

CD4⁺ T lymphocytes produce adiponectin in response to transplants

Sreedevi Danturti,^{1,2} Karen S. Keslar,¹ Leah R. Steinhoff,¹ Ran Fan,¹ Nina Dvorina,¹ Anna Valujskikh,¹ Robert L. Fairchild,¹ and William M. Baldwin III¹

¹Department of Immunology, Cleveland Clinic, Cleveland, Ohio, USA. ²Department of Biological, Geological, and Environmental Sciences, Cleveland State University, Cleveland, Ohio, USA.

Adiponectin is a pleiotropic cytokine with diverse immunomodulatory effects on macrophages and lymphocytes. In the current paradigm, lymphocytes and macrophages respond to adiponectin that is produced by adipocytes and other parenchymal cells. Using a model of chronic arterial inflammation in cardiac transplants, we found that T cells derived from the recipient migrate to the heart and produce adiponectin locally. The evidence that T cells produce significant amounts of adiponectin is based on 3 experimental approaches. First, CD4⁺ T cells isolated from the blood and spleen after cardiac transplantation express mRNA for adiponectin. Second, reconstitution of T cell-deficient recipients with transgenic CD4⁺ T cells that express receptors for donor antigens results in arterial infiltrates containing T cells and increased mRNA expression for adiponectin in cardiac transplants. Third, CD4⁺ T cells isolated from the allograft secrete adiponectin *in vitro*. Taken together, these data indicate that adiponectin-competent cells originating in the recipient migrate into the transplant. Establishing T cells as a source of adiponectin provides a new dimension, to our knowledge, to the modulatory effects of adiponectin on immune responses.

Introduction

Adiponectin is a pleiotropic cytokine that has been associated with protective effects on the cardiovascular system (1). In healthy individuals, adiponectin circulates in large amounts (3–30 $\mu\text{l/ml}$). Originally described as being produced exclusively by adipose tissue (2), adiponectin has more recently been found to be produced by smooth muscle cells (3), cardiac myocytes (4), endothelial cells (5), and epithelial cells (6). Adiponectin binds to 2 signaling receptors: ADIPOR1 and ADIPOR2. These receptors are abundantly expressed on skeletal muscle and liver, but also on leukocytes, vascular endothelial cells, and smooth muscle cells (7). T-cadherin is a critical receptor on myocardial cells for the cardioprotective effects of adiponectin in ischemia reperfusion (1). As a result, adiponectin has been found to modulate many aspects of cardiac and vascular inflammation.

The expression of ADIPOR1 and ADIPOR2 on macrophages is dynamic and is linked to macrophage polarization in response to cytokines. Adiponectin receptor expression is decreased on macrophages during inflammation compared with wound healing (8). Moreover, adiponectin induces divergent responses from inflammatory macrophages and wound-healing macrophages. Macrophages isolated from adiponectin-KO mice have higher expression of proinflammatory cytokines, such as TNF α , monocyte chemoattractant protein-1 (MCP-1; CCL2), and IL-6, compared with WT mice. In contrast anti-inflammatory mediators, such as arginase-1 and IL-10, are significantly lower in KO mice when compared with WT mice (9).

Numerous studies have correlated low levels of adiponectin in the circulation with vascular inflammation (10). However, the significance of this correlation varies greatly among studies. In genetically modified mice, adiponectin deficiency increases neointimal formation in arteries that are subjected to mechanical endovascular injury (11) or external vascular cuff injury (12). Replacement of adiponectin to deficient mice inhibits TNF α -induced monocyte adhesion to vessels by decreasing expression of the adhesion molecules (E-selectin, ICAM-1, and VCAM-1) on endothelial cells (13).

With the appreciation that a range of tissues can produce and respond to adiponectin, the potential for adiponectin to exert paracrine and autocrine effects has gained attention. Paracrine or autocrine effects of endogenously produced adiponectin have been reported for skeletal muscle, cardiomyocytes, and arterial smooth muscle cells (3, 4, 14).

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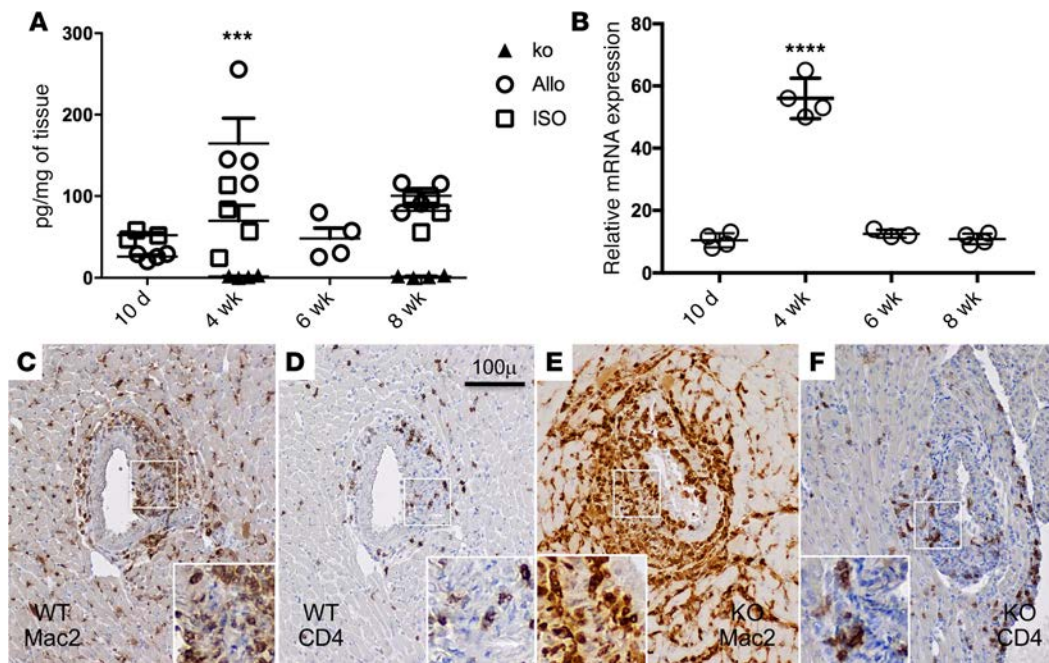


Figure 1. Adiponectin and macrophage infiltrates are increased in cardiac allografts 4 weeks after transplantation. (A) Adiponectin protein was measured by ELISA in tissue homogenates from cardiac isografts (open squares) and allografts (open circles) in WT mice, as well as allografts in adiponectin-KO mice (closed triangles) at 10 days to 8 weeks ($n = 4$ per group). (B) Message for adiponectin in the allografts of WT mice was calculated as fold change compared with isografts ($n = 4$ per group). The \pm SEM is represented in each group. Significant differences are indicated as **** $P < 0.00001$ and *** $P < 0.0001$ by 1-way ANOVA. (C and D) IHC for Mac2⁺ macrophage and CD4⁺ T cell infiltrates in WT cardiac allografts in WT recipients. (E and F) Mac2⁺ macrophage infiltrates and CD4⁺ T cell infiltrates in adiponectin-KO allografts to knock out recipients. Original magnification of C–F is 100 \times ; scale bar: 100 μ m (inserts are enlargements of the boxed areas encompassing the arterial wall). Positive cells are identified by brown precipitate.

The availability of mice with complete genetic elimination of adiponectin provides an opportunity to examine the different sources of adiponectin in vascular inflammatory responses. To dissect the impact of local and systemic sources of adiponectin, we transplanted hearts between informative combinations of adiponectin-KO mice. Hearts were transplanted from male to female C57BL/6 (B6) mice to induce a progressive chronic inflammatory injury in the coronary artery over a 6–8 week time frame as previously reported (15). We were able to exploit transgenic mice that express T cell receptors specific for antigens encoded on the Y chromosome of B6 males (the H-Y antigen) to determine sources of adiponectin production (16). In addition, we developed in vitro assays to quantify production of adiponectin by isolated T cell populations. Our combined experiments support the concept that, over time, CD4⁺ T cells evolve into a biologically significant source of adiponectin that regulates the development of chronic inflammation in coronary arteries.

Results

Adiponectin levels peak at 4 weeks after transplantation in male hearts allografted to female recipients. Expression of adiponectin protein was assessed by ELISA on tissue homogenates of isografts and allografts. From 10 days to 8 weeks after transplantation, the adiponectin levels increased gradually from about 40 pg/mg to 100 pg/mg of tissue in the control isografts. In the allografts, adiponectin was in the control range at 10 days but peaked at 4 weeks (115–260 pg/mg) and returned to control range at 6 and 8 weeks (Figure 1A). As expected, adiponectin was undetectable in allografts of KO mice.

In order to determine whether some of the adiponectin protein contained in the graft is produced locally, expression of adiponectin mRNA in the allografts was assayed by RT-PCR. Similar to the levels of protein, expression of adiponectin mRNA peaked at 4 weeks after transplantation in the allografts when compared with isografts (Figure 1B).

Macrophage infiltrates of cardiac allografts are expanded in the absence of adiponectin. As previously reported, cardiac allografts from WT male B6 donors to WT female B6 recipients elicit an acute interstitial cellular infiltrate that is predominantly composed of CD4⁺ T cells and macrophages (15, 16). After this

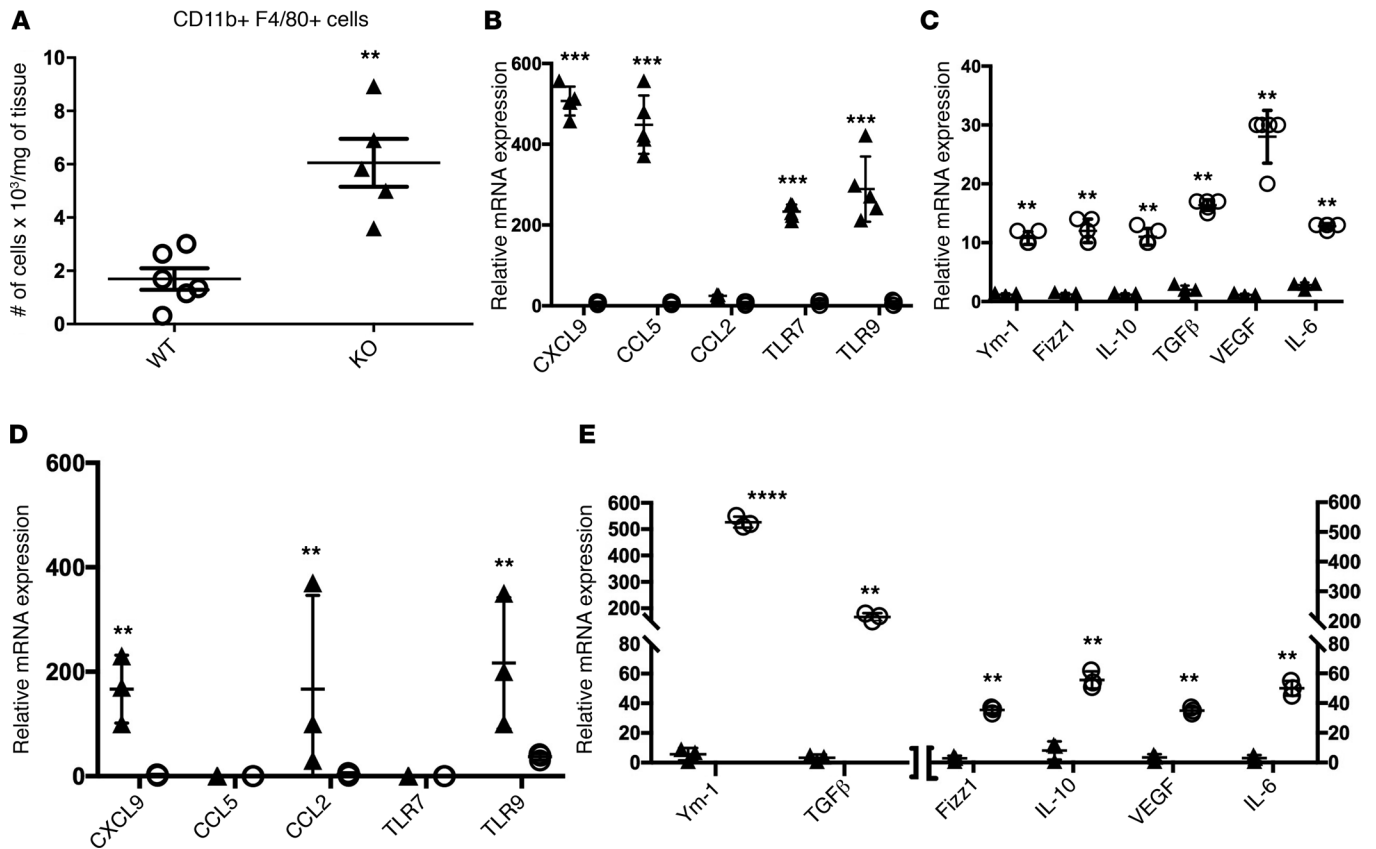


Figure 2. Adiponectin modifies the expression of cytokines by macrophages. (A) Cells infiltrating cardiac allografts were isolated, and macrophages were identified by expression of CD11b and F4/80 in flow cytometry. The number of CD11b⁺ and F4/80⁺ cells per mg of tissue were calculated for allografts to WT ($n = 6$) and adiponectin-KO ($n = 5$) recipients. (B and C) Isolated infiltrating macrophages expressed increased message for CXCL9, CCL5, CCL2, TLR7, and TLR9 in the adiponectin-KO mice (closed triangles) compared with the WT mice (open circles). Expression of Ym-1, Fizz1, IL-10, TGFβ, VEGF, and IL-6 were decreased in the adiponectin-KO mice compared with WT mice. (D and E) Isolated macrophages were cultured for 3 days in the presence of 10 μg/ml recombinant adiponectin (open circles) or absence of adiponectin (closed triangles). In the absence of adiponectin, macrophages maintained increased expression of CXCL9, CCL2, and TLR9. Adiponectin induced an increase in Ym1, TGFβ, Fizz1, IL-10, VEGF, and IL-6. Each symbol represents 1 animal, and bars indicate ± SEM. RT-PCR results are expressed as fold changes normalized to β actin. Significant differences are indicated as **** $P < 0.00001$, *** $P < 0.0001$, and ** $P < 0.005$. Student t test nonparametric was performed for A. A 2-way ANOVA was performed with the Bonferroni-Dunn method as a post hoc correction for multiplicity of gene testing for B-E.

resolved, a progressive arterial infiltrate composed primarily of macrophages developed. The macrophage infiltrates were extensive by 4 weeks after transplantation when adiponectin levels peaked (Figure 1C). By 4 weeks, only moderate numbers of CD4⁺ T cells are interspersed within the macrophages (Figure 1D), even though these cells are required to orchestrate the macrophage infiltrates (15, 16).

Cardiac allografts from adiponectin-KO male B6 donors to adiponectin-KO female B6 recipients resulted in more macrophages around arteries by 4 weeks, but T cell numbers were largely unchanged (Figure 1, E and F). Planimetry measurements of the allografts at 4 weeks confirmed that the periarterial macrophage infiltrates were more extensive in the absence of adiponectin (data not shown).

The infiltrates of macrophages into the allografts of WT and adiponectin-KO mice at 4 weeks were assessed directly by digesting harvested grafts to prepare single cell suspensions for phenotypic analysis by flow cytometry. Adiponectin-KO mice had a 3-fold increase in number of infiltrating macrophages expressing CD11b and F4/80 compared with the WT allografts at 4 weeks (Figure 2A). RT-PCR on RNA isolated from the infiltrating macrophages demonstrated increased expression of chemokines (CXCL9, CCL5, CCL2) and TLR7 and TLR9 in the adiponectin-KO mice compared with WT mice at 4 weeks (Figure 2B). At the same time, macrophages isolated from adiponectin-deficient allografts had decreased expression of eosinophil chemotactic factor-lymphocyte (ECF-L; Ym-1), Fizz1 (found in inflammatory zone), IL-10, TGFβ, VEGF, and IL-6 (Figure 2C).

We reported previously that macrophage infiltrates in allografts are associated with extensive

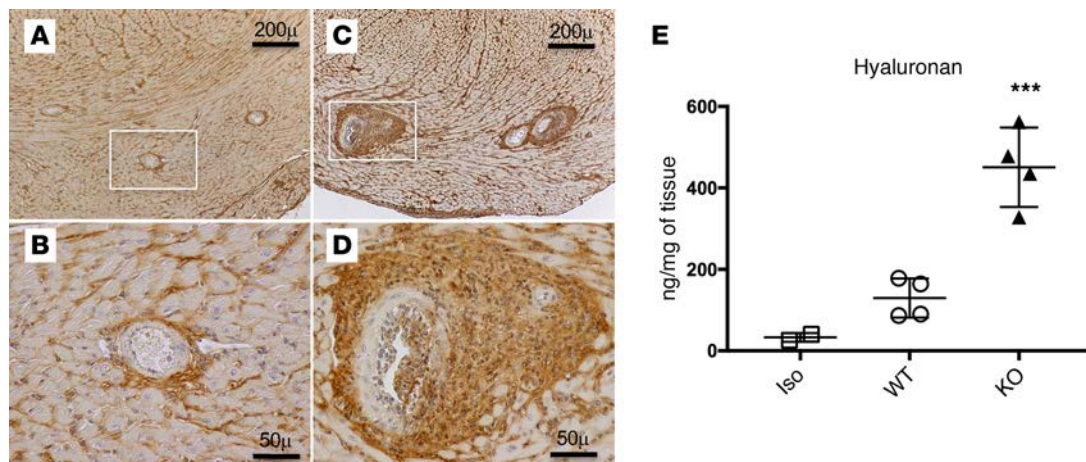


Figure 3. Hyaluronin is increased in cardiac allografts in the absence of adiponectin. (A–D) Immunohistology demonstrated increased areas of hyaluronin (brown) associated with macrophage infiltrates in cardiac allografts in adiponectin-KO mice (C, low magnification, and D, high magnification of boxed artery) compared with WT mice (A, low magnification, and B, high magnification of boxed artery) (E) Hyaluronan protein measured by ELISA in tissue homogenates from isografts (open squares; $n = 3$), WT allografts (open circles; $n = 4$), and adiponectin-KO allografts (closed triangles; $n = 4$) at 4 weeks. Each symbol represents 1 animal in each group, and bars indicate \pm SEM. Significant differences are indicated as $***P < 0.0001$ by 1-way ANOVA.

extracellular matrix remodeling, as evidenced by accumulation of hyaluronan in the arterial compartments (15). Immunohistology confirmed that the macrophages were enmeshed in hyaluronan (Figure 3, A–D). Hyaluronan was quantified by ELISA on graft homogenates and found to be increased about 3-fold in the absence of adiponectin (Figure 3E).

Adiponectin modifies the expression of cytokines by macrophages. In order to directly test the effects of adiponectin on macrophages in allografts, transplants were harvested at 4 weeks after transplantation to adiponectin-KO recipients, and F4/80-expressing macrophages were separated with magnetic beads from single cell suspensions. These cells were cultured in macrophage conditioning medium in the presence or absence of recombinant adiponectin. After 3 days of culture in the absence of adiponectin, isolated macrophages maintained high expression of CXCL9, CCL2, and TLR9 (Figure 2D). In contrast, expression of CXCL9, CCL2, and TLR9 were decreased by recombinant adiponectin, and expression of YM-1, Fizz1, IL-10, TGF β , VEGF, and IL-6 were increased (Figure 2E).

T cells are a significant source of adiponectin. The finding that mRNA for adiponectin was expressed in allografts indicated that either cells intrinsic to the heart or infiltrating cells produce adiponectin. Therefore, we performed a series of experiments to determine the source of adiponectin.

The potential cardiac sources of adiponectin were tested by transplanting hearts from WT males into female adiponectin-KO recipients. The reverse combination was performed to test whether infiltrating recipient cells were the source of adiponectin message. All the grafts were harvested 4 weeks after transplantation. RT-PCR demonstrated that KO hearts transplanted to WT recipients expressed high amounts of message for adiponectin, but only low levels of adiponectin were expressed in the reverse combination (Figure 4A). KO hearts transplanted to WT recipients also contained elevated amounts of adiponectin protein compared with WT hearts transplanted to KO recipients (Figure 4B). Moreover, the pattern of cytokines expressed in adiponectin-KO hearts transplanted to WT recipients was similar to WT hearts allografted to WT recipients with low amounts of CXCL9, CCL5, CCL2, TLR7, and TLR9 but high amounts of YM-1, Fizz1, IL-10, TGF β , VEGF, and IL-6 (Figure 4, C and D). These data indicate that cells from the recipient were infiltrating the grafts and producing adiponectin locally.

Therefore, circulating leukocytes were isolated from the blood of WT recipients 4 weeks after transplantation using magnetic beads, and RNA was isolated from the negatively separated populations. Preliminary studies on separated cell populations revealed that T cells expressed adiponectin (data not shown). In subsequent experiments, CD4 $^{+}$ and CD8 $^{+}$ T cells were isolated by negative selection, and the isolated CD4 $^{+}$ T cell populations from both the blood and spleen were found to express adiponectin (Figure 5A). The CD4 $^{+}$ T cell population also expressed CD4, which validated the isolation procedure. The CD8 $^{+}$ populations did not produce adiponectin but expressed CD8.

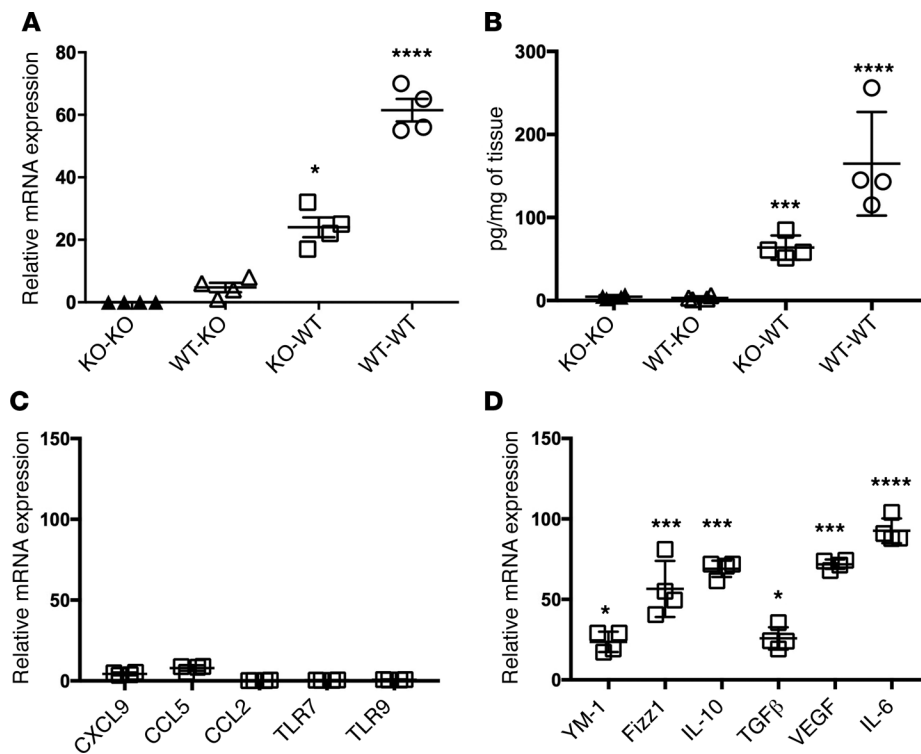


Figure 4. Adiponectin is produced by cells infiltrating allografts and modulates cytokine production by macrophages. (A) Message for adiponectin in tissue homogenates of allografts from adiponectin-KO male donors to KO female recipients (closed triangles), WT male donors to adiponectin-KO female recipients (open triangles), KO male donors to WT female recipients (open squares), and WT male donors to WT female recipients (open circles) at 4 weeks ($n = 4$ per group). (B) Adiponectin protein measured by ELISA in the same allografts. (C and D) Message for CXCL9, CCL5, CCL2, TLR7, and TLR9 and message for Ym1, Fizz1, IL-10, TGF β , VEGF, and IL-6 in tissue lysates of adiponectin-KO hearts transplanted into WT recipients. Each symbol represents 1 animal, and bars indicate \pm SEM. Significant differences are indicated as **** $P < 0.00001$, *** $P < 0.0001$, and * $P < 0.05$ by 1-way ANOVA.

After identifying that CD4⁺ T cells express message for adiponectin, CD4⁺ T cells were isolated from the blood spleen and allograft at serial times after transplantation. Low amounts of adiponectin message were detected in CD4⁺ cells isolated from blood and spleen of naive mice. The expression of mRNA increased first in CD4⁺ T cells in the blood and then in the spleen. Expression of mRNA for adiponectin remained elevated in both the blood and spleen through 4 weeks (Figure 5B).

In order to determine whether CD4⁺ cells secrete adiponectin protein in response to transplants, CD4⁺ cells were isolated from the spleen and graft 4 weeks after transplantation and stimulated polyclonally with bead-conjugated antibodies to CD3 and CD28 for 1 day. CD4⁺ T cells isolated from both spleen and graft produced adiponectin in response to polyclonal stimulation (Figure 5C).

Frequency of CD4⁺ T cells secreting adiponectin in spleen and transplants. Crosslinking of CD3 and CD28 causes polyclonal stimulation of all T cells and does not realistically reflect antigen-specific responses of T cells. Therefore, isolated CD4⁺ cells were stimulated with the relevant alloantigen, and the CD4⁺ T cells that secreted adiponectin were enumerated in an ELISPOT assay. Significantly more cells from the allograft than the spleen (about 100 cells vs. 10 cells per million cells) produced adiponectin in response to H-Y antigen (Figure 6A). This suggests that CD4⁺ T cells secrete adiponectin in the allograft in response to specific antigenic stimulation.

To confirm that antigen-specific T cells are a source of adiponectin in allografts in vivo, hearts were transplanted from WT male B6 mice into female B6 Rag1-deficient recipients (B6.RAG^{-/-}), which lack mature T and B cells. One week later, we reconstituted the recipients with T cells in limited numbers (1×10^3 or 2×10^4 CD4⁺) from transgenic female B6 mice that express T cell receptors for H-Y peptides in the context of H2-IA^b (Marilyn mice). A third group of mice received 2×10^4 CD4⁺ T cells from naive WT B6 males. The mice were euthanized 4 weeks after cell transfer, and adiponectin protein was measured by ELISA. The recipients reconstituted with 2×10^4 Marilyn cells expressed high amounts of adiponectin compared with mice that received either the naive CD4⁺ T cells or 1×10^3 Marilyn cells (Figure 6B). Significant numbers of T cells were detected in arteries by immunohistology in the group reconstituted with 2×10^4 Marilyn cells but not in the other two groups (Figure 6, C and D).

Discussion

The initial model of adiponectin as a protein with endocrine functions that is secreted exclusively by adipose tissue and circulates to modulate target cells and tissues (2) has been modified, as various parenchymal cells have

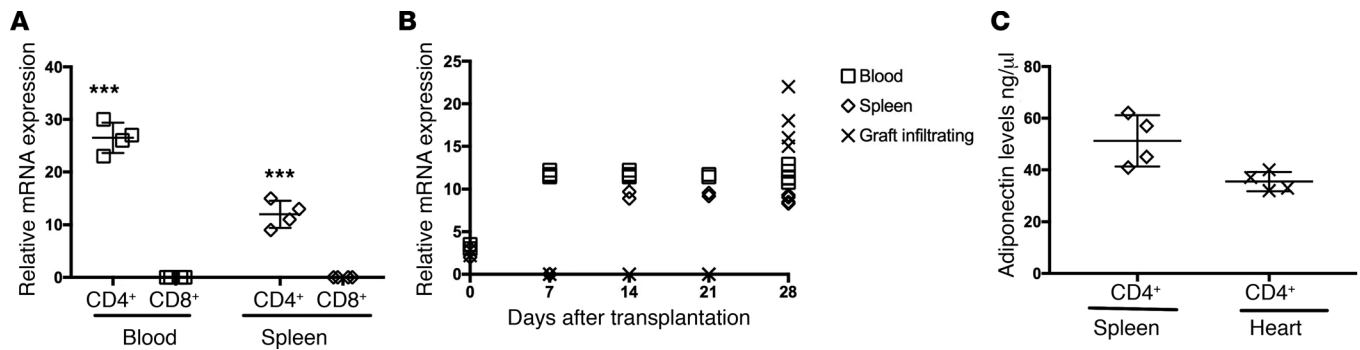


Figure 5. Adiponectin message and protein is expressed by T cells isolated from blood, spleen, and allografts. (A) Adiponectin message in circulating CD4⁺ and CD8⁺ T cells isolated by negative magnetic bead separation from blood (open squares) and spleen (open diamond) of WT recipients of WT allografts. Bars indicate \pm SEM with significant differences ($***P < 0.005$) calculated by 1-way ANOVA. (B) Adiponectin message in CD4⁺ T cells isolated from blood (open squares), spleen (open diamonds), and graft (crosses) 0, 7, 14, 21, and 28 days after transplantation. Each symbol represents 1 animal in each group. (C) WT CD4⁺ T cells isolated from both spleen (open diamond) and graft (cross) 4 weeks after transplantation produced adiponectin in response to polyclonal stimulation with bead-conjugated antibodies to CD3 and CD28 for 1 day. Each symbol represents 1 animal in each group, and bars indicate \pm SEM.

been demonstrated to produce adiponectin. The production of adiponectin by cardiomyocytes (4) and vascular smooth muscle cells (3) supports the potential for adiponectin to function in a paracrine or autocrine mode in the heart. Our data expand the range of adiponectin interactions by demonstrating that, under chronic stimulation, CD4⁺ T cells can produce significant amounts of adiponectin at the site of antigen engagement.

Based on our current findings, we have developed a working model for the production of adiponectin by T cells. In our model, the multiple strong signals released by the tissue injury caused by major surgery and revascularization of an ischemic organ polyclonally expand CD4⁺ T cells that can produce adiponectin in the circulation and spleen. As these cells circulate through the transplant, antigen-specific CD4⁺ T cells are retained. Migration of the CD4⁺ T cells into the graft and engagement of specific antigen initiates local production of adiponectin. Local production of adiponectin by infiltrating T cells is biologically significant because this provides a mechanism for high-molecular weight adiponectin to leave the vascular compartment and enter the interstitial tissue compartment (17). Concentrated quantities of adiponectin in the interstitium can then modulate the macrophage production of cytokines and the remodeling of extracellular matrix.

Our findings build upon previous reports that human lymphocytes produce minor amounts of adiponectin that are increased after polyclonal stimulation with mitogen (18). Of direct relevance to the ischemia reperfusion inherent in transplantation, Jin et al. found that adiponectin is upregulated in kid-

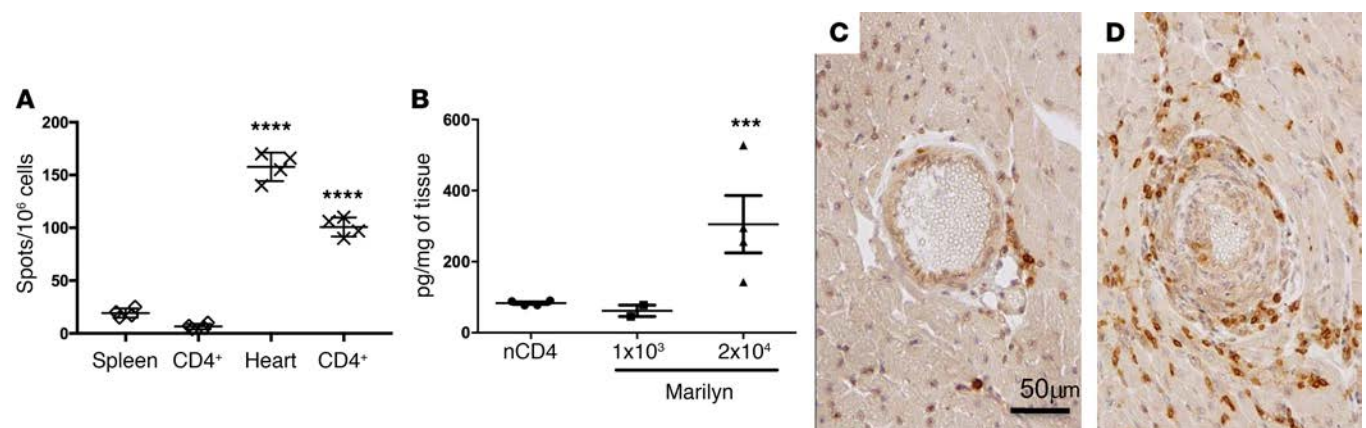


Figure 6. T cell secretion of adiponectin is induced by specific antigen. (A) Total leukocytes and CD4⁺ T cells isolated from spleen and grafts were tested in an ELISPOT assay to determine the frequency of cells producing adiponectin. (B) Adiponectin protein measured by ELISA in tissue homogenates from allografts to RAG^{-/-} mice that were reconstituted with either 2×10^4 naive CD4⁺ T cells (nCD4), 1×10^3 Marilyn cells, or 2×10^4 Marilyn cells ($n = 4$). (C) Immunohistology for CD3⁺ cells in allografts to RAG^{-/-} mice that were reconstituted with 1×10^3 Marilyn cells or (D) 2×10^4 Marilyn cells. (Scale bar = 50 μ m). Significant differences are indicated as $****P < 0.00001$ and $***P < 0.0001$ by 2-way ANOVA. Bars indicate \pm SEM.

Table 1. Taqman assay numbers for all the genes measured

Gene	Assay ID
MIG(CXCL9)	TaqMan Gene Expression Assay ID# Mm00434946_m1
RANTES(CCL5)	TaqMan Gene Expression Assay ID# Mm01302427_m1
MCP-1(CCL2)	TaqMan Gene Expression Assay ID# Mm00441242_m1
TLR7	TaqMan Gene Expression Assay ID# Mm00446590_m1
TLR9	TaqMan Gene Expression Assay ID# Mm00446193_m1
VEGF	TaqMan Gene Expression Assay ID# Mm01281449_m1
TGFβ	TaqMan Gene Expression Assay ID# Mm00441724_m1
YM-1	TaqMan Gene Expression Assay ID# Mm00657889_mH
Fizz1	TaqMan Gene Expression Assay ID# Mm00445109_m1
IL-6	TaqMan Gene Expression Assay ID# Mm00446190_m1
IL-10	TaqMan Gene Expression Assay ID# Mm00439616_m1
CD4	TaqMan Gene Expression Assay ID# Mm00442754_m1
CD8	TaqMan Gene Expression Assay ID# Mm01182107_g1
Adiponectin	TaqMan Gene Expression Assay ID# Mm00456425_m1

neys of mice subjected to transient occlusion of the renal artery (19). This study used BM chimeras to demonstrate that hematopoietic cells were one source of adiponectin in the kidney subjected to ischemia-reperfusion injury. In our experiments, adiponectin message was increased in allografts compared with isografts about 10-fold and almost 60-fold by 4 weeks after transplantation.

The finding that CD4⁺ T cells are a greater source of adiponectin than CD8⁺ T cells in our model of male hearts allografted into a female recipients likely reflects the underlying mechanism of the immune response to the H-Y antigen. Many studies in mice and humans have demonstrated that peptides from the H-Y antigen are presented in the groove of MHC class II molecules to CD4⁺ T cells (16, 20).

We have reported previously that T cells are required to orchestrate the progressive macrophage infiltrates that are a prominent component of chronic arterial pathology in cardiac allograft rejection (15). Several studies have associated wound-healing macrophages with aspects of chronic rejection in cardiac and renal allografts (21–23). The mediators that modulate macrophages in transplants have not been elucidated. Our new data are consistent with reports that adiponectin polarizes macrophages toward an antiinflammatory phenotype (9).

In summary, our data indicate that, as chronic inflammation progresses, production of adiponectin by T cells increases in sites of inflammation and becomes a significant modulator of macrophage responses.

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Methods

Mice. Male and female C57BL/6J (H2^b), male and female adiponectin-KO (Adipoq^{tm1Chan}) mice, and male B6.129S7-Rag1^{tm1MOM/J} (SCID) mice were purchased from Jackson Laboratories and used at 8–12 weeks of age. Female C57BL/10NA-(Tg) TCR Marilyn-(KO) Rag2 N11,N2 mice (H-2^b, Marilyn), age 6–8 weeks, were obtained as a gift from Polly Matzinger (NIH, Bethesda, Maryland, USA) and Oliver Lantz (INSERM, Paris, France).

Heterotopic heart transplantation. Heterotopic heart transplantation was performed as previously described (15, 16). Briefly, the donor aorta and pulmonary artery were anastomosed to the recipient abdominal aorta and inferior vena cava, respectively. Graft function was monitored weekly by abdominal palpation. Recipients were euthanized at designated intervals after transplantation, at which time a thin slice of the graft was fixed for immunohistology, and other segments were immediately snap frozen in liquid nitrogen or placed in media for analysis of graft-infiltrating cells.

IHC. Full cross-sections through the base of the cardiac grafts were fixed in 60% methanol/10% glacial acetic acid solution, embedded in paraffin, and sectioned at 5 μm (15). The following primary reagents were used: polyclonal rabbit antibody to CD3 (Abcam; ab5690), monoclonal rabbit antibody to CD4 (Abcam; clone EPRI9514), monoclonal rat antibody to Galectin-3 (Mac-2; Cedarlane, catalog CL8942ap), and biotinylated hyaluronic acid binding protein (Millipore, catalog 385911-50UG). Secondary reagents were rabbit HRP-Polymer (Biocare Medical, catalog RHRP520) for CD3 and CD4, and rat HRP-Polymer (Biocare Medical, catalog RT517) for Mac-2. ABC Elite Kit (Vector Labs) was used for biotinylated hyaluronic acid binding protein. Reactions were visualized using DAB substrate (Vector Labs). Sections were counterstained with hematoxylin (Richard-Allen Scientific).

Quantification of adiponectin and hyaluronan in allografts. Portions of grafts were homogenized reconstituted in protease inhibitor cocktail (Sigma-Aldrich, catalog P2714). Then 1 ml of 1.5% Triton X-100 in PBS was added before shaking for 30 minutes at 4° C. After pelleting cell debris, the supernatants were collected and total protein concentration quantified by Coomassie Plus Protein Assay Reagent kit (Pierce Thermo Fisher Scientific, catalog 23200). Protein concentrations were determined using ELISA kit for adiponectin and hyaluronan (R&D Systems, catalog MRP300 and DHYALO).

RNA purification and RT-PCR. Snap-frozen portions of grafts were crushed and homogenized, and RNA was isolated using fibrous tissue kits (Qiagen, catalog 74704). RNA was reverse transcribed using

the High-Capacity cDNA Archive kit (Applied Biosystems, catalog 4368814). Reverse transcription and real-time PCR were performed using commercially available reagents, Taqman probes, and 7500 Fast Real-Time Thermocycler (Applied Biosystems). The ddCT method was used for relative quantification of message expression, calibrated to the indicated control sample, and normalized to β actin. Taqman assay numbers for all the genes measured are listed in Table 1.

Isolation of graft-infiltrating cells. Harvested tissue was weighed and incubated 1 hour at 37°C in RPMI with type II collagenase (Sigma-Aldrich, catalog C6885-1G) before pressing through a 40- μ m filter. The collected cells were washed twice in RPMI and counted.

Enumeration of graft-infiltrating macrophages by flow cytometry. Isolated graft-infiltrating cells were phenotyped with fluoresceinated CD45 (clone 30-F11) and CD11b (clone M1/70) from BD Biosciences, and with F4/80 (clone BM8) from eBiosciences using a FACSCalibur cytometer (BD Biosciences) and FlowJo analysis software (Tree Star Inc.). For each sample, 200,000 gated events were accumulated. The total number of CD11b and F4/80 double-positive cells was calculated by (the total number of leukocytes in the sample counted using a hemocytometer) \times (% of CD45⁺ cells that were CD11b and F4/80 double-positive by flow cytometry)/100. The data are reported as the number of CD11b and F4/80 double-positive cells/mg of tissue.

PCR analysis on macrophages isolated from grafts. Cells isolated from grafts were stained for PE F4/80 (BD Biosciences, catalog 12-4801), and the cell pellet was incubated with anti-PE beads (Miltenyi Biotec, catalog 130-048-801). F4/80 cells were positively selected by passing through MS columns (Miltenyi Biotec, catalog 130-042-201). RNA was extracted using RNeasy Mini kits (Qiagen, catalog 74104). RNA was reverse transcribed using the High-Capacity cDNA Archive kit (Applied Biosystems, catalog 4368814). RT-PCR and real-time PCR were performed using commercially available reagents, Taqman probes, and 7500 Fast Real-Time Thermocycler (Applied Biosystems).

Culture of graft-infiltrating macrophages with adiponectin. Separated F4/80 cells were cultured in RPMI medium supplemented with 10% FCS, 40 μ g/ml gentamicin, 2 mM glutamine, 1 mM sodium pyruvate, and 10 mM HEPES buffer (all from Sigma-Aldrich). Aliquots (200 μ l) containing 5×10^5 macrophages were cultured in 96-well round-bottom plates with either adiponectin 10 μ g/ml (R&D Systems, catalog 5095-AC-050) or no adiponectin. After culturing the cells for 3 days at 37°C in 5% CO₂, cells were washed with PBS/0.05% Tween-20 (Sigma-Aldrich, catalog P7949). RNA extraction, RT-PCR, and real time PCR were performed as above.

Isolation of CD4⁺ and CD8⁺ T cells for PCR analysis and in vitro polyclonal stimulation. CD4⁺ and CD8⁺ T cells were purified by negative selection with Mouse EasySep cell isolation kits (Stemcell Technologies, catalogs 19852 and 19853). Isolated T cells were stimulated with Dynabeads Mouse T-Activator CD3/CD28 for T Cell Expansion and Activation (ThermoFisher Scientific) according to manufacturer's instructions. RNA extraction, RT-PCR, and real time PCR were performed as above.

ELISPOT assays to enumerate cells secreting adiponectin. ELISPOT assays to enumerate the percentage of CD4⁺ T cells secreting adiponectin in the spleen, blood, and graft recipients were performed as previously described (16). Briefly, CD4⁺ T cells were isolated from the blood, spleen, and grafts using magnetic beads (Stemcell Technologies, catalog 19852). These responder cells and mitomycin C-treated self-, donor, and third-party stimulator cells populations were cocultured for 24 hours at 37°C in serum-free HL-1 media in 96-well Multiscreen-IP (Millipore, Billerica) plates coated with capture antibodies to adiponectin mAb (R&D Systems, catalog DY1119-841353). Then, biotinylated-antibody to adiponectin (R&D Systems, catalog DY1119-841354) was added as a detection reagent for 2 hours at RT. After extensive washes, the plates were incubated with Streptavidin-Alkaline Phosphatase for 1 hour at RT, followed by BCIP/NBT substrate. The numbers of spots per well and the cumulative spot-size distribution were analyzed using an ImmunoSpot Series 2 Analyzer (Cellular Technology Ltd.). The results were calculated as numbers of adiponectin secreting cells per 1×10^6 cells.

Statistics. Data are represented as a mean with \pm SEM. Statistical analyses were performed using Graph Pad Prism 7.0 software (GraphPad Software Inc.). Significance was either determined by 2-tailed, type-2 Student's *t* test or with 1-way ANOVA or 2-way ANOVA, with *P* values of less than 0.05 considered significant. In Figure 2, the Bonferroni-Dunn method was used as a post hoc correction for multiplicity of gene testing.

Study approval. All animal studies were approved by the Cleveland Clinic institutional animal care and use committee.

Author contributions

SD and LRS performed the experiments. SD wrote the first draft of the paper, performed statistical analyses, and prepared figures. KSK advised and reviewed PCR analyses. RF performed the micro-surgery. ND validated antibodies and performed the immunohistology. AV and RLF helped design experiments and reviewed the final manuscript. WMB formulated the concept and revised the draft and the final version of the manuscript.

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Address correspondence to: William M. Baldwin III, Lerner Research Institute NB30, 9500 Euclid Avenue, Cleveland, Ohio 44195, USA. Phone: 216.445.2384; Email: baldwiw@ccf.org.

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