

Blockade of $\alpha 4$ Integrin Signaling Ameliorates the Metabolic Consequences of High-Fat Diet–Induced Obesity

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OBJECTIVE—Many prevalent diseases of advanced societies, such as obesity-induced type 2 diabetes, are linked to indolent mononuclear cell–dependent inflammation. We previously proposed that blockade of $\alpha 4$ integrin signaling can inhibit inflammation while limiting mechanism-based toxicities of loss of $\alpha 4$ function. Thus, we hypothesized that mice bearing an $\alpha 4$ (Y991A) mutation, which blocks signaling, would be protected from development of high-fat diet–induced insulin resistance.

RESEARCH DESIGN AND METHODS—Six- to eight-week-old wild-type and $\alpha 4$ (Y991A) C57Bl/6 male mice were placed on either a high-fat diet that derived 60% calories from lipids or a chow diet. Metabolic testing was performed after 16–22 weeks of diet.

RESULTS— $\alpha 4$ (Y991A) mice were protected from development of high-fat diet–induced insulin resistance. This protection was conferred on wild-type mice by $\alpha 4$ (Y991A) bone marrow transplantation. In the reverse experiment, wild-type bone marrow renders high-fat diet–fed $\alpha 4$ (Y991A) receptor animals insulin resistant. Furthermore, fat-fed $\alpha 4$ (Y991A) mice showed a dramatic reduction of monocyte/macrophages in adipose tissue. This reduction was due to reduced monocyte/macrophage migration rather than reduced monocyte chemoattractant protein-1 production.

CONCLUSIONS— $\alpha 4$ integrins contribute to the development of HFD-induced insulin resistance by mediating the trafficking of monocytes into adipose tissue; hence, blockade of $\alpha 4$ integrin signaling can prevent the development of obesity-induced insulin resistance. *Diabetes* 57:1842–1851, 2008

Obesity leads to insulin resistance that results in type 2 diabetes (1) and that contributes to hypertension and cardiovascular disease (2). Mononuclear cell–mediated inflammation in obese adipose tissue plays a pathogenetic role in insulin resistance (3,4). Thus, there is great interest in the possibility of using anti-inflammatory strategies to ameliorate obesity-induced insulin resistance.

Blockade of leukocyte adhesion is a proven therapeutic strategy for a wide variety of inflammatory diseases (5). In

particular, inhibiting $\alpha 4$ integrins or their counter-receptors (vascular cell adhesion molecule-1 [VCAM-1] and mucosal addressin cell adhesion molecule-1 [MadCAM-1]) blocks inflammatory responses mediated by mononuclear leukocytes (6). $\alpha 4$ integrin antagonists are of proven benefit in several human inflammatory diseases (7,8). These antagonists, such as the monoclonal antibody natalizumab, block ligand binding function, thus producing a complete loss of $\alpha 4$ integrin function. Lack of $\alpha 4$ integrins is embryonic lethal and results in defective placentation, heart development, and hematopoiesis (9–11). Furthermore, natalizumab therapy has been associated with fatal progressive multifocal leukoencephalopathy in humans, possibly because of defective T-cell trafficking to the brain (12,13). Thus, currently available $\alpha 4$ integrin antagonists are of proven value in mononuclear cell–mediated diseases; however, complete loss of $\alpha 4$ integrin function is associated with developmental defects and abnormal hematopoiesis.

As noted above, whereas $\alpha 4$ integrin antagonists show promise for several autoimmune and inflammatory diseases, mechanism-based toxicities may limit their use, particularly in low-grade chronic inflammatory conditions, such as obesity-induced insulin resistance. We recently proposed an alternative strategy—blockade of $\alpha 4$ integrin signaling—to perturb functions involved in inflammation, while limiting mechanism-based adverse effects (14). $\alpha 4$ integrin signaling involves the binding of paxillin to the $\alpha 4$ integrin tail, and a point mutation ($\alpha 4$ Y991A) that selectively blocks this interaction reduces $\alpha 4$ -mediated leukocyte migration (15) and adhesion strengthening in flowing blood (16) while sparing $\alpha 4$ -mediated static cell adhesion (17). Furthermore, mice bearing an $\alpha 4$ (Y991A) mutation are viable and fertile and have intact lympho-hematopoiesis and humoral immune responses; however, they exhibit defective recruitment of mononuclear leukocytes in experimental inflammation (18). Here, we report that the $\alpha 4$ (Y991A) mutation reduces mononuclear leukocyte infiltration of white adipose tissue (WAT) in high-fat diet–induced obese mice and hence reduce high-fat diet–induced insulin resistance. Thus, we establish that blocking $\alpha 4$ integrin signaling can ameliorate the metabolic consequences of high-fat diet–induced obesity.

RESEARCH DESIGN AND METHODS

Animals and animal care. The $\alpha 4$ (Y991A) mice were previously described and have been backcrossed nine times onto the C57Bl/6 background (18). We fed male mice (aged 6–8 weeks) either on high-fat diet, containing 60% fat by weight (D12492; Research Diets) or on chow diet (10% fat; D12450B; Research Diets) for 16–22 weeks. All experiments were approved by the University of California San Diego Institutional Animal Care and Use Committee.

Glucose and insulin tolerance tests. We carried out glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs) as described previously (19) (see

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TABLE 1
Plasma measurements of lipids and adipokines in wild-type and $\alpha 4$ (Y991A) mice

Genotype	Normal chow diet		High-fat diet	
	Wild type	Y991A	Wild type	Y991A
<i>n</i>	6	5	10	9
Plasma free fatty acids ($\mu\text{mol} \cdot \text{l}^{-1} \cdot \text{ml}^{-1}$)	0.71 \pm 0.23	0.44 \pm 0.07	0.89 \pm 0.15	0.61 \pm 0.09
Total cholesterol (mg/dl)	132.2 \pm 5.9	130.0 \pm 2.7	238.7 \pm 10.5*	203.0 \pm 27.9*
Plasma triglycerides (mg/dl)	41.2 \pm 2.5	42.7 \pm 2.4	42.4 \pm 2.6	46.6 \pm 4.6
MCP-1 (pg/ml)	22.0 \pm 3.5	25.67 \pm 6.1	43.25 \pm 16.6	47.0 \pm 20.5
Adiponectin ($\mu\text{g} \cdot \text{ml}^{-1} \cdot \text{g}^{-1}$)	1.13 \pm 0.22	1.37 \pm 0.04	0.63 \pm 0.05*	0.68 \pm 0.03*
Leptin (pg/ml)	3,699 \pm 465	4,129 \pm 723	14,038 \pm 1,219*	10,222 \pm 1,878*
IL-6 (pg/ml)	0.63 \pm 0.01	0.89 \pm 0.09	1.51 \pm 0.68	1.62 \pm 0.44
Resistin (pg/ml)	900.3 \pm 60.6	1,104.3 \pm 67.4	1,446.4 \pm 108.6	1,270.8 \pm 68.6
TNF- α (pg/ml)	2.61 \pm 0.28	2.41 \pm 0.31	3.16 \pm 0.27	3.28 \pm 0.49

Data are means \pm SE. *n* values per group are indicated. *Significant difference between diet within genotype. None of these parameters were statistically different between genotypes.

supplemental methods in the online appendix available at <http://dx.doi.org/10.2337/db07-1751>.

Whole-blood and plasma measurements. Total white blood cell number and differential counts were assessed by standard techniques (ACP Diagnostic Lab, University of California, San Diego). We measured plasma insulin by radioimmunoassay (Linco Research) and determined free fatty acids by colorimetric assay (Wako). Plasma cytokines were measured by the core laboratories of the Diabetes and Endocrinology Research Consortium (University of California, Los Angeles; LINCOplex assay for Mouse Cytokines, <http://www.derc.med.ucla.edu/core.htm>). Plasma cholesterol and triglyceride levels were measured by enzymatic methods using an automated bichromatic analyzer (Abbot Diagnostics). All of these measurements were performed on 11 wild-type and 9 $\alpha 4$ (Y991A) mice for high-fat diet and 6 wild-type and 5 $\alpha 4$ (Y991A) mice for chow diet (Table 1).

Histochemistry. Adipose tissue was fixed overnight in 10% formaldehyde, dehydrated in ethanol bath, and paraffin-embedded. Sections were stained with hematoxylin-eosin (H-E) for observation of adipose tissue structure.

Pancreas isolation and determination of islet area. Pancreata were isolated and fixed in 4% formalin overnight. Paraffin sections were generated and stained with H-E. Pictures were taken of H-E-stained pancreas sections, and the area of Langerhans islets and the total area of the pancreas were measured using ImageJ software (NIH freeware). Islet area was depicted as percentage of total pancreas area. Sections of four to seven mice per group and at least six different pancreas areas per mouse were analyzed for statistical significance using unpaired two-tailed Student's *t* test. *P* values <0.05 were considered significant.

Bone marrow transplantation. We injected bone marrow obtained from wild-type and $\alpha 4$ (Y991A) mice (4×10^6 cells) through the tail vein into male C57BL/6 (4 months) and $\alpha 4$ (Y991A) (4–6 months) mice that had been irradiated (10 Gy) 4 h before. Mice were allowed 4 weeks for reconstitution of donor marrow, which we verified by PCR (18).

Isolation of stromal vascular cells. Epididymal fat pads were minced in PBS and digested with collagenase (Roche) and DNase I (Sigma) for 1 h at 37°C. Suspensions were then filtered through a 100- μm nylon cell strainer and then spun at 1,000*g* for 10 min. The preadipocyte/adipocyte-enriched fraction was removed. Isolated stromal vascular cells were resuspended in red blood cell lysis buffer (10 mmol/l KHCO₃, 150 mmol/l NH₄Cl, and 0.1 mmol/l EDTA, pH 8) for 5 min at room temperature. Cell suspension was centrifuged for 5 min at 500*g*, and stromal vascular cells were resuspended in PBS at 10⁶ cells/100 μl .

Flow cytometry. Cells were harvested from WAT, peripheral blood, and bone marrow and incubated in Fc blocker (rat anti-mouse CD16/32) for 20 min at room temperature. The cells were then incubated with fluorescein isothiocyanate (FITC)-Ly-6G (1/200) and phycoerythrin (PE)-7/4 (1/50) (BD Biosciences/PharMingen, San Diego, CA). Negative control staining was performed with FITC-rat IgG2a/k and PE-rat IgG2a. Cell staining was analyzed with FACScan flow cytometer using CellQuest software (BD Biosciences Systems).

Cell migration assay. Cell migration was assayed in a modified Boyden chamber system using a 24-well transwell plate (8- μm pore size; Corning) coated with 5 $\mu\text{g}/\text{ml}$ VCAM-1 (R&D Systems). Monocyte chemoattractant protein-1 (MCP-1) (R&D Systems) was added in the lower chamber at 1 nmol/l. Cells harvested from wild-type or $\alpha 4$ (Y991A) bone marrow were grown for 5–7 days in the presence of granulocyte macrophage-colony-stimulating factor (5 μl of 0.1 mg/ml stock solution). Bone marrow-derived macrophages (2×10^4) were kept in suspension in 1% serum containing medium for 1 h at room temperature. Cells were then added to the top

chamber and incubated overnight at 37°C. Filters were fixed and stained with crystal violet, and migrated cells in the lower chamber were enumerated.

RNA isolation and RT-PCR. Total RNA was isolated from WAT using RNeasy Lipid Tissue kit (Qiagen). RT-PCRs were carried out as described in the online appendix (20).

Statistical analysis. Means and SEs were calculated for all dependent measures. Data were analyzed for statistical significance using the one- or two-tailed Student's *t* test. Significance was set at *P* \leq 0.05. For Fig. 1*B*, change scores for the chow diet and high-fat diet data were determined using the total area under the curve (AUC) (21).

RESULTS

$\alpha 4$ (Y991A) mutation is protective against the development of high-fat diet-induced glucose intolerance and insulin resistance. To investigate the role(s) of integrin $\alpha 4$ interaction with paxillin in $\alpha 4$ -mediated functions in vivo, we previously generated and analyzed mice bearing a point mutation in the $\alpha 4$ integrin tail (Y991A) that inhibits paxillin binding with little detectable effect on the binding of other proteins (18). To examine the potential effect of this mutation on a model of human type 2 diabetes, these $\alpha 4$ (Y991A) mice and wild-type controls were placed on a 60% fat diet for 16–22 weeks. On this diet, wild-type animals developed impaired glucose tolerance (Fig. 1*A*). In contrast, mice bearing the $\alpha 4$ (Y991A) mutation were partially protected against high-fat diet-induced glucose intolerance (Fig. 1*A*; Supplemental Fig. 1*A*). The increased glucose tolerance in the high-fat diet-fed $\alpha 4$ (Y991A) animals was not due to greater insulin release because these mice released significantly less insulin than high-fat diet-fed wild-type animals (*P* = 0.036) (Fig. 1*B*). The wild-type mice showed a greater increase in pancreatic β -cells than those bearing the $\alpha 4$ (Y991A) mutation (Fig. 1*C*) in response to the high-fat diet. Because increased β -cells is an early manifestation of insulin resistance (22), these findings strongly suggested that the $\alpha 4$ (Y991A) mutation helped preserve sensitivity to insulin in fat-fed animals. This idea was confirmed by the finding that the high-fat diet-fed mutant mice showed a much greater insulin-induced drop in blood glucose than the high-fat diet-fed wild-type mice (Fig. 1*D*). Thirty minutes after administration of 0.85 units/kg insulin, plasma glucose decreased by 43% in the mutant fat-fed animals compared with a 28% decline in wild-type animals. In contrast, the chow-fed animals of both genotypes exhibited a 37% decline at 30 min (0.75 units/kg insulin injected). At later time points, there was a statistically insignificant trend of greater insulin-induced drop in chow-fed $\alpha 4$ (Y991A) animals ver-

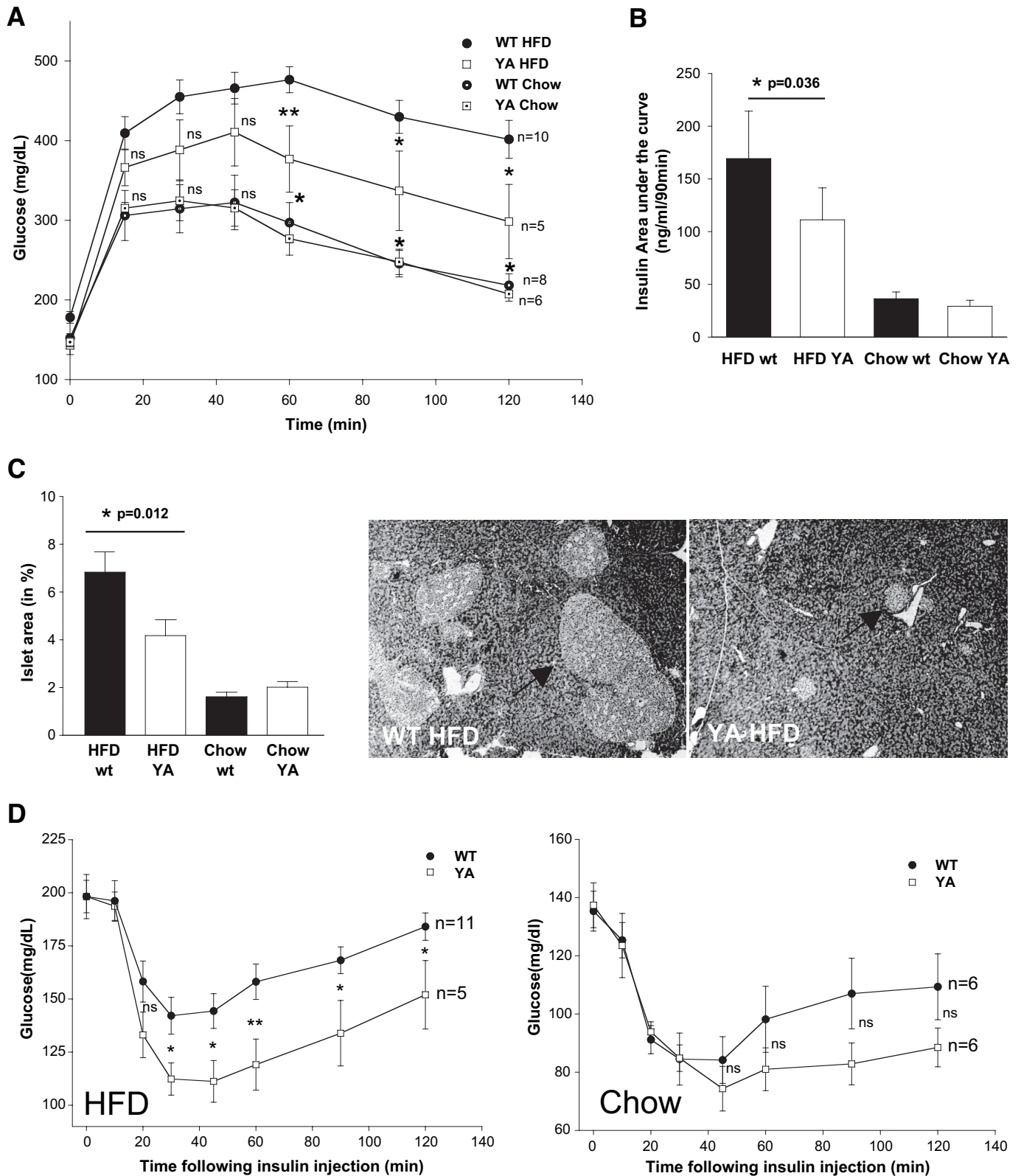


FIG. 1. $\alpha 4$ (Y991A) mutation protects mice against the development of high-fat diet-induced glucose intolerance and insulin resistance. **A:** In vivo glucose homeostasis was assessed by GTT in wild-type (●) and $\alpha 4$ (Y991A) mice (□) on high-fat diet (plain symbols) and normal chow (dotted symbols). The results shown are means \pm SE for each time point. **B:** Plasma insulin concentrations during GTT were collected. They are represented as total AUC, which was calculated using the trapezoidal method (see RESEARCH DESIGN AND METHODS for details). **C:** Size of pancreatic β -cell islets (right) in wild-type (closed) and $\alpha 4$ (Y991A) mice (open) was measured. Representative H-E staining of wild-type and $\alpha 4$ (Y991A) pancreas is also shown (left). Arrowheads indicate pancreatic islets. **D:** ITT was performed in wild-type (●) and $\alpha 4$ (Y991A) mice (□) on high-fat diet (HFD; left) and normal chow (Chow; right). Plasma glucose was significantly higher in wild-type mice fed high-fat diet than in all other groups during both the GTTs and ITTs. No significant differences in plasma insulin during the GTT were found between the wild-type and $\alpha 4$ (Y991A) mice on chow diet. Plasma insulin and percentage of pancreatic β -cell islets were significantly higher in the wild-type mice after high-fat diet. *n* values per group are indicated. **P* < 0.05; ***P* < 0.01; ns, not significant.

sus wild type. Thus, the $\alpha 4$ (Y991A) mutation protects against high-fat diet–induced insulin resistance.

The $\alpha 4$ (Y991A) mutation did not affect caloric intake or weight gain. Both wild-type and $\alpha 4$ (Y991A) mice exhibited the same caloric intake on high-fat diet (Fig. 2A, *left*), which was about 1.5-fold higher than on chow diet (Fig. 2A, *right*). Consistent with similar caloric intake, no differences in weight gain were observed between wild-type and $\alpha 4$ (Y991A) mice (Fig. 2B, *left* and *right*). Animals were weight matched before starting the 16 weeks of diet [average starting weight for high-fat diet, 27.8 ± 0.6 and 27.3 ± 1.0 g for wild type and $\alpha 4$ (Y991A), respectively; for chow diet, 27.8 ± 1.3 and 28.8 ± 1.6 g for wild type and $\alpha 4$ (Y991A), respectively]. Gross histological analysis of WAT isolated from high-fat diet–fed wild-type and $\alpha 4$ (Y991A) mice showed no statistical difference in adipocyte size or number (Fig. 2C; Supplemental Fig. 1B).

Plasma levels of free fatty acids and triglycerides were similar between wild-type and $\alpha 4$ (Y991A) mice on both diets (Table 1). Wild-type mice exhibited increased plasma cholesterol on high-fat diet (238.7 ± 10.5 mg/dl) in comparison with those on chow diet (132.2 ± 5.9 mg/dl). The $\alpha 4$ (Y991A) mice on high-fat diet exhibited a statistically insignificant trend to a lesser rise in cholesterol (203.0 ± 27.9 vs. 130.0 ± 2.7 mg/dl, high-fat diet vs. chow diet), which may reflect a decrease in LDL levels. Plasma adiponectin concentrations decreased to similar levels in both high-fat–fed wild-type and $\alpha 4$ (Y991A) mice. Plasma leptin and resistin levels increased after high-fat diet, but no differences were found between genotypes (Table 1). High-fat diet–fed $\alpha 4$ (Y991A) mice exhibited similar plasma expression of proinflammatory cytokines, such as interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), and MCP-1, compared with wild-type mice (Table 1).

Bone marrow–derived cells are responsible for protection from high-fat diet–induced insulin resistance. Even though $\alpha 4$ integrin is not present at the surface of adipocytes (data not shown), it is widely expressed (23,24) and is particularly prominent in the functioning of mononuclear leukocytes. Moreover, bone marrow–derived mononuclear cells contribute to insulin resistance (3,4,25). To determine whether the protection against high-fat diet–induced insulin resistance in the $\alpha 4$ (Y991A) mice is mediated through bone marrow–derived cells, we performed bone marrow transplantation (BMT) experiments. Six- to eight-week-old lethally irradiated (10 Gy) wild-type male mice received bone marrow cells from either wild-type or $\alpha 4$ (Y991A) donor mice via tail vein injection. Recipient mice were allowed 4 weeks for recovery and reconstitution of the transplanted bone marrow and were then placed on high-fat diet for 16–22 weeks before metabolic experiments. Posttransplant chimerism was evaluated by PCR on both groups. No wild-type $\alpha 4$ was detected in blood collected from mice receiving bone marrow from $\alpha 4$ (Y991A) mice [$\alpha 4$ (Y991A)-BMT]. Mice transplanted with wild-type and $\alpha 4$ mutant [$\alpha 4$ (Y991A)-BMT] bone marrow gained equal amounts of weight on high-fat diet compared with normal chow (data not shown). Levels of plasma fatty acids, cholesterol, triglycerides, adiponectin, and leptin were similar between genotypes (data not shown).

Wild-type mice that received $\alpha 4$ (Y991A) bone marrow were partially protected against high-fat diet–induced glucose intolerance (Fig. 3A) and insulin resistance (Fig. 3B) compared with wild-type animals that received wild-type bone marrow. When animals were fed a normal diet,

glucose tolerance was similar in mutant and wild-type transplanted mice (Fig. 3A), but there was a statistically insignificant trend toward increased insulin sensitivity in chow-fed $\alpha 4$ (Y991A) compared with wild-type marrow recipients (Fig. 3C). In reverse BMT experiments, i.e., transplantation of wild-type bone marrow into lethally irradiated $\alpha 4$ (Y991A) mice, no mutant allele was detected in $\alpha 4$ (Y991A) animals receiving wild-type bone marrow, confirming complete reconstitution of wild-type bone marrow in these animals. Wild-type marrow made $\alpha 4$ (Y991A) acceptor mice susceptible to glucose intolerance (Supplemental Fig. 2). Thus, bone marrow–derived cells are responsible for the observed protection of $\alpha 4$ (Y991A) mice from high-fat diet–induced insulin resistance.

The $\alpha 4$ (Y991A) mutation leads to a decrease in adipose tissue monocyte/macrophages in high-fat diet–fed mice. The foregoing experiments showed that the effect of the $\alpha 4$ (Y991A) mutation was manifest through bone marrow–derived cells. Among bone marrow–derived cells, $\alpha 4$ integrin is highly expressed on most mononuclear leukocytes (26). Monocyte/macrophages are bone marrow–derived mononuclear cells that mediate the inflammatory response to high-fat diet, and macrophages contribute to the pathogenesis of obesity-induced insulin resistance (25,27). We therefore used flow cytometry to quantify the presence of monocyte subpopulations, defined by the level of expression of surface markers 7/4 and Ly6-G (28) in the stromal vascular fraction (SVF) of WAT isolated from wild-type and $\alpha 4$ (Y991A) mice on chow or high-fat diet. We used a combination of Ly-6G and 7/4 markers, described by Tsou et al. (28), which define a cell population uniformly positive for F4/80, CD11b, and CC chemokine receptor 2 for CCL2/MCP-1 (CCR2) and phenotypically identical to the inflammatory monocytes previously described (29–32).

The accumulation of macrophages, associated with obesity-induced insulin resistance, occurs predominantly in epididymal WAT (25,33); hence we studied WAT from this site. High-fat diet–fed $\alpha 4$ (Y991A) mice exhibited a marked reduction in the number of monocytes ($7/4^{\text{hi}}\text{Ly-6G}^{\text{neg}}$) compared with high-fat diet–fed wild-type mice (0.8 ± 0.13 vs. $2.88 \pm 0.49\%$) (Fig. 4A, *top left panel*) in WAT SVF. This phenomenon was also observed (Fig. 4A, *bottom left panel*) with $7/4^{\text{dim}}\text{Ly-6G}^{\text{neg}}$ cells (mixed monocyte/lymphocytes) (Y991A $3.77 \pm 1.43\%$ vs. wild type $7.6 \pm 1.11\%$). In contrast, when fed a normal diet, wild-type and $\alpha 4$ (Y991A) mice had similar percentages of both $7/4^{\text{hi}}\text{Ly-6G}^{\text{neg}}$ and $7/4^{\text{dim}}\text{Ly-6G}^{\text{neg}}$ cells in their WAT SVF (Fig. 4A, *right panels*). These results were confirmed by the reduction in mRNA for F4/80, a macrophage marker, in WAT from fat-fed $\alpha 4$ (Y991A) mice compared with wild-type mice (Supplemental Fig. 1C). Thus, the $\alpha 4$ (Y991A) mutation leads to a reduction in monocyte/macrophage accumulation in the WAT SVF in response to high-fat diet.

WAT monocyte/macrophages derive in part from peripheral blood monocytes, which in turn are derived from the bone marrow. To investigate the cause of the reduced monocyte/macrophage accumulation in WAT of $\alpha 4$ (Y991A) mice, we quantified monocyte subpopulations in the peripheral blood and in the bone marrow of wild-type and $\alpha 4$ (Y991A) mice. In the high-fat diet–fed mice, there was a reduction in the percentage (1.4 ± 0.28 vs. $2.9 \pm 0.38\%$ for Y991A and wild type, respectively) and absolute number of monocytes in the peripheral blood of $\alpha 4$ (Y991A) mice (Fig. 4A and B). In contrast, there was no significant difference in the

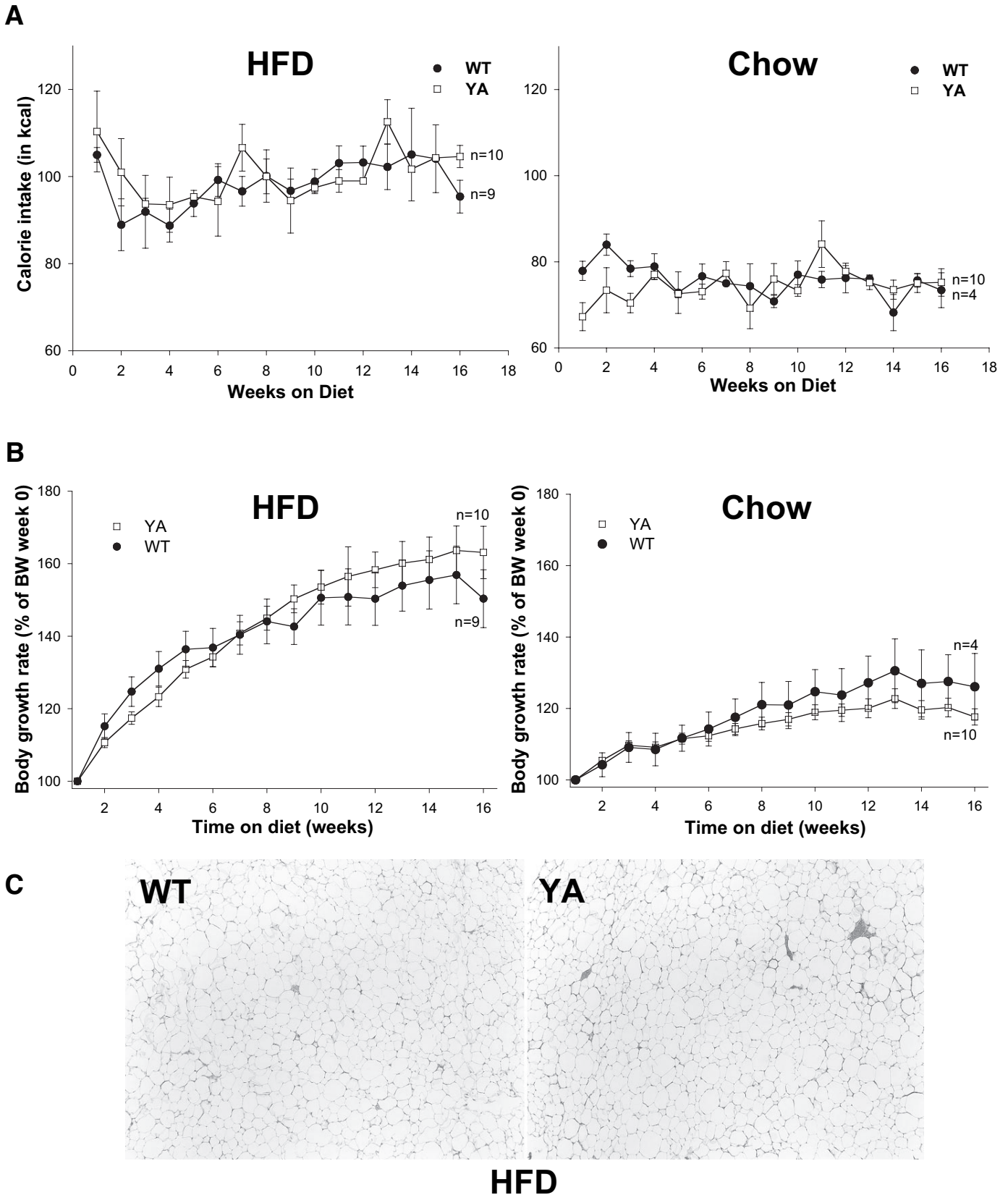


FIG. 2. Normal caloric intake, weight gain, and adipocyte size in $\alpha 4(Y991A)$ mice compared with wild-type mice after high-fat diet. Calorie intake (A) and weight gain (B) were measured in wild type (○) and $\alpha 4(Y991A)$ (■) mice on high-fat diet (HFD; left) and normal chow (chow; right). C: H-E staining of WAT isolated from wild type (WT) and $\alpha 4(Y991A)$ (YA) mice was performed. No significant difference was observed between genotypes within diet. *n* values are indicated.

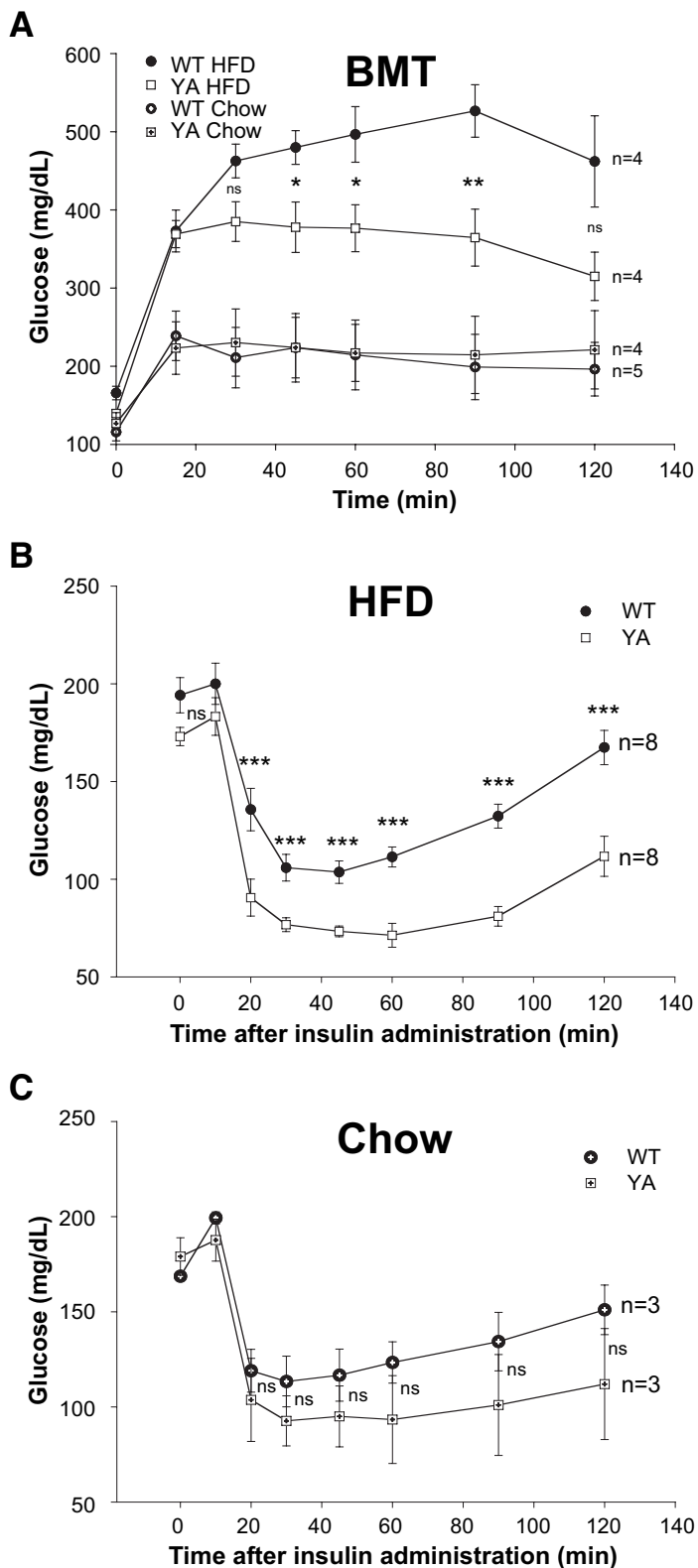


FIG. 3. Bone marrow cells from $\alpha 4(Y991A)$ mice are sufficient for protection against high-fat diet-induced insulin resistance. GTTs (A) and ITTs (B and C) were performed in wild-type mice that received bone marrow from either wild-type (●) or $\alpha 4(Y991A)$ donors. Recipient animals were on high-fat diet (HFD; plain symbols) or normal chow (Chow; dotted symbols). The results shown are means \pm SE for each time point. Plasma glucose was significantly lower in high-fat diet-fed wild-type mice that received $\alpha 4(Y991A)$ bone marrow than in all other groups during both the GTTs and ITTs. *n* values per group are indicated. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; ns, not significant.

percentage of pure or mixed monocyte populations in the bone marrow of high-fat diet-fed wild-type and $\alpha 4(Y991A)$ mice (Fig. 4A). Similarly, a slight monocytopenia was observed in the peripheral blood of normal chow-fed $\alpha 4(Y991A)$ mice (1.71 ± 0.13 vs. $2.39 \pm 0.12\%$ for Y991A and wild type, respectively), and there was no significant difference in abundance of $7/4^{hi}Ly-6G^{neg}$ and $7/4^{dim}Ly-6G^{neg}$ cells in the bone marrow (Fig. 4). Taken together, these data suggest that the $\alpha 4(Y991A)$ mutation does not impair monocyte development. Rather, they suggest that the mutation reduces mobilization of these cells. These data do not define the relative contributions of reduced egress from the bone marrow and entry into the adipose tissue in high-fat diet-fed mice in the observed reduced macrophage accumulation in $\alpha 4(Y991A)$ WAT.

$\alpha 4(Y991A)$ monocyte/macrophages exhibit reduced chemotaxis toward MCP-1. MCP-1 is an important monocyte chemoattractant and is particularly implicated in the mobilization of monocytes from the bone marrow and the infiltration of adipose tissue with monocyte/macrophages (34,35). Thus, we hypothesized that the decreased $7/4^{hi}Ly-6G^{neg}$ and $7/4^{dim}Ly-6G^{neg}$ cells in WAT of high-fat diet-fed $\alpha 4(Y991A)$ mice could be due to impaired migration in response to this chemokine. $\alpha 4(Y991A)$ bone marrow-derived macrophages showed reduced $\alpha 4$ integrin-dependent MCP-1-driven migration relative to wild-type cells (Fig. 5A). Thus, impaired $\alpha 4$ integrin-dependent monocyte/macrophage migration can account for the decreased number of monocyte/macrophages detected in WAT of fat-fed $\alpha 4(Y991A)$ mice.

Chemokines, such as MCP-1, are produced by macrophages, endothelial cells, and adipocytes (4,36,37). Deficiency in MCP-1 production and/or secretion could also contribute to an impairment of monocyte/macrophages migration into adipose tissue (4). Circulating MCP-1 was increased approximately twofold after high-fat diet, but no significant difference was observed between genotypes (Table 1). The relative expression of MCP-1 was also evaluated in the WAT of $\alpha 4(Y991A)$ and wild-type mice by RT-PCR (Fig. 5B). As expected from the circulating level of MCP-1, no difference was observed in MCP-1 mRNA expression in epididymal WAT between genotype in high-fat diet-fed mice (Fig. 5B). Thus, we ascribe the reduction in monocyte/macrophages in WAT in high-fat diet-fed mice to an impaired migratory response to MCP-1 rather than to reduced production of this chemokine.

The amelioration of insulin resistance in high-fat diet-fed $\alpha 4(Y991A)$ mice appears to depend on reduction of monocyte/macrophages in WAT; these cells are the source of cytokines, such as TNF- α and IL-6. Abdominal adipose gene expression levels of TNF- α , IL-6, plasminogen activator inhibitor 1 (PAI-1), and leptin are positively linked with insulin resistance. Levels of proinflammatory cytokines TNF- α and IL6 were strikingly reduced (70 and 55%, respectively) in $\alpha 4(Y991A)$ compared with wild-type WAT (Fig. 5C). In contrast, we observed similar levels for fat-derived peptides leptin and PAI-1 in wild-type and $\alpha 4(Y991A)$ WAT. Thus, the reduction in monocyte/macrophages in WAT in high-fat diet-fed $\alpha 4(Y991A)$ mice leads to reduced production of pro-inflammatory cytokines (TNF- α and IL-6) in WAT.

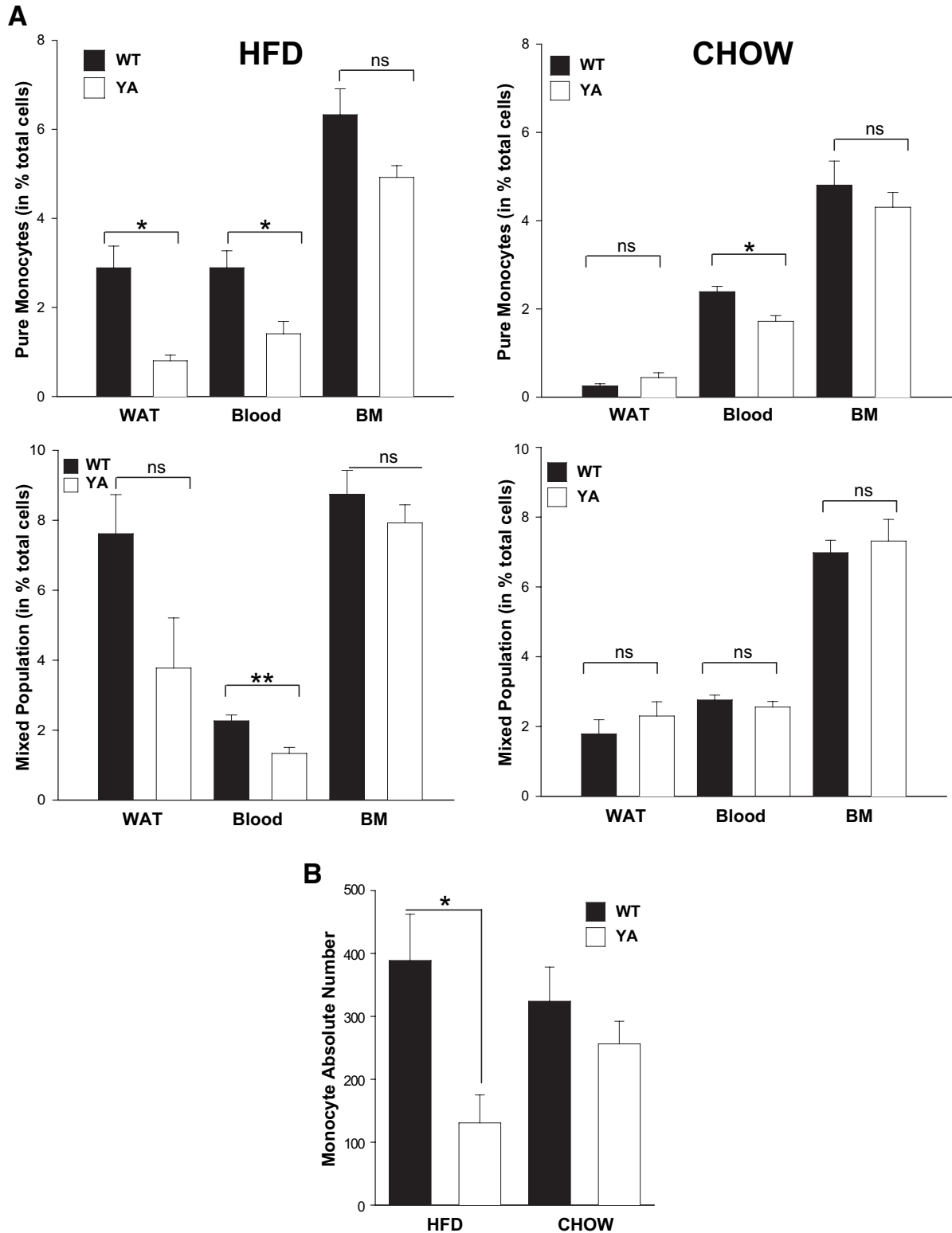


FIG. 4. High-fat diet-fed $\alpha 4$ (Y991A) mice exhibit a decreased number of adipose tissue and peripheral blood monocyte/macrophages. **A:** Stromal vascular cells of WAT, peripheral blood (blood) cells, and bone marrow (BM) cells were isolated from wild-type (■) and $\alpha 4$ (Y991A) (□) mice were stained for 7/4 and Ly-6G surface markers. Mice were fed with either high-fat diet (left) or normal chow (right). Quantification of 7/4^{hi}Ly-6G^{neg} (pure monocytes; top) and 7/4^{dim}Ly-6G^{neg} (mixed leukocyte population; bottom) cells was performed by flow cytometry. WAT of high-fat diet-fed $\alpha 4$ (Y991A) mice contain a decreased number of pure monocyte (7/4^{hi}Ly-6G^{neg}) cells compared with wild type. The mixed population (7/4^{dim}Ly-6G^{neg}) also shows a decrease in the high-fat diet-fed $\alpha 4$ (Y991A) WAT, although it does not reach significance. A slight monocytopenia (7/4^{hi}Ly-6G^{neg}) was measured in normal chow-fed $\alpha 4$ (Y991A) mice, which was aggravated upon high-fat diet feeding. The decreased 7/4^{dim}Ly-6G^{neg} cell number was observed only in peripheral blood of high-fat diet-fed $\alpha 4$ mutant mice. No significant differences were observed in monocyte/macrophage numbers in the bone marrow in the two genotypes regardless of diet. Values are means \pm SE. **B:** Quantification of monocytes from wild-type (■) and $\alpha 4$ (Y991A) (□) hemograms is shown. Cell morphology was used to identify monocytes. Note that $\alpha 4$ (Y991A) monocytopenia worsen when mice are fed a high-fat diet. Values are means \pm SE. *n* values per group are indicated. **P* < 0.05; ***P* < 0.01; ns, not significant.

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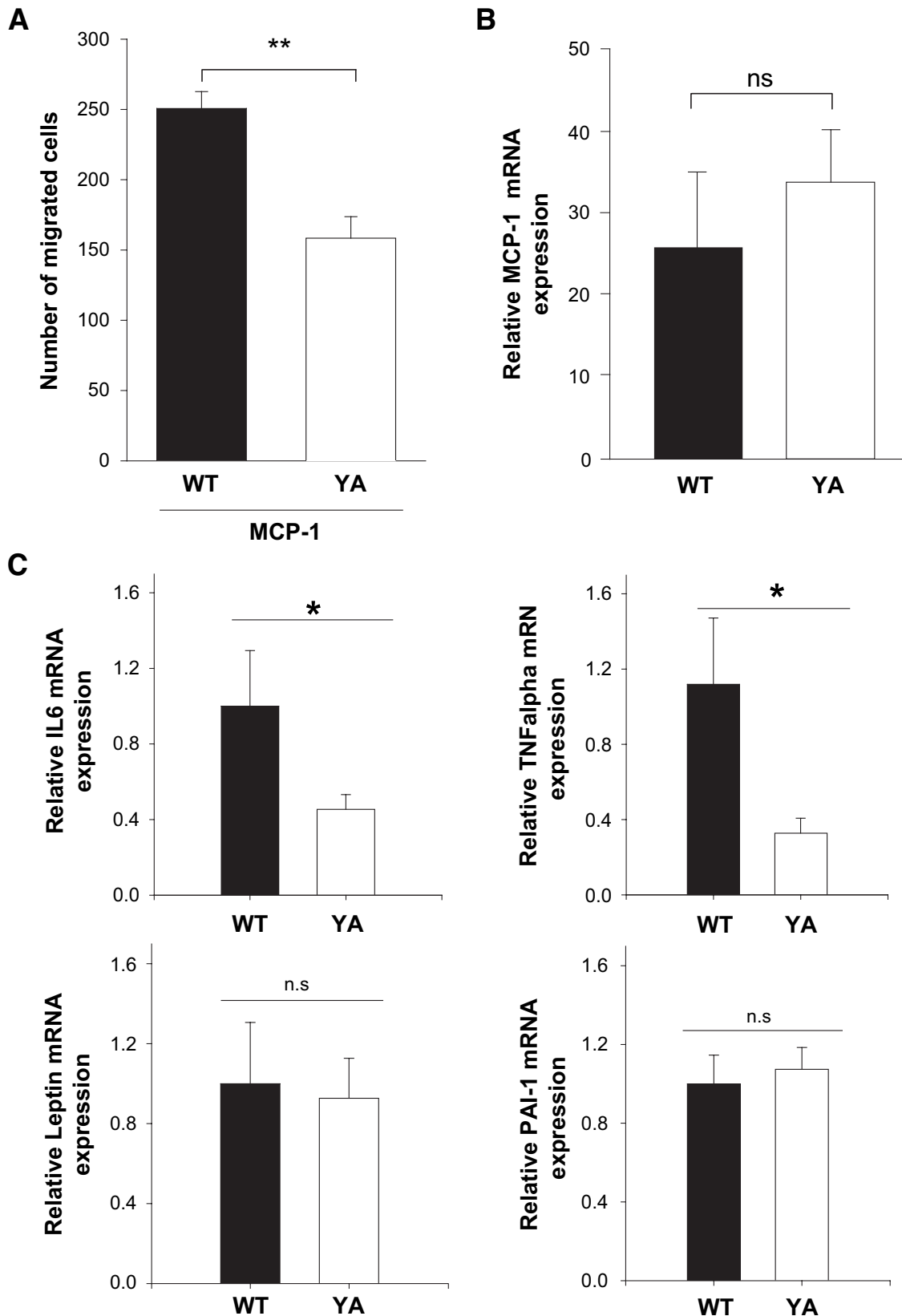


FIG. 5. $\alpha 4$ (Y991A) monocyte/macrophages show reduced migration in vitro in response to stimulation with MCP-1. **A:** Bone marrow–derived macrophages isolated from wild-type and $\alpha 4$ (Y991A) mice were added to the top chamber of a VCAM-1–coated transwell. Chemoattractant, MCP-1 (1 nmol/l), was added in the lower chamber. Cells were allowed to migrate for 16 h. After fixation, cells were stained with crystal violet, and the total number of migrated cells (bottom chamber) was enumerated. **B:** Levels of MCP-1 mRNA were determined in vivo in epididymal WAT from wild-type and $\alpha 4$ (Y991A) mice by using real-time RT-PCR. The data were normalized to the expression of V-ATPase. No significant difference was seen between genotypes. **C:** Levels of IL-6, PAI-1, TNF- α , and leptin mRNA were evaluated in vivo in WAT from wild-type and $\alpha 4$ (Y991A) mice by real-time RT-PCR. Both TNF- α and IL-6 levels are decreased in $\alpha 4$ (Y991A) compared with wild-type. Values are means \pm SE ($n = 14$). ** $P < 0.01$; ns, not significant.

DISCUSSION

$\alpha 4$ integrins are proven therapeutic targets in chronic inflammatory diseases, such as multiple sclerosis; however, complete blockade of $\alpha 4$ integrin function can result in defects in hematopoiesis, heart, and placental development (9,11,38,39) and is associated with progressive multifocal leukoencephalopathy (13). Chronic low-grade inflammation contributes to the development of insulin resistance (40), and the adipose tissue macrophage is a principal cell type responsible (27). Here, we report that mice bearing the $\alpha 4$ (Y991A) mutation are protected from high-fat diet-induced glucose intolerance and insulin resistance. The mutation did not block development of monocytes in the bone marrow but impaired their migration in response to MCP-1, leading to a combination of reduced egress into the blood and diminished accumulation in adipose tissue. Reduction of $\alpha 4$ (Y991A) monocyte/macrophages in WAT consequently diminished pro-inflammatory cytokine (IL-6 and TNF- α) production, which can explain the amelioration of insulin resistance in these mice. This is the first study showing a role for integrin signaling in the pathogenesis the metabolic consequences of diet-induced obesity.

$\alpha 4$ integrins are important in the pathogenesis of high-fat diet-induced insulin resistance because they mediate the localization of monocyte/macrophages to adipose tissue. In particular, we found that a point mutation that impairs $\alpha 4$ integrin signaling led to markedly improved glucose tolerance and insulin sensitivity in high-fat diet-fed mice. The $\alpha 4$ (Y991A) mice become obese on a high-fat diet, but they remained insulin-sensitive. This insulin-sensitive phenotype can be conferred by transplanting bone marrow from $\alpha 4$ (Y991A) mice into irradiated wild-type host animals. In the reverse experiment, fat-fed $\alpha 4$ (Y991A) mice receiving wild-type bone marrow were insulin resistant. Thus, bone marrow-derived cells are responsible for this protective effect, most likely by limiting accumulation of inflammatory adipose tissue macrophages. Recent studies have begun to classify macrophage subpopulations with differing roles in insulin resistance; future studies will be required to define effects of the $\alpha 4$ (Y991A) mutation on these subpopulations (41–44). It is noteworthy that improvement of glucose tolerance in mutant mice fed a high-fat diet is not complete. This implies that other factors could contribute to glucose intolerance induced by high-fat diet. These results add insulin resistance/type 2 diabetes to diseases in which $\alpha 4$ integrins may serve as therapeutic targets.

Consistent with our results, MCP-1 deficiency or deletion of the MCP-1 receptor (CCR2) reduced monocyte egress from the bone marrow (28) and accumulation of macrophages in adipose tissue (4), and MCP-1 or CCR2 KO mice are partially protected from high-fat diet-induced insulin resistance (4,45). Thus, deletion of CCR2 or its main ligand leads to a similar phenotype as shown here for the $\alpha 4$ (Y991A) mice. Furthermore, we demonstrated a defect in CCR2-driven chemotaxis in $\alpha 4$ (Y991A) monocytes. These relationships suggest that CCR2-mediated monocyte recruitment is linked to the binding of paxillin to the $\alpha 4$ integrin cytoplasmic domain.

The phenotypes observed in the $\alpha 4$ (Y991A) and $\alpha 4$ (Y991A)-BMT animals are remarkably similar to those observed in mice deficient in *Sorbs1* gene, which encodes cbl-associated protein (Cap) (19). These mice are also protected from high-fat diet-induced insulin resistance,

and this protection can be transferred to wild-type mice by BMT of Cap-null bone marrow. Furthermore, Cap deletion resulted in reduced numbers of monocytes/macrophages in both blood and adipose tissue, and Cap knockdown led to decreased migration in the RAW264.7 macrophage cell line. Previously, Cap was shown to mediate signals for the formation of stress fibers and focal adhesions through interaction with the focal adhesion kinase p125^{FAK}, an effector directly linked to paxillin binding to $\alpha 4$ integrin (15), and Cbl is required for macrophage spreading and migration. Thus, the relationship between Cap and $\alpha 4$ -paxillin interaction in the pathogenesis of insulin resistance is an area of potential future interest.

Chronic inflammation can be a primary mediator of obesity-induced insulin resistance, and inflammation is recognized as one of the contributors to the metabolic syndrome (or syndrome X) and type 2 diabetes (46). This study establishes a new role for $\alpha 4$ signaling in the development of high-fat diet-induced insulin resistance through its action on monocyte/macrophage trafficking. It also suggests that blockade of $\alpha 4$ signaling can improve insulin sensitivity and reduce inflammation, which could translate into clinical benefit in type 2 diabetes.

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