots were diluted with PBS to a final concentration of 10% DMSO and administrated by daily intraperitoneal injection at 30 mg/kg for BADGE, or at 1 mg/kg for GW9662, from one day prior to the experiment and continued for up to 2 weeks. In the FVB AA model, some mice were injected with cyclosporine A (CsA, 50 mg/kg/day, Sigma) starting 1 hour after the LN injection, and continued for 5 days as immunosuppression. At the end of the experiments, the mice were euthanized by CO_2 inhalation.

Methods, including peripheral blood (PB) and BM cell counting, flow cytometry, RNA isolation and gene expression analysis by PCR array, protein extraction and immunoblotting, cytokine measurement, histology using confocal microscopy, calcium flux assay and cell culture are detailed in the *Online Supplementary Methods*.

Statistics

To compare gene expression in adipogenesis and inflammasome pathways between AA and BADGE-treated AA mice, two-way hierarchical cluster analysis was performed for genes that had more than 2-fold changes, based on PCR array data using Ward's method (JMP version 10.0.0, SAS Institute, Cary, NC, USA). Differences of CBCs, total BM cell number, cytokines, and flow cytometry data between different groups were analyzed by student's t-test, Mann-Whitney test, or one-way ANOVA test using Prism software (GraphPad Software, La Jolla, CA, USA). Significant difference was set at $P < 0.05$ for all the statistical tests. Data were expressed as mean ± SEM.

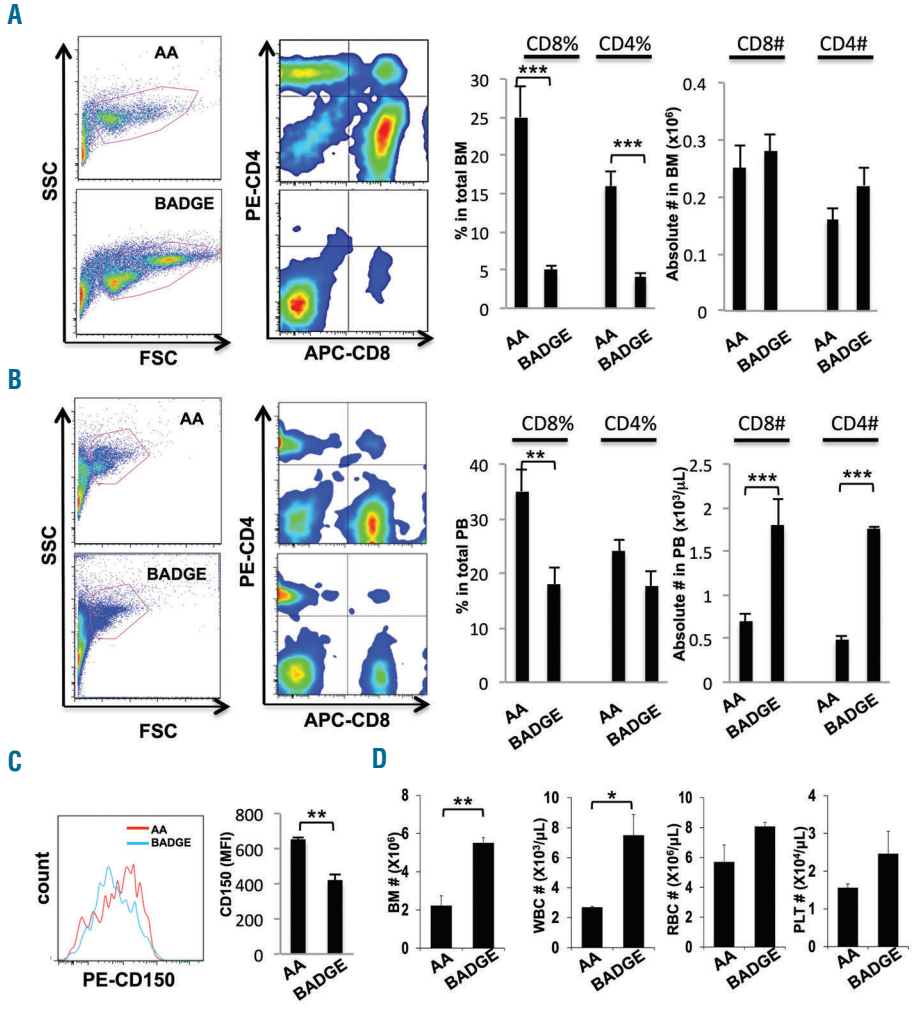

Figure 3. T cell activation in BADGE-treated AA mice. Altered $CD4^+$ and $CD8^+$ T cells in BM (A) and PB (B) in BADGE-treated AA mice. Representative data at 2 weeks are shown. Data were gated on all nucleated cells. Results of percentage and absolute numbers are summarized beside the flow cytometry graphs. AA (n=15), BADGE-treated (n=16). (C) Comparison of CD150 expression on BM T cells in BADGE-treated and control AA mice. The histograms were gated on viable $CD3^+$ T cells. Representative data of at least 3 separate experiments are shown. (D) The effect of delayed BADGE treatment on PB cell count and BM hematopoiesis. AA (n=6), BADGE-treated (n=7). *P<0.05; **P<0.01.

Results

PPARγ antagonists ameliorated pancytopenia and BM destruction in AA mice

Naveiras *et al.'s* study suggested that adipocytes were negative regulators of hematopoiesis.[7] We speculated that PPARγ antagonists could ameliorate the immune-mediated marrow failure model by inhibiting adipogenesis. We induced BM failure by the injection of B6 LN cells into sublethally irradiated CB10 recipients, which were matched at MHC H2 antigens but differed in multiple minor histocompatibility antigens (miHAs). In this adaptation of "runt" disease, all mice uniformly develop progressive and fatal pancytopenia, accumulating a large number of adipocytes in the BM - closely resembling human AA, and without evidence of graft-*versus*-host disease.[16,17] BM destruction in this model is mediated by miHA H60-reactive T cells.[17] We treated recipient mice with PPARγ antagonists BADGE or GW9662, or control vehicle. On day 14, mice were sacrificed and evaluated in PB by cell counts

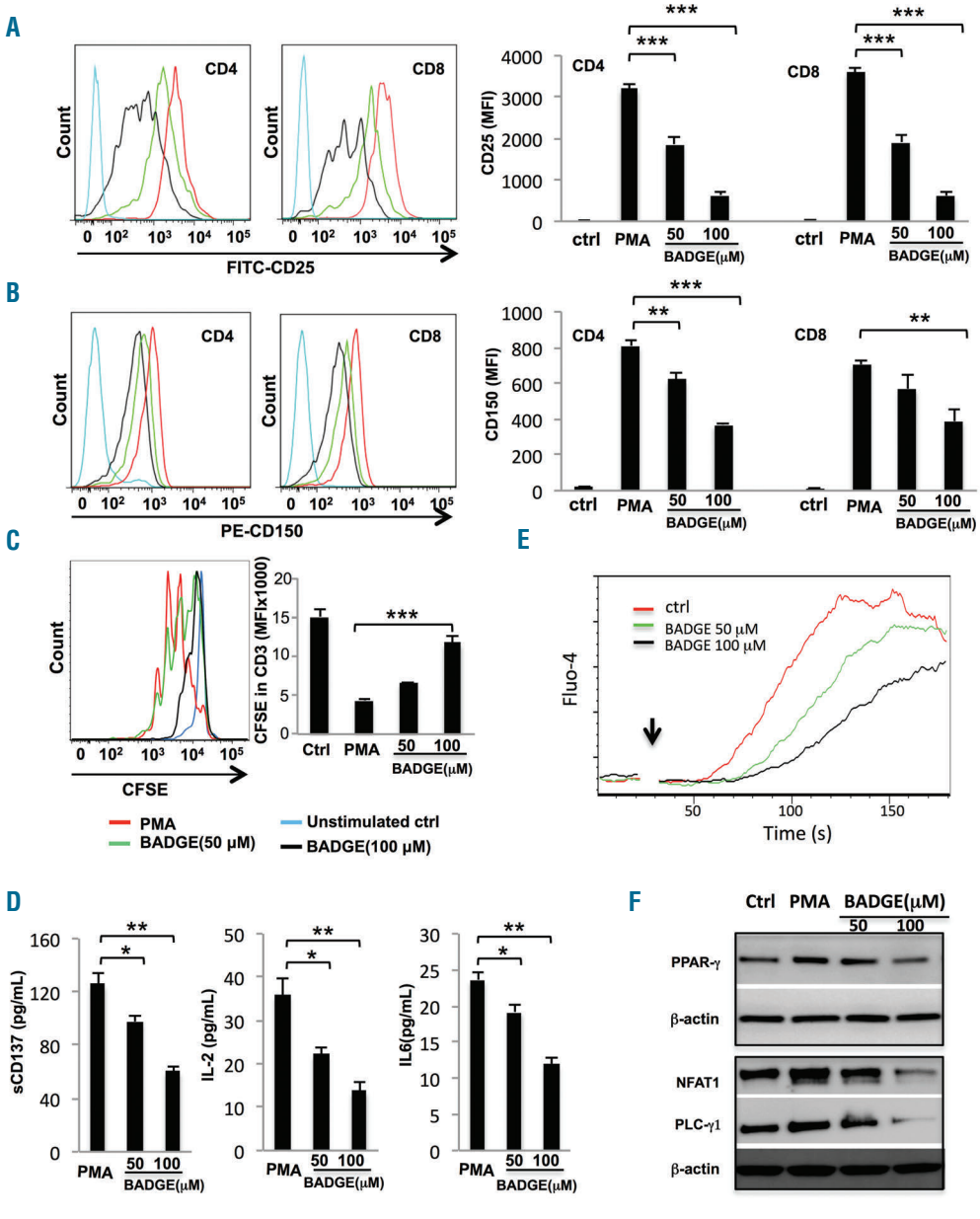

Figure 4. T cell activation and proliferation are inhibited by BADGE. Dose response of BADGE on CD25 (A) and CD150 (B) expression of $CD4^+$ and $CD8^+$ T cells. Mouse lymph node T cells were stimulated with PMA (5 ng/ml) and ionomycin (500 nM) in the presence of BADGE (50 or 100 μM) or control vehicle DMSO for 16 hours. (C) Dose response of BADGE on proliferation of $CD3^+$ T cells. Mouse T cells were labeled with CFSE and stimulated with PMA (5 ng/ml) and ionomycin (500 nM), and then incubated with BADGE (50 or 100 μM) or control vehicle DMSO for 3 days. Statistic analysis of flow cytometry data is shown on the right. All the data were gated on viable cells ($7AAD^-$). (D) T cell related cytokines in the supernatants of PMA-stimulated T cell culture in the presence of BADGE (50 or 100 μM) or control vehicle DMSO for 16 hours (n=5 for each group). (E) Calcium influx is altered by BADGE. Mouse LN cells were cultured with or without BADGE (50 or 100 μM) for 4 hours, and were then loaded with Fluo-4 at 37°C for 30 minutes. The cells were added to biotinylated anti-CD3 antibodies, and crosslinked with streptavidin at 20 sec of the analysis (arrow) to initiate calcium flux. (F) Protein levels of PPARγ, PLCγ1, and NFAT1 in PMA-stimulated T cells in the presence or absence of BADGE for 16 hours. β-actin was used as a loading control. Representative results (A, B, C, E) or images (F) of at least three experiments are shown.

and in BM by estimating cellularity and morphologic examination of marrow adipocytes.

Confocal microscopy showed massive expansion of adipocytes in the BM of AA mice. Overall BM cellularity, estimated by the density of 4',6-diamidino-2-phenylindole (DAPI) staining (nuclear, blue color), was very low. In contrast, mice treated with PPARγ antagonists had many fewer adipocytes (green color, boron-dipyrromethene dye -BODIPY) in the BM and much higher marrow cellularity (Figure 1A, left). By conventional staining, the BM structure of AA mice showed extensive disruption and replacement by adipocytes, which occupied the "empty" space, whereas the BM of BADGE- or GW9662-treated mice showed less empty space and more hematopoietic cellularity (Figure 1A, right). Blood leukocyte and platelet counts were higher in BADGE-treated mice compared with AA mice (Figure 1B). BM nucleated cell counts in both BADGE- and GW9662-treated mice were significantly higher than counts in the AA group. Using flow cytometry, we found the frequency of Lin^- cells and the absolute number of $Lin^-Sca1^+c\text{-}kit^+$ (LSK) stem cells in BM were higher in BADGE-treated mice than in controls (Figure 1C).

To test for a dose effect of BADGE in the AA mouse

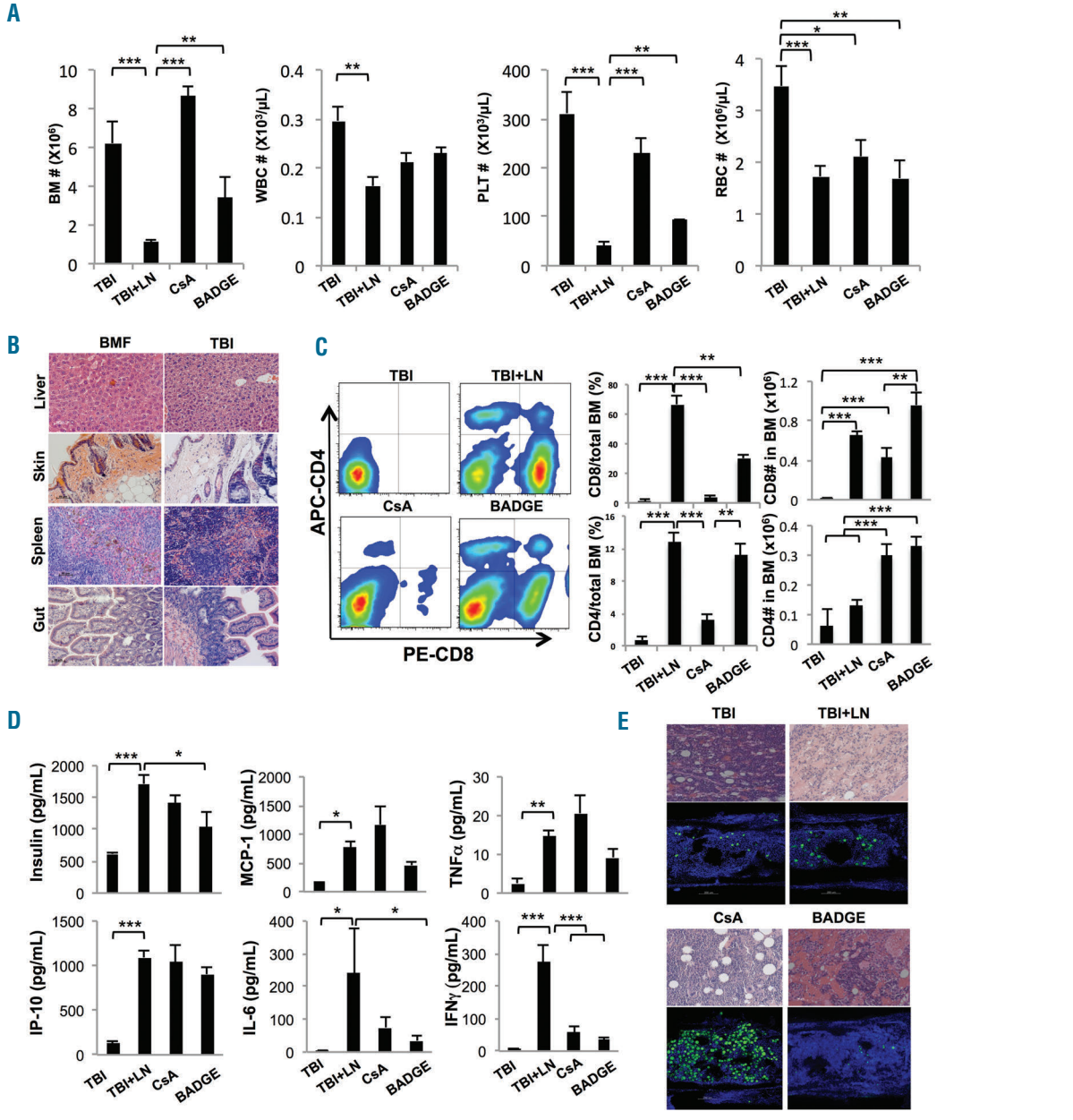

Figure 5. ***In vivo*** **effect of BADGE on T cells in FVB BM failure model.** (A) CBCs and BM cell numbers of FVB BM failure mice. (B) H&E staining of liver, spleen, gut, and skin in BM failure and control mice. Original magnification, x100. (C) Frequencies and absolute numbers of $CD8^+$ and $CD4^+$ T cells in the BM of LN-infused FVB mice. (D) Impact of BADGE and CsA on plasma cytokine levels in FVB BM failure model. TBI control, LN-infused, CsA-treated, and BADGE-treated mice (n=10 for each group) at 2 weeks. Error bars indicate SEM. **P*<0.05; ***P*<0.01; ****P*<0.001. (E) H&E staining and confocal microscopic imaging show BM structure and adipocytes in the sternums of FVB TBI control, LN-infused, CsA-treated, and BADGE-treated mice at 2 weeks. Representative pictures of each group of at least three separate experiments are shown. Original magnification, x 200 (H&E), x 100 (confocal).

model, we compared BADGE at low dose (15 mg/kg), standard dose (30 mg/kg), and high dose (60 mg/kg). Low dose BADGE had no effect, and high dose BADGE appeared to produce no added activity for the promotion of hematopoiesis and inhibition of BM adipogenesis, as compared to standard dose BADGE (Figure 1D,E), suggesting that an optimal dose of PPARγ antagonists is needed to inhibit BM adipogenesis.

To visualize hematopoietic stem and progenitor cells (HSPCs, Figure 1F) and megakaryocytes (Figure 1G) in the BM, we stained fixed sternums with anti-CD117 and anti-CD41 antibodies, respectively, followed by confocal imaging. In TBI alone mice, HSPC, megakaryocytes, and a few adipocytes were present in BM; in AA mice, the numbers of HSPCs and megakaryocytes were markedly reduced and adipocytes were abundant. In BADGE-treated mice, HSPCs and megakaryocytes were well preserved but with many fewer adipocytes, consistent with platelet counts on CBC and LSK cells in flow cytometry data. In both TBI only and BADGE-treated mice, HSPCs were distributed along the edge of the bones (Figure 1F).

Altered immunity of PPARγ antagonist-treated AA mice

In our AA mice model, T cell immunity is the cause of BM destruction. In order to determine if treatment with PPARγ antagonists altered the immunological status of AA mice, we measured plasma cytokine levels in a multiplex assay. Insulin, an adipogenesis-related hormone, was higher in AA mice than in TBI controls; BADGE- or GW9662-treatment corrected insulin levels to normal. AA mice showed higher levels of the inflammatory cytokine monocyte chemoattractant protein-1 (MCP-1) and Th1 cytokines such as IFNγ, IFNγ-induced protein 10 (IP-10), and TNFα than did TBI control mice. BADGE- or GW9662-treatment reduced plasma MCP-1 as compared to levels in AA mice; BADGE reduced IFNγ and IP-10 levels, and GW9662-treatment reduced TNFα levels as well. BADGE- or GW9662-treatment tended to decrease IL-6 levels (Figure 2A).

We extracted RNA from whole femurs and performed PCR-based arrays, focusing on adipogenesis (Figure 2B) and inflammasome (Figure 2C) pathways (gene expression levels are presented as heat maps). Compared with AA mice, expression levels of many adipogenesis genes decreased by

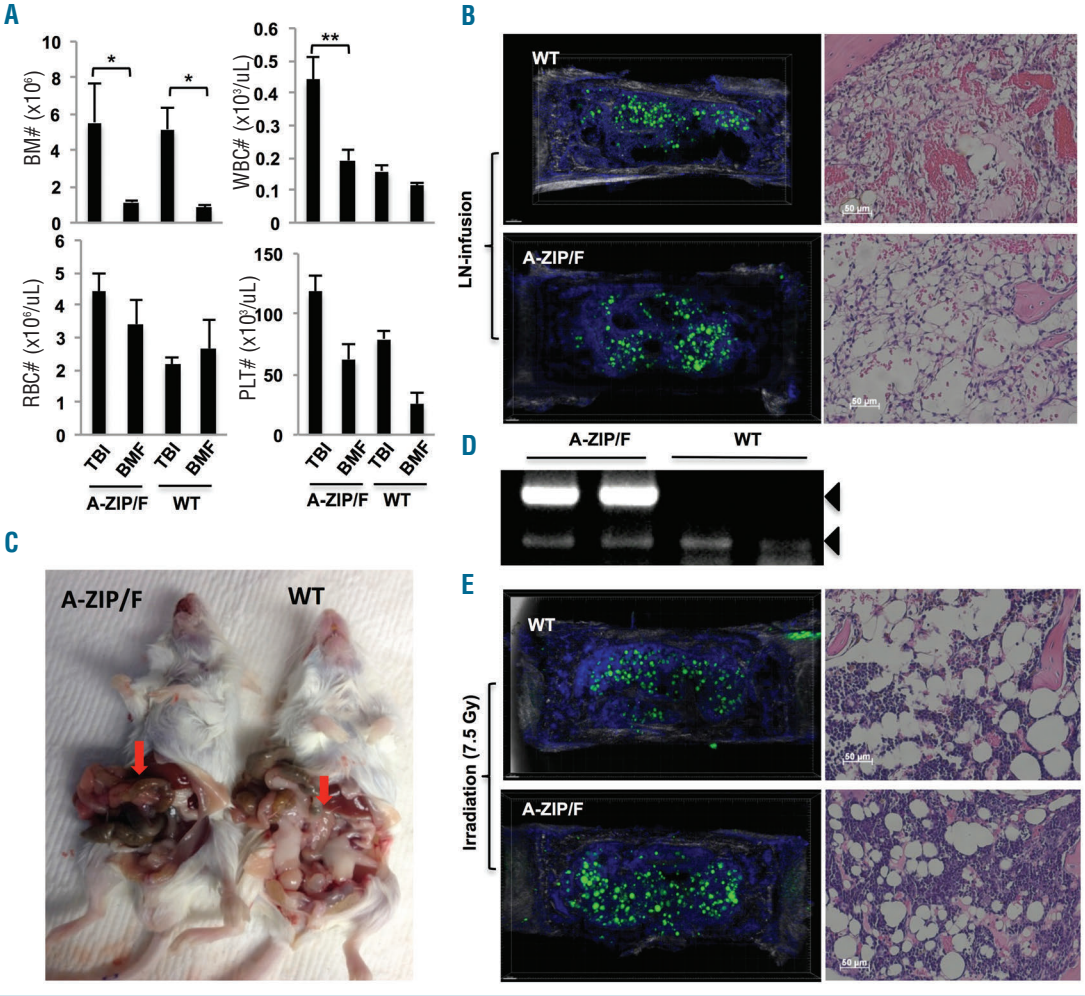

Figure 6. Marrow and body fat in "fatless" mice with bone marrow failure. (A) CBCs and BM cell numbers of A-ZIP/F "fatless" and wild-type (WT) mice with bone marrow failure (BMF) induced by injection of DBA lymph node (LN) cells post total body irradiation (TBI, 6.8 Gy). N=5 for each group. Error bars indicate SEM. *P<0.05; **P<0.01; ***P<0.001. (B) Confocal images and H&E staining of BM fat in immune-mediated BMF with "fatless" and wild-type mice. (C) Lack of body fat in "fatless" mice compared with wild-type mice. (D) Genotyping of "fatless" and wild-type mice. PCR results show upper band (332 bp, A-ZIP/F transgene) and lower band (200 bp, internal positive control). (E) Confocal images and H&E staining of BM fat in irradiation (7.5 Gy)-mediated BMF with "fatless" and wild-type mice. All data shown are at 2 weeks post BMF. Original magnification, x 200 (H&E), x 100 (confocal).

3- to 20-fold in BADGE-treated mice. The affected genes were known PPARγ targets, pro-adipogenesis, and adipokine-related genes. For example, expression levels of adipose angiotensinogen (*Agt*), *Nrob2, Sirt1, Ncoa2, Adig, Bmp2*, and *Acacb* were more than 5-fold lower in BADGE-treated mice. Expression of cell cycle- and proliferation-related genes *E2f1* and *Mapk14* increased 6- and 3-fold, respectively, perhaps reflecting active hematopoietic cell repopulation in the BM of BADGE-treated mice (Figure 2B). Inflammasome genes include four family members (*Aim2, Ipaf, Nlrp1,* and *Nlrp3*); BADGE only affected Ipaf, as expression levels of its components *Naip1, Naip5*, and *Nlrc4*, were reduced more than 3-fold, and no changes were observed in the genes of other inflammasome family members. Expression of inflammation-related genes including *Il6*, *Tnfsf4*, and *Tnf* was also markedly decreased in the BADGE-treated group compared with control AA mice, while expression of the anti-inflammation related gene *Nlrp12* (10-fold) was elevated (Figure 2C). Decreased *Il6* and Tnf expression at mRNA levels in treated mice was concordant with plasma protein levels (Figure 2A).

Gene expression levels, as determined by PCR array, were validated by immunoblot in order to confirm protein levels of PPARγ and AGT in BM. PPARγ isoform 2, an adipocyte-specific master regulator, was highly expressed in the BM of AA mice; both PPARγ isoforms 1 and 2 were greatly reduced in BADGE-treated mice, confirming that BADGE inhibited PPARγ expression in the model. AGT, one of the adipogenesis regulatory hormones and a PPARγ target protein, was not visible in TBI control CB10 mice, but was present at high levels in AA mice. BADGE treatment reduced AGT protein levels (Figure 2D), consistent with PCR array data (Figure 2B). Furthermore, immunoblotting results demonstrated that T cells isolated from the BM of BADGE-treated mice had decreased PPARγ protein levels compared to that from control AA mice (Figure 2E).

In order to determine whether PPARγ antagonist affected T cell populations, and especially T cells infiltrating the marrow of AA mice, we performed flow cytometry of PB and nucleated cells manually flushed from the BM. In AA mice, there was massive expansion of $CD8^+$ and $CD4^+$ T cells in the BM as expected; in contrast, BADGE reduced the frequencies of both $CD8^+$ and $CD4^+$ T cells significantly, while the absolute numbers of $CD8^+$ and $CD4^+$ T cells were not reduced (Figure 3A). In the PB of AA mice, $CD8^+$ T cells were markedly expanded, and BADGE largely reduced $CD8^+$ T cell frequencies, with less effect on $CD4^+$ T cells (Figure 3B). We measured expression of CD150, an activation marker for T cells, on residual BM $CD3^+$ T cells by flow cytometry: BADGE suppressed CD150 expression compared with control AA mice (Figure 3C), suggesting inhibition of T-cell activation by BADGE. In these experiments, PPARγ was administered beginning one day prior to LN cell infusion. To exclude the possibility that the drug might affect donor T cells homing to recipient BM, we delayed BADGE administration to one day after LN cell infusion. Amelioration of BM failure (as well as blockade of adipogenesis) was still observed (Figure 3D), suggesting that the effect of BADGE on T cells might be the inhibition of T cells directly, rather than altering T cell homing.

PPARγ antagonist inhibited T cell activation and proliferation in vitro

In our AA mice, BM adipogenesis was a consequence of marrow destruction mediated by T cells. *In vivo* data provided evidence of T cell inhibition by PPARγ antagonist. To test whether immunosuppression was a direct effect of the antagonist, we assessed the action of BADGE on T cell function *in vitro*. We stimulated mouse LN T cells with phorbol myristate acetate (PMA) and ionomycin and then incubated cells with different concentrations of BADGE for 16 hours, followed by flow cytometry analysis. CD25, an activation marker on both $CD4^+$ and $CD8^+$ T cells, was greatly decreased in the presence of BADGE, in a dose dependent manner (Figure 4A). Similarly, expression of T cell activation marker CD150 was also inhibited by BADGE (Figure 4B), parallel to the results of *in vivo* experiments (Figure 3C). Higher concentrations of BADGE (> 100 μM) were cytotoxic, as determined by flow cytometry with 7-amino-actinomycin D viability staining, as previously reported.[14]

PPARγ antagonists not only inhibited T cell activation but also suppressed T cell proliferation (Figure 4C): PMA plus ionomycin promoted T cell proliferation, reflected by the multiple divisions seen using carboxyfluorescein succinimidyl ester (CFSE) signal, while BADGE prevented T cells from dividing.

To confirm the early effect of BADGE on T cell activation, we collected culture supernatants and measured cytokines. BADGE decreased levels of PMA-induced secretion of soluble CD137, IL-2, and IL-6 by T cells (Figure 4D).

Cytoplasmic calcium changes in T cells accompany activation by multiple signaling pathways. To investigate if the inhibition of T cell activation by BADGE is related to this process, we performed calcium flux assay. Stimulation with CD3 antibody crosslinked with streptavidin induced marked calcium flux in mouse LN cells, but pre-incubation with BADGE was inhibitory of cation shift in a dose-dependent fashion (Figure 4E).

The effects of BADGE on T cell activation, proliferation, and cytokine secretion were related to PPARγ expression levels. As shown in immunoblotting, BADGE treatment decreased PPARγ in T cells in a dose-dependent fashion (Figure 4F). Similarly, BADGE also suppressed phospholipase C gamma 1 (PLCγ1) and the nuclear factor of activated T-cells 1 (NFAT1) expression in activated T cells (Figure 4F).

In vivo effect of BADGE on T cells in FVB BM failure model

To test if the effect of BADGE could be replicated in another mouse model (MHC-matched but non-MHC mismatched), we created a second immune-mediated BM failure model using FVB strain mice. As described in Naveiras *et al.*,[7] transplantation of BM cells from donor DBA/1 mice rescued lethally irradiated FVB mice. Because the donor and recipient mice are MHC-matched ($H2^q$) but non-MHC mismatched, these strains have been reported to differ in patterns of expressed genes, among which some are quantitative trait loci related to immune diseases.[18,19] We tested whether the injection of LN cells (mainly T cells) from donor DBA mice could induce T cell-mediated BM destruction of FVB mice, as in the CB10 AA model.[17] We infused 10×10^6 DBA LN cells into sublethally irradiated (6.8 Gy) FVB mice. At 2 weeks, mice that had received LN cells developed pancytopenia and BMs were hypocelluar (Figure 5A) without apparent graft-*versus*-host disease (Figure 5B). When mice were treated with the immunosuppressive drug CsA, BM failure was prevented; when FVB

BM failure mice were treated with BADGE, marrow cellularity improved (Figure 5A). On flow cytometry, there was an expansion of T cells, especially $CD8^+$ T cells, in the BM of control LN-infused mice (Figure 5C). CsA or BADGE significantly decreased the frequency of infiltrating T cells in the BM, although the absolute numbers of $CD8^+$ and $CD4^+$ T cells in BM remained elevated (Figure 5C).

Plasma levels of insulin, MCP-1, TNFα, IL-6, and IFNγ were increased when BM failure was induced, suggesting an inflammatory microenvironment. Both BADGE and CsA decreased levels of IFNγ significantly, while BADGE also corrected insulin and IL-6 levels and tended to decrease other inflammatory cytokines such as TNFα and MCP-1 (Figure 5D).

Low dose irradiation was associated with the appearance of only a few adipocytes in the BM of TBI control mice (Figure 5E). We anticipated adipocytes to be increased in the BM of LN-injected recipient mice due to BM destruction, but surprisingly this did not occur (Figure 5E). In contrast, CsA-treated mice had much higher BM cellularity and also many more adipocytes in their BM compared with marrow from TBI control mice and untreated BM failure mice, while almost no change in BM adipocytes was observed in BADGE-treated mice (Figure 5E). Lack of correlation between marrow fat cell content and BM failure indicated that BADGE might act by inhibiting T cell function rather than by suppressing BM adipogenesis in this model.

BM and body adipogenesis in "fatless" mice

Ideally, mice genetically incapable of forming adipocytes would be useful in our BM failure model, in order to separate the mechanisms of anti-adipogenesis and immunosuppression by PPARγ antagonists. We attempted such experiments with inbred lipoatrophic A-ZIP/F1 "fatless" transgenic mice.[20] The injection of DBA/1 LN cells into A-ZIP/F "fatless" mice indeed induced BM failure at 2 weeks, producing the expected hypocellularity of BM and decreased peripheral blood counts (Figure 6A). To our surprise we observed adipocytes in the marrow (Figure 6B) with the induction of BM failure, although we confirmed the absence of body fat in these mice (Figure 6C) and the genotype as previously described[20] (Figure 6D). BM fat was similarly present in non-immune-mediated marrow failure induced by irradiation (Figure 6E). The different distribution of body fat and marrow fat in both immune- and irradiation-mediated BM failure models suggests different mechanisms between BM and systemic adipogenesis under physiologic and stressed conditions.

Discussion

We observed that PPARγ antagonists ameliorated the severity of BM failure in an immune-mediated AA mouse model, and we hypothesized that PPARγ antagonists might modulate T cell function in addition to suppressing BM adipogenesis under these circumstances. In support of our *in vivo* data, *in vitro* experiments showed that T cell activation and proliferation, as well as cytokines, were significantly diminished in the presence of PPARγ antagonists. Treatment with BADGE in a FVB BM failure model, in which very few adipocytes accumulated in the BM, further confirmed the effect of PPARγ antagonists on T cell functions.

BM failure is a disorder of hematopoiesis. Cells of the perivascular niche (vascular endothelial cells) and endosteal niche (osteoblasts and their progenitors) are known to play active roles in the regulation of hematopoiesis.[21-23] Osteoblasts, an important component of the endosteal niche, originate from mesenchymal stromal cells (MSCs), the common pluripotent precursor shared with adipocytes. Differentiation of MSCs to osteoblasts or to adipocytes is regulated by intracellular and extracellular cytokines and transcription factors, including PPARγ.[22,23] An inverse reciprocal relationship exists between adipogenesis and osteoblastosis. Most growth factors and cytokines that promote osteoblastosis negatively regulate adipogenesis, and vice versa. BM failure is often accompanied by active adipogenesis. A clinical study has shown that an increased volume of BM adipocytes is strongly correlated with reduced bone formation.[24] In recently published work, the numbers of HSPCs were reduced by up to 3-fold in adipocyte-rich tail vertebrae, as compared to adipocyte-poor thoracic vertebrae.[7] Lipoatrophic A-ZIP/F1 "fatless" transgenic mice, which are genetically incapable of forming adipocytes, showed markedly stimulated osteogenesis that resulted in bone formation after lethal irradiation.[7] Thus, it was attractive to postulate that BM adipocytes might have a negative role in the regulation of hematopoiesis, in contrast with osteoblasts' supportive function in hematopoiesis. Adipogenesis involves a complex network of transcription factors, PPARγ and C/EBPs are the master regulators of these processes. In particular, PPARγ signaling is understood to be both necessary and sufficient for fat cell formation.[25]

In this current work, we show that expression of PPARγ (particularly the PPARγ2 isoform) is increased in our CB10 AA mouse model with the accumulation of fat cells in BM. Thus, the inhibition of PPARγ and suppression of adipogenesis are implicated functionally. However, there are controversies concerning the effects of PPARγ antagonists on adipogenesis. Some studies have concluded that BADGE blocks adipogenesis from a mouse preadipocyte cell line and from normal isolated MSCs (IC_{50} of approximately 100 μM).[26] Conversely, in other experiments, BADGE and GW9662 did not antagonize PPARγ signaling nor prevent adipogenesis *in vitro* and *in vivo*.[27-30] In one study, BADGE actually promoted adipogenesis at nanomolar levels, while higher concentrations of BADGE were cytotoxic.[28] We found that low dose BADGE had either no therapeutic effect or even worsened BM failure, whereas high dose BADGE did not add to hematological improvement. Both PPARγ agonists and antagonists have been reported to be cytotoxic at high doses.[28,31] For example, thiazolidinediones, which are clinically used as specific high affinity ligands for PPARγ, have shown a series of unexpectedly severe adverse effects, including fatal hepatitis, heart failure due to water retention, increased risk of cardiovascular events, and a higher incidence of bladder cancer.[31] These cytotoxic effects may restrict their utility as well as hinder evaluation of molecular mechanisms. Overall, the effects of PPARγ antagonists vary greatly depending on drug concentrations, as well as on biological or culture conditions. Our observation suggests that fresh preparation, short-term (< 2 weeks) preservation, and timely administration are critical for efficacy of BADGE or GW9662, probably due to their short active chemical half-life.

BM adipogenesis often has an inverse correlation with hematopoiesis, but this is not a consistent relationship. For example, adipose-derived MSCs efficiently support hematopoiesis *in vivo* and *in vitro*.[32,33] Herein, we demonstrate that the injection of DBA/1 LN cells into FVB mice (different at non-MHC) induced severe BM destruction without the accumulation of large numbers of adipocytes in the BM, different from other BM failure models; possibly, more severe BM damage was the result of a higher irradiation dose, a larger LN cell inoculum, and/or a stronger bystander effect of injected T cells, which may have led to a more efficient elimination of HSC and stromal cells, including adipocyte precursors. CsA suppressed T cells effectively, and allowed for the recovery of HSC and stromal cells including adipocyte precursors, leading to the coexistence of improved BM cellularity and the expansion of BM adipocytes. Although BADGE also suppressed T cells, the efficacy was not as potent as CsA, as evidenced by residual T cells in BM, documented by flow cytometry. We attempted to separate the mechanisms of anti-adipogenesis and immunosuppression by PPARγ antagonists by inducing BM failure with inbred lipoatrophic A-ZIP/F1 "fatless" transgenic mice.[20] However, we noted that for both immune- and irradiation-mediated BM failure, adipocytes still accumulated in BM despite the phenotype of missing body fat and confirmed genotype, suggesting that subcutaneous and BM adipogenesis are critically different. The underlying molecular biology in these circumstances is under further investigation.

Although an immunomodulatory role of PPARγ signaling has been inferred from experiments with agonists or antagonists *in vitro*, applications to animal models have been limited.[7,9-12,15,34] Most rodent studies have explored extramedullary adipocytes, focusing on obesity or metabolic syndrome rather than immune models.[15,34] PPARγ plays pleiotropic roles in inflammation, and the inflammatory reaction may be stimulated or suppressed by the presence of PPAR ligands[35] and PPARγ antagonists. Competitive radioligand binding studies showed BADGE to be a ligand for PPARγ with micromolar affinity, and functionally BADGE is a pure antagonist for this receptor.[26] Therefore, the effects of BADGE on adipogenesis or immunomodulation are complicated. The study by Dworzanski *et al.* used very high concentrations of BADGE (120 mg/kg),[35] at which the toxic and inflammatory effects might be dominated. In our BM failure models, BADGE at 30-50 mg/kg seems optimal to suppress immune reaction. Our initial intention in these experiments was to clarify the role of BM adipocytes in AA by inhibiting PPARγ signaling. However, we unexpectedly found that, while alloreactive T cell activation in response to multiple miHAs appeared to be ameliorated by PPARγ antagonism, the inhibition of adipocyte formation was not a generalizable activity when related to marrow failure. Previous studies showed that PPARγ agonists inhibited T cell proliferative response and IL-2 secretion by activated T cells.[9,10,12,13] However, our results indicated that PPARγ antagonists also had similar effects on T cell activation and IL-2 secretion; the antagonists directly inhibited calcium flux and suppressed expression of PPARγ and NFAT1. In addition, PPARγ antagonists were reported to have potent antiproliferative effects on hematopoietic and cancer cell lines by induction of apoptosis, in caspase-dependent or –independent mechanisms.[8,11,14] It is noteworthy that both agonists and antagonists induced apoptosis in these cells, effects not necessarily related to PPARγ expression levels.[11] Based on our results, we conclude that PPARγ antagonists act as negative regulators of T cells in addition to their inhibition of BM adipogenesis. Our findings hold implications for the application of PPARγ antagonists in immune-mediated pathophysiologies both in the laboratory and in the clinic.

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