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Adiponectin Inhibits Allograft Rejection in Murine Cardiac Transplantation

Yoshihisa Okamoto[1,3,4,5], **Thomas Christen**[1], **Koichi Shimizu**[1], **Ken-ichi Asano**[2], **Shinji Kihara**[3], **Richard N. Mitchell**[2], and **Peter Libby**[1]

[1]Division of Cardiovascular Medicine, Brigham and Women's Hospital, Harvard Medical School

[2]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School

[3]Department of Metabolic Medicine, Graduate School of Medicine, Osaka University

[4]Department of Internal Medicine, Social Insurance Kinan Hospital

[5]Department of Bioregulation, Nippon Medical School, Graduate School

Abstract

Background—Low levels of plasma adiponectin, an adipocytokine that possesses anti-inflammatory and anti-atherogenic properties, frequently observed among obese subjects correlate with higher prevalence of several cardiovascular diseases. This study investigated whether adiponectin modulates allograft rejection in MHC class II—mismatched cardiac transplants.

Methods—We heterotopically transplanted Bm12 allografts into adiponectin-deficient (APN−/−, C57BL/6 background) or wild-type (APN+/+) mice. Some APN−/− mice received adiponectin reconstitution by adenovirus. Histological analyses assessed allograft rejection, and real-time RT-PCR evaluated the genes for cytokines/chemokines associated with the immune and inflammatory responses. In addition, we tested the effect of adiponectin on proliferation and cytokine/chemokine production in mouse T lymphocytes stimulated in vitro with anti-CD3 antibodies.

Results—Allografts transplanted to APN−/− mice showed severe acute rejection relative to transplants in APN+/+ hosts accompanied by increased accumulation of CD4- and CD8-positive T lymphocytes and Mac3-positive macrophages. Adiponectin provision by adenovirus in APN−/− mice reversed these exacerbated responses to allografting. The rejected allografts in APN−/− mice contained significantly higher levels of TNFα, IFNγ, and RANTES. Moreover, adiponectin significantly suppressed proliferation and production of TNFα, IFNγ, RANTES, MCP-1, and IP-10 in mouse T lymphocytes stimulated in vitro with anti-CD3 antibodies.

Address for Correspondence: Peter Libby, M.D. Division of Cardiovascular Medicine Brigham and Women's Hospital, Harvard Medical School NRB 741, 77 Avenue Louis Pasteur Boston, MA 02115, U.S.A. Tel: 1-617-525-4350 Fax: 1-617-525-4399 plibby@rics.bwh.harvard.edu.
[1]Division of Cardiovascular Medicine, Brigham and Women's Hospital, Harvard Medical School: NRB 7th Floor, 77 Avenue Louis Pasteur, Boston, MA 02115, U.S.A.
[2]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School: NRB 7th Floor, 77 Avenue Louis Pasteur, Boston, MA 02115, U.S.A.
[3]Department of Metabolic Medicine, Graduate School of Medicine, Osaka University: 2-2 Yamada-oka, Suita, Osaka, 565-0871, Japan
[4]Department of Internal Medicine, Social Insurance Kinan Hospital: 46-70 Shinjo-cho, Tanabe, Wakayama, 646-8588, Japan
[5]Department of Bioregulation, Nippon Medical School, Graduate School: 1-396 Kosugi-cho, Nakahara-ku, Kawasaki, Kanagawa, 211-8533, Japan

Authorship: Yoshihisa Okamoto[1,2,3,4] Thomas Christen[3,4], Koichi Shimizu[3], Ken-ichi Asano[3,4], Shinji Kihara[1], Richard N. Mitchell[1,2,4], and Peter Libby[1,2,4]
Participated in research design[1], in the writing of the paper[2], in the performance of the research[3], and in data analysis[4]
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Conclusions—These observations provide new mechanistic insight into immunoregulation in allograft recipients relative to obesity, an increasingly prevalent risk factor. Adiponectin may offer a new therapeutic target for allograft rejection after cardiac transplantation.

Keywords

Adiponectin; Cardiac transplantation; Rejection

Obesity, a condition associated with chronic systemic inflammation, constitutes a well-known risk factor for cardiovascular disease (1) (2). Grady et al. recently reported that postoperative obesity also increases the risk for morbidity and mortality after cardiac transplantation (3). However, the mechanism(s) that link obesity and rejection after heart transplantation remains unclear.

Adipose tissue secretes adipocytokines whose dysregulation associates closely with a variety of pathophysiologic abnormalities. In particular, obese individuals have low plasma levels of adiponectin, an anti-inflammatory and anti-atherogenic adipocytokine (4). Adiponectin appears to mitigate atherosclerosis, ischemic myocardial damage, and cardiac hypertrophy (5-7). No prior study has examined the effect of adiponectin in the setting of cardiac transplantation. This study therefore examined the effect of adiponectin on cardiac allograft rejection *in vivo* and *in vitro*.

Materials and Methods

Mouse Cardiac Transplantation

B6.C-H2<bm12>KhEg (bm12; H-2bm12, Jackson Laboratory, Bar Harbor, ME) donor hearts were transplanted heterotopically into B6 background adiponectin-deficient (APN−/−) (8) or littermate wild-type (APN+/+) mice (MHC class II mismatch) (9). For adiponectin reconstitution experiments, we injected adenovirus-expressing mouse adiponectin (Ad-APN) or β-galactosidase (Ad-βgal) into APN−/− mice through the tail veins 3 days before transplantation (2.0×10^8 plaque-forming unit / mouse). Plasma adiponectin levels were measured with mouse/Rat Adiponectin ELISA Kit (B-Bridge International, Mountain View, CA). Grafts were harvested 3 weeks (for gene analyses with quantitative RT-PCR) or 4 weeks (for histological analyses) after transplantation. Protocols for animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Harvard Medical School.

Histological Analyses

Hematoxylin-and-eosin and elastic fiber staining of paraffin-embedded tissue section were performed. Blinded observers scored parenchymal rejection (PR, 0 to 4, based on extent of inflammatory infiltrate and associated myocyte injury)(10). To quantify T lymphocytes and macrophages, cryosections were immunostained with antibodies against CD4, CD8, or Mac-3 (BD Biosciences, San Jose, CA) (11). Eight randomly chosen microscopic fields were examined to assess infiltrated CD4- or CD8-positive cells, as well as Mac-3 positive area (11).

Gene Expression Analysis by Quantitative RT-PCR

Total RNA isolated from allografts was reverse-transcribed, and real-time quantitative PCR with cDNA was performed (4). mRNA levels were quantified and expressed relative to GAPDH as an internal control. The Supplementary Table lists the sequence of primers used.

Splenocyte Proliferation and Proinflammatory Cytokine Production

For splenocyte proliferation assays, 2.0×10^5 naive splenocytes were treated with various concentrations of mouse recombinant adiponectin (BioVendor, Candler, NC) for 24 hours, washed, then stimulated in 96-well plates precoated with anti-CD3 mAb (1 μg/ml, BD Biosciences). Cells were cultured at 37°C in a 5% CO_2 incubator for 4 days. After 4 days of stimulation (day 4), a portion of the culture supernatants was removed, and assayed for mouse inflammatory cytokine using a cytometric bead array kit (BD Biosciences) as well as ELISA kits for mouse RANTES and IP-10 (R&D, Minneapolis, MN). Lymphocyte proliferation was monitored by pulsing the wells for 6 h with 1 μCi/well of [^{3}H]thymidine (New England Nuclear, Boston, MA). Proliferation was measured as incorporated radioactivity (cpm) (10).

Statistical Analyses

Data are shown as mean ± SEM. Groups were compared using the Student t-test. Between-group comparison of means was performed by ANOVA, followed by t-test. A value of $p<0.05$ was regarded as a significant difference.

Results and Discussion

Four weeks after transplantation, palpation and inspection revealed markedly reduced contraction force in allografts transplanted to APN−/− mice. We consequently harvested all the allografts before or at that time point to perform a meaningful comparison of the extent and severity of rejection between control and experimental groups. Allograft histology, even within hours of cessation of contraction, is markedly confounded by a diffuse acute inflammatory response to necrobiotic tissue, and therefore does not provide an accurate assessment of rejection. These qualitative observations suggested marked functional impairment at earlier timepoints in allografts implanted into APN −/− hosts versus control wild-type animals. Even in the contracting APN−/− hosts, markedly increased numbers of leukocytes accumulated in allografts relative to wild-type APN+/+ mice; this was also accompanied by early tissue necrosis and hemorrhage, presumably related to rejection involving the allograft vasculature (Figure 1A and Supplementary Figure 1, upper panels). APN deficiency in recipients markedly exacerbated parenchymal rejection (PR) when compared at the same timepoints in viable beating hearts (APN+/+: 2.15±0.24, APN−/−: 3.92±0.08, $p<0.0001$, n=10 and 6 respectively, Figure 1B). We also injected adiponectin-expressing adenovirus into APN−/− mice (APN−/− +Ad-APN) to test the hypothesis that provision of adiponectin would reduce PR. When mice were killed, circulating adiponectin levels in APN−/− +Ad-APN were 19.9±2.4 μg/ml (within the physiological range) while those in APN+/+, APN−/− and APN−/− + Ad-β-gal were 16.7 ±0.5, <0.05, <0.05 μg/ml, respectively. Reconstitution of mouse adiponectin to APN−/− mice substantially ameliorated PR (Figure 1A and Supplementary Figure 1, lower panels) and significantly reduced PR scores (APN−/− + Ad-β-gal: 3.40±0.25 vs. APN−/− +Ad-APN: 1.70 ±0.26, $p<0.01$, n=5 each, Figure 1B). These findings demonstrate that adiponectin mitigates parenchymal rejection of allografts. The coronary arteries of allografts in APN−/− recipient mice showed marked lumenal accumulation of leukocytes yielding subtotal occlusion; adiponectin supplementation reversed this abnormality (Supplementary Figure 1B). We do not interpret the pathology as graft arterial disease (GAD) because GAD lesions contain a more fibrous component, consisting of vascular smooth muscle cells and extracellular matrix, and develop at later time points (usually 8-12 weeks after transplantation) in MHC class II—mismatched mice (12). However, this coronary occlusion due to leukostasis may cause myocardial ischemia leading to subsequent graft failure. Moreover, adiponectin may directly protect cardiomyocytes, as previously suggested (7).

Post-transplantation, parenchymal graft rejection involves both adaptive and innate immune responses. During parenchymal rejection, inflammatory cells, including T lymphocytes and

macrophages, infiltrate the allografts and produce various cytokines and chemokines. Adiponectin deficiency in the recipient led to significantly greater accumulation of CD4 or CD8 positive T lymphocytes and Mac-3 positive macrophages in the transplanted hearts (CD4: 283±13.8 vs. 828±148.6 cells/mm^2, $p<0.05$; CD8: 296±114.2 vs. 950±83.2 cells/mm^2, $p<0.01$; Mac-3: 3.62±1.19 vs. 8.41±0.95 % $p<0.05$, n=4 and 6 respectively, Figure 1C-1E). Adiponectin reconstitution by adenovirus significantly reduced infiltration of these cells compared vs. control (CD4: 678±89.0 vs. 233±41.8 cells/mm^2, $p<0.01$; CD8: 756±65.2 vs. 185±14.6 cells/mm^2, $p<0.0001$; Mac-3: 7.52±0.73 vs. 3.21±0.53 %, $p<0.01$, n=5 each, Figure 1C-1E).

TNFα and IFNγ stimulate inflammation while RANTES and IP-10 recruit T lymphocytes and MCP-1 attracts mononuclear phagocytes. In addition, previous studies suggested the reciprocal association between adiponectin and RANTES or IP-10 and recruitment of T lymphocytes (5,13). Allografts in APN−/− mice have significantly higher mRNA levels for TNFα(100.0 ±19.5 vs. 300.7±23.5%, n=5 each, p<0.001, Figure 2A) and IFNγ (100.0±20.3 vs. 441.3 ±85.6%, n=5 each, p<0.01, Figure 2B) compared with allografts in APN+/+ mice, as determined by real-time quantitative RT-PCR. Furthermore, APN−/− recipients had significantly higher levels of RANTES mRNA (CCL5, 100.0±15.7 vs. 268.5±46.0, n=5 each, p<0.01, Figure 2C), although APN−/− recipients tend to contain more mRNA for MCP-1 (CCL2) and IP-10 (CXCL10) (MCP-1; 100.0±7.6 vs. 301.3.±87.4, n=5 each, p=0.051, Figure 2D: IP-10; 100.0±22.7 vs. 385.9±129.7, n=5 each, p=0.062, Figure 2E). However, no difference emerged in expression of mRNAs that encode CCR1, CCR2, and CXCR3, cognate receptors for RANTES, MCP-1, and IP-10, between allografts in APN+/+ and APN−/− mice (Figure 2F-2H). For this analysis, we used allografts harvested at 3 weeks after transplantation. Allografts in APN−/− recipients harvested at 4 weeks contained diffuse regions of necrosis related to the loss of viability as reflected by loss of contractility (as shown in Figure 1A and Supplementary Figure 1, upper panels); such necrotic specimens did not allow the isolation of high-quality RNA. We therefore decided not to use 4-week samples for real-time quantitative RT-PCR analysis. Moreover, in the pathogenesis of allograft rejection, chemokine expression (e.g., RANTES, MCP-1, and IP-10) would logically precede leukocyte infiltration. Our data indicate that within 3 weeks of transplantation, the presence of recipient adiponectin correlated with suppression of chemokine expression within the allograft, with a consequent diminution of leukocyte infiltration at later timepoints (Figure 1C-1D, and Figure 2F-2H). We interpret these results to suggest that adiponectin can limit allograft rejection by suppressing the expression of local cytokine/chemokine ligands that mediate inflammation and immune cell recruitment.

To test the hypothesis that adiponectin directly limits T lymphocyte proliferation and cytokine/chemokine production, we stimulated splenocytes from APN+/+ mice for four days with anti-CD3 antibody, a polyclonal activator of T cells. Adiponectin, within a physiological range, significantly reduced T lymphocyte proliferation in a concentration-dependent manner (by 78.2% at concentration of 10 μg/ml, p<0.001, n=6, Figure 3A). Moreover, based on assay of supernatants from these anti-CD3 stimulated splenocytes, adiponectin significantly suppressed production of cytokines/chemokines such as TNF-α, IFNγ, RANTES, MCP-1, and IP-10 in a concentration-dependent manner (by 96.0, 99.5, 85.2, 81.5, and 83.8%, at concentration of 10 μg/ml vs. anti-CD3 stimulation alone, p<0.01, <0.0001, <0.001, <0.01, and <0.0001, n=3, respectively, Figure 3B). Since reduced lymphocyte apoptosis can also increase accumulation of T lymphocytes in allograft, we assessed T cell apoptosis by TUNEL staining. Histological analysis by double staining of TUNEL and CD4 or CD8 did not demonstrate increased death of T cells (data not shown). These findings suggest that adiponectin inhibits the accumulation of T lymphocytes in MHC class II mismatched cardiac allograft by suppressing T lymphocyte proliferation as well. Since adiponectin circulates in the bloodstream and may modulate immunological reactions in the spleen, the spleen may play a part in the effect of adiponectin on cardiac allograft rejection observed in the present study.

Taken together, higher plasma adiponectin levels may prove beneficial for outcome among recipients of cardiac transplant. Several drugs such as thiazolidinediones and statins increase plasma levels of adiponectin (14-16). Interestingly, such drugs may also protect against cardiac allograft rejection and transplant vasculopathy (9,17,18). Over all, the present study suggests that adiponectin reduces allograft rejection by blocking lymphocyte proliferation and recruitment. These observations provide new mechanistic insight into immunoregulation in allograft recipients in relation to obesity, a risk factor of increasing prevalence. Moreover, adiponectin may represent a new therapeutic target for allograft rejection after cardiac transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APN, adiponectin
MHC, Major Histocompatibility Complex
RT-PCR, Real-Time Polymerase Chain Reaction
PR, Parenchymal Rejection
GAPDH, glyceraldehyde 3-phosphate dehydrogenase
TNFα, Tumor Necrosis Factor α,
IFNγ, Interferon γ,
RANTES, Regulated upon Activation Normal T Expressed and presumably Secreted
MCP-1, Monocyte Chemotactic Protein-1
IP-10, Interferon γ inducible protein-10
TUNEL, TdT-mediated dUTP-biotin Nick End Labeling

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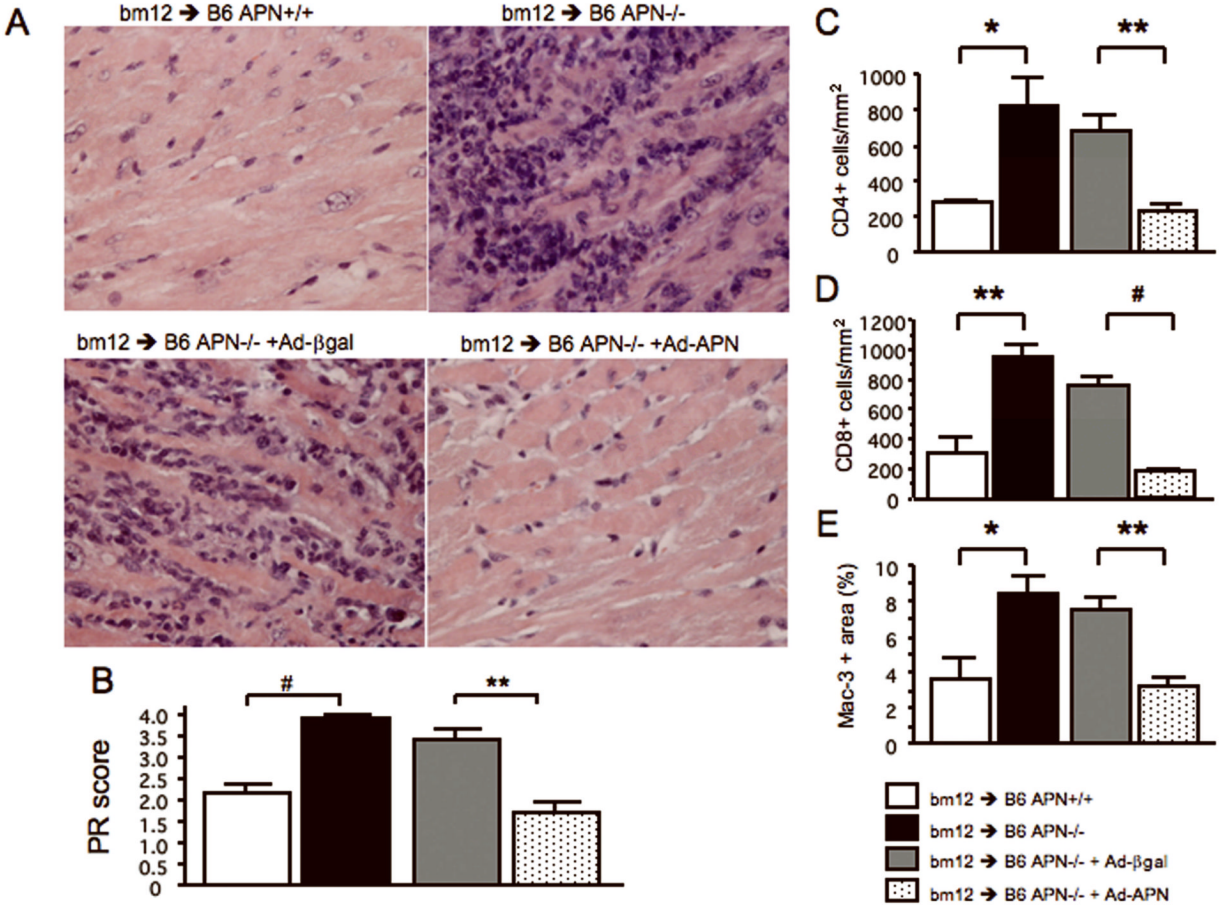

Figure 1.

Representative sections of allografts stained with Hematoxylin-Eosin [4 weeks after transplantation with high magnification (A)]. PR (B) in allografts (transplanted to APN+/+, APN−/−, APN−/−+Ad-βgal, and APN−/−+Ad-APN mice; n=10, 6, 5, and 5 respectively) were assessed by blinded pathologic evaluation. Immunostaining for mouse CD4 (C), CD8 (D), and Mac-3 (E) in allografts (transplanted to APN+/+, APN−/−, APN−/−+Ad-βgal, and APN−/−+Ad-APN mice; n=4, 6, 5, and 5 respectively). The numbers of CD4- (C) and CD8- (D) positive cells and Mac-3-positive area are expressed as number of cells per mm^2, and the percentage of positively stained areas for Mac-3 (E), respectively. Results are shown as mean ± SEM. *$p<0.05$, **$p<0.01$ and $^{\#}p<0.0001$.

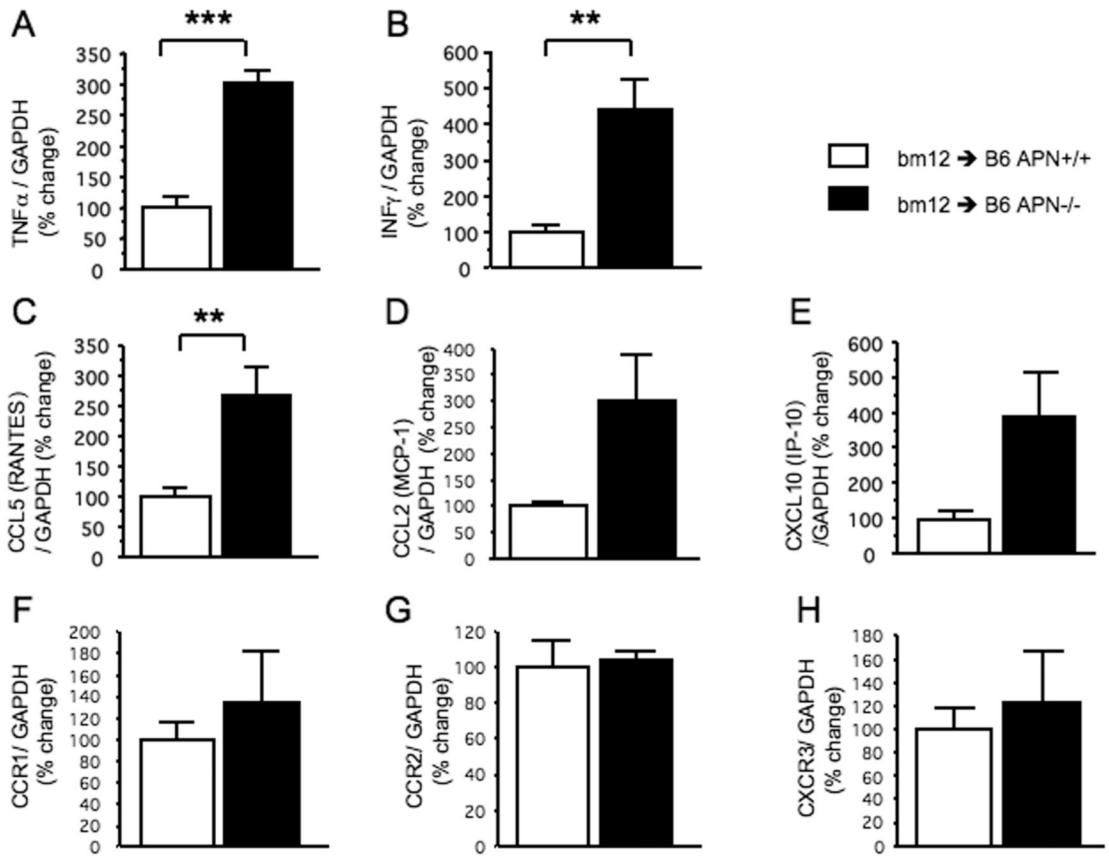

Figure 2.

Real-time quantitative RT-PCR determined mRNA expression levels for inflammatory cytokines; TNFα (A), IFNγ (B), chemokines; CCL5 (RANTES, C), CCL2 (MCP-1,D), and CXCL10(IP-10, E), and chemokine receptors; CCR1 (F), CCR2 (G), and CXCR3 (H). The mRNA levels encoded by these genes were quantified and expressed after adjusting relative to those of GAPDH as an internal control (n=5 each group). Results are shown as mean ± SEM. $^{**}p<0.01$, $^{***}p<0.001$.

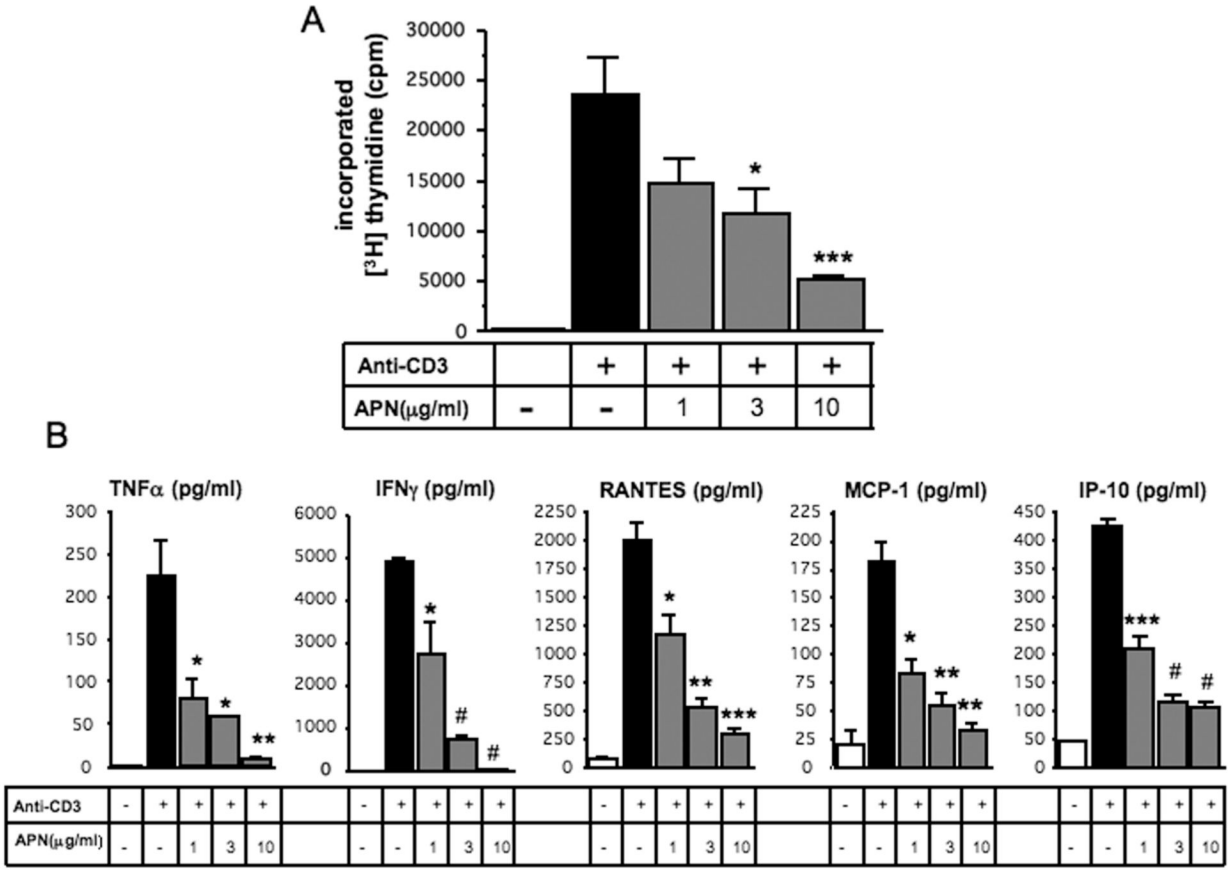

Figure 3.

Splenocytes from C57BL/6 (APN+/+) mice were stimulated for 4 days with anti-CD3 antibody following treatment with the indicated concentrations of mouse adiponectin. Proliferation was evaluated by [^{3}H]thymidine incorporation (A). TNFα, IFNγ, MCP-1, RANTES, and IP-10 were measured in the supernatant of these splenocytes as described in Materials and Methods (B). Results are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 and $^{\#}p$<0.0001.