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PPAR-δ senses and orchestrates clearance of apoptotic cells to promote tolerance

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Macrophages rapidly engulf apoptotic cells to limit the release of noxious cellular contents and to restrict autoimmune responses against self antigens. Although factors participating in recognition and engulfment of apoptotic cells have been identified, the transcriptional basis for the sensing and the silent disposal of apoptotic cells is unknown. Here we show that peroxisome proliferator–activated receptor-δ (PPAR-δ) is induced when macrophages engulf apoptotic cells and functions as a transcriptional sensor of dying cells. Genetic deletion of PPAR-δ decreases expression of opsonins such as complement component-1qb (C1qb), resulting in impairment of apoptotic cell clearance and reduction in anti-inflammatory cytokine production. This increases autoantibody production and predisposes global and macrophage-specific *Ppard*$^{-/-}$ mice to autoimmune kidney disease, a phenotype resembling the human disease systemic lupus erythematosus. Thus, PPAR-δ has a pivotal role in orchestrating the timely disposal of apoptotic cells by macrophages, ensuring that tolerance to self is maintained.

In vertebrates, resident and recruited macrophages are the professional phagocytes that rapidly clear apoptotic cells[1]. This functions to protect neighboring cells from the noxious contents of dying cells and prevents activation of the immune system by liberated cellular contents[1,2]. Indeed, defects in apoptotic cell clearance make mice and humans susceptible to the autoimmune disease systemic lupus erythematosus[3–6]. Despite the importance of apoptotic cell disposal, how macrophages transcriptionally coordinate this process remains poorly understood.

Two distinct groups of proteins facilitate the recognition and uptake of apoptotic cells by macrophages. First, factors secreted by macrophages, termed opsonins, serve as a bridge to link unique chemical moieties exposed on apoptotic cells to macrophage cell surface receptors[3–6]. C1q, protein S, growth arrest–specific-6, thrombospondin-1 and milk fat globule-epidermal growth factor-8 (Mfge-8) are examples of opsonins that enhance the recognition and phagocytosis of apoptotic cells by macrophages[4,5]. Cell surface receptors that bind opsonins comprise the second category of proteins involved in the uptake of dying cells. Members of this diverse group include the macrophage scavenger receptors, integrin and complement receptors, tyrosine kinase Mer, calreticulin and immunoglobulin and mucin domain–containing protein TIM-4 (refs. 4–7). In support of their pivotal role in apoptotic cell clearance, mice deficient in opsonins such as *C1qa* or *Mfge8* or in the engulfment receptor c-mer proto-oncogene tyrosine kinase *Mertk* show heightened susceptibility to autoimmune disease on certain genetic backgrounds[8–11].

Engulfment of apoptotic cells brings large amounts of cellular lipids, including oxidized fatty acids and oxysterols, into the macrophage. We postulated that PPARs, the genetic sensors of native and oxidized fatty acids[12,13], are ideally suited to sense this lipid influx. Because we and others have previously shown that PPARs regulate the macrophage program of alternative activation[14–16], it raised the question of whether these lipid sensors also orchestrate tolerogenic responses in macrophages.

RESULTS

PPAR-δ orchestrates timely disposal of apoptotic cells

To investigate the role of PPARs in apoptotic cell clearance, we profiled the expression of all three mouse PPARs (α, δ and γ) in primary bone marrow–derived macrophages (BMDMs) after apoptotic cell feeding (**Supplementary Fig. 1a**). PPAR-δ messenger RNA, but not PPAR-γ mRNA, was induced ~200% after apoptotic cell feeding (**Fig. 1a**), whereas PPAR-α mRNA was undetectable in macrophages (data not shown). Immunoblotting confirmed that ingestion of apoptotic cells led to a ~190% increase in PPAR-δ protein abundance (**Fig. 1b**). To exclude the contribution of apoptotic cell–derived PPAR-δ to the signals detected in macrophages, we analyzed the expression of PPAR-δ protein in thymocytes. Intracellular staining failed to detect substantial amounts of PPAR-δ protein in thymocytes (**Fig. 1c**). In contrast, expression of PPAR-δ protein was specifically increased in macrophages fed apoptotic thymocytes (**Fig. 1c**). Moreover, we failed to detect expression of lymphocyte-specific proteins, such as the Src

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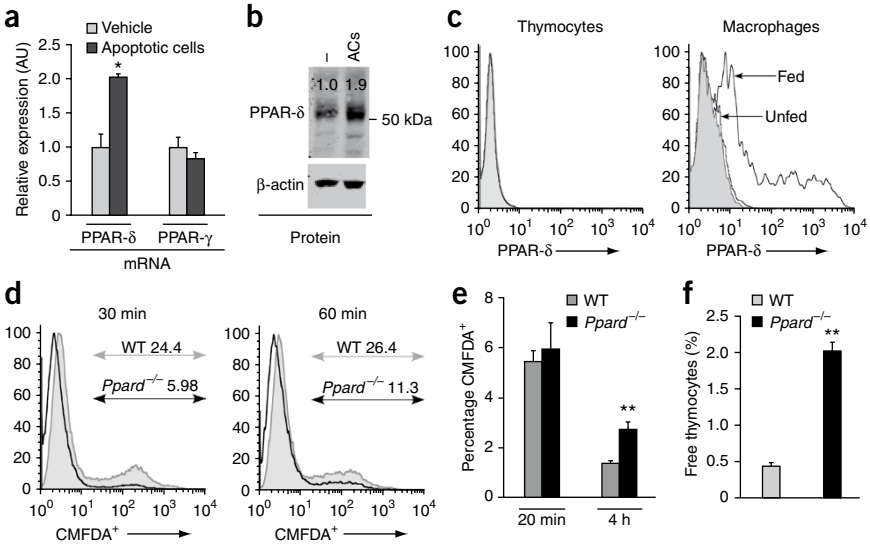

Figure 1 PPAR-δ orchestrates timely disposal of apoptotic cells. (**a**,**b**) Expression of PPAR-δ mRNA (**a**) and protein (**b**) in macrophages fed apoptotic cells. (**c**) Intracellular staining for PPAR-δ in thymocytes and macrophages fed apoptotic cells. Isotype control, gray histogram; PPAR-δ, unshaded histograms. (**d**) Impaired phagocytosis of apoptotic cells in *Ppard*$^{-/-}$ macrophages. Experiments were repeated five to six independent times, and a representative experiment is shown. (**e**,**f**) Delayed clearance of apoptotic thymocytes by splenic (**e**) and resident peritoneal (**f**) macrophages in *Ppard*$^{-/-}$ mice ($n = 4$ or 5). Data are presented as means ± s.e.m. $^{*}P < 0.05$ and $^{**}P < 0.01$. ACs, apoptotic cells; AU, arbitrary units; WT, wild-type.

kinase Lck or Cd3, in macrophages fed apoptotic thymocytes (**Supplementary Fig. 1b**,**d**), whereas Lck and Cd3 proteins were readily detectable in thymocytes (**Supplementary Fig. 1b**,**c**). Lastly, phagocytosis of latex beads, necrotic cells or opsonized sheep red blood cells (sRBCs), which are taken up via distinct pathways[6,17], did not induce a marked increase in PPAR-δ mRNA (**Supplementary Fig. 1e**) or protein (**Supplementary Fig. 1f**,**g**). Altogether, these data demonstrate that phagocytosis of apoptotic cells induces expression of PPAR-δ in macrophages, suggesting its potential involvement in coordinating the macrophage's transcriptional response to apoptotic cells.

To test this postulate, we fed BMDMs from wild-type and *Ppard*$^{-/-}$ mice apoptotic thymocytes and quantified phagocytosis. Because doublet discrimination can distinguish between engulfed and bound cells, we monitored engulfment of fluorescence-labeled apoptotic cells by flow cytometry[18]. Strikingly, compared to wild-type macrophages, in *Ppard*$^{-/-}$ macrophages phagocytosis of apoptotic cells was lower by ~75% and ~60% at 30 and 60 min, respectively (**Fig. 1d**). We obtained similar results when we quantified phagocytosis by microscopy (**Supplementary Fig. 2a**). Furthermore, on an individual cell basis, *Ppard*$^{-/-}$ macrophages were less efficient at taking up apoptotic thymocytes, as evidenced by the lower percentage of cells containing more than two thymocytes (**Supplementary Fig. 2b**,**c**). This observed phagocytic defect was specific for apoptotic cells, because phagocytosis of opsonized apoptotic thymocytes, opsonized sRBCs or necrotic thymocytes was unaffected in *Ppard*$^{-/-}$ macrophages (**Supplementary Fig. 2d–f**). This impairment in phagocytosis was also independent of PPAR-δ's known role in energy metabolism (**Supplementary Fig. 2g**)[19,20].

We undertook two independent approaches to investigate apoptotic cell clearance *in vivo*. First, we injected wild-type and *Ppard*$^{-/-}$ mice with fluorescence-labeled apoptotic thymocytes and monitored splenic clearance[9]. Although we found similar numbers of apoptotic thymocytes in spleens 20 min after injection (**Fig. 1e**), the relative clearance of apoptotic cells was delayed in *Ppard*$^{-/-}$ mice. At 4 h, approximately twofold more fluorescent cells were present in the spleens of *Ppard*$^{-/-}$ mice (1.38% versus 2.7%) (**Fig. 1e**). Similarly, resident peritoneal macrophages of *Ppard*$^{-/-}$ mice showed a lower capacity for clearance of fluorescence-labeled apoptotic cells (**Fig. 1f**). Six hours after intraperitoneal injection of apoptotic thymocytes, the number of free thymocytes recovered from the peritoneum of *Ppard*$^{-/-}$ mice was approximately fivefold higher than in wild-type mice (0.43% versus 2.02%) (**Fig. 1f**). These findings demonstrate that PPAR-δ promotes timely disposal of apoptotic cells in various macrophage populations.

PPAR-δ regulates opsonin gene expression in macrophages

To identify the molecular targets by which PPAR-δ regulates apoptotic cell clearance, we monitored expression of opsonins and their cognate receptors by microarray analysis. Clustering analyses revealed that a number of genes encoding opsonins, including *C1qa*, *C1qb*, *C1qc*, *Gas6* (encoding growth arrest specific-6), *Mfge8* and *Thbs1* (encoding thrombospondin-1), were downregulated in *Ppard*$^{-/-}$ macrophages (**Fig. 2a**). In contrast, the expression of macrophage receptors that recognize these 'eat-me' signals was largely unchanged, with the notable exception of *Mertk* (**Fig. 2a**). Quantitative PCR (qRT-PCR) analysis confirmed that expression of *C1qa*, *C1qb*, *Mfge8*, *Mertk* and *Thbs1* was 60–70% lower in *Ppard*$^{-/-}$ BMDMs (**Fig. 2b**). In agreement, immunoblot analysis revealed a ~300% decrease in C1qb protein abundance in *Ppard*$^{-/-}$ macrophages (**Fig. 2c**). To determine whether activation of PPAR-δ can enhance opsonin gene expression in macrophages, we treated wild-type BMDMs with the PPAR-δ agonist GW0742 (ref. 21), and we monitored opsonin gene expression by qRT-PCR. Indeed, PPAR-δ activation induced *C1qa*, *C1qb*, *Mfge8* and *Thbs1* by ~200–300% (**Fig. 2d**) in a PPAR-δ-dependent manner (**Supplementary Fig. 3b**). Finally, because mutations in C1q are responsible for the monogenic form of systemic lupus erythematosus in humans[22], we investigated whether *C1qb* is a direct transcriptional target of PPAR-δ. *In silico* analysis revealed a consensus PPAR-δ binding site in the *C1qb* gene located at ~−1.6 kilobases. Deletion analysis of the mouse promoter affirmed that PPAR-δ–RXR heterodimers directly regulate *C1qb* gene expression (**Fig. 2e** and **Supplementary Fig. 3a**).

We next tested whether pharmacologic activation of PPAR-δ enhances phagocytosis of apoptotic cells. Notably, treatment of wild-type mice with GW0742 enhanced the ability of CD11b^{+} splenic macrophages to phagocytose labeled apoptotic thymocytes by ~200% (**Fig. 3a**). To extend these findings to human cells, we treated primary human monocyte-derived macrophages with GW0742. Both opsonin gene expression (**Fig. 3b**) and apoptotic cell uptake (**Fig. 3c**) were enhanced in human macrophages stimulated with GW0742, indicating conservation of this pathway between mice and humans.

Kupffer cells are the primary source of the opsonins that circulate in the serum[23–25], prompting us to investigate whether opsonin expression was lower in livers of *Ppard*$^{-/-}$ mice. Indeed, we found substantially lower expression of *C1qa*, *C1qb*, *Thbs1* and *Mfge8*

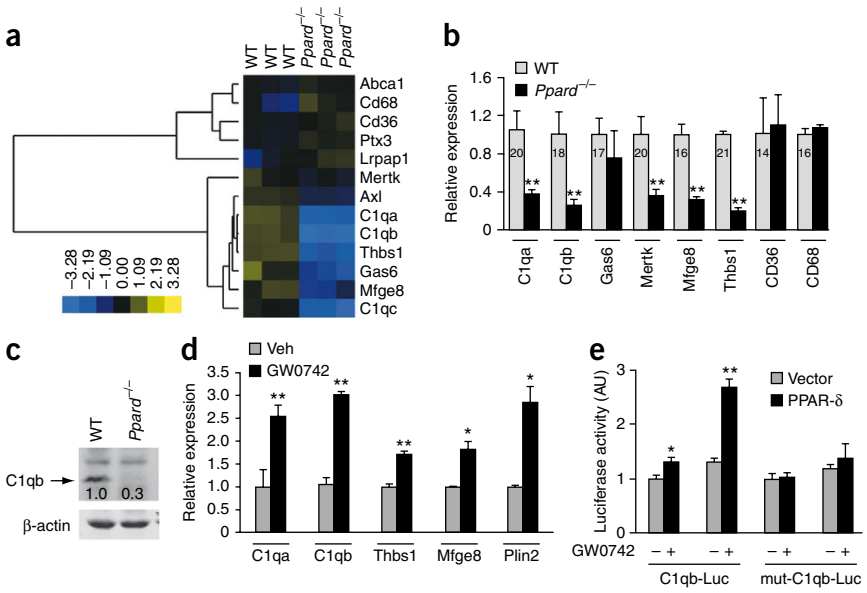

Figure 2 PPAR-δ regulates expression of opsonins in macrophages. (**a**) Clustering analysis of microarray data from WT and *Ppard*$^{-/-}$ macrophages. (**b**) Relative expression of opsonins and receptors in WT and *Ppard*$^{-/-}$ macrophages, as assessed by qRT-PCR. (**c**) Reduced expression of C1qb protein in *Ppard*$^{-/-}$ macrophages. (**d**) Induction of opsonin gene expression by PPAR-δ agonist (GW0742 100nM) in wild type macrophages ($n = 3$ or 4). (**e**) Activation of C1qb promoter by PPAR-δ. C1qb promoter fragments containing or lacking the PPAR response element (C1qb-Luc or mut-C1qb-Luc, respectively) were transfected into CV-1 cells, and luciferase activity was assayed 18 h later. Data are presented as means ± s.e.m. $*P < 0.05$ and $**P < 0.01$. The cycle time for the highest expressing gene is indicated inside its corresponding bar.

(~40–60%) in livers of *Ppard*$^{-/-}$ mice (**Supplementary Fig. 3c**). Conversely, treatment of wild-type mice with GW0742 increased hepatic opsonin gene expression by ~50–90% (**Supplementary Fig. 3d**). Congruent with these observations, circulating levels of C1qb (**Fig. 3d**) and, to a lesser extent, Mfge8 (**Supplementary Fig. 3e**) were lower in *Ppard*$^{-/-}$ mice. To determine whether the reduction in opsonin abundance contributes to the phagocytic defect of *Ppard*$^{-/-}$ macrophages, we performed a series of experiments using sera from wild-type and *Ppard*$^{-/-}$ mice. The observed phagocytic defect in *Ppard*$^{-/-}$ macrophages was largely rescued by sera from wild-type mice (**Fig. 3e**), whereas incubation of wild-type macrophages with *Ppard*$^{-/-}$ sera reduced their phagocytic capacity by ~35% (**Fig. 3e**). Similarly, addition of purified human C1q protein to *Ppard*$^{-/-}$ macrophages restored their phagocytic activity to ~90% of wild-type macrophages (**Fig. 3f**). We thus conclude that PPAR-δ orchestrates the prompt disposal of dying cells in mouse and human macrophages by inducing expression of opsonins.

PPAR-δ is a transcriptional sensor of apoptotic cells

Apoptotic cells contain large amounts of native and oxidized fatty acids, leading us to ask whether they might activate PPAR-δ to enhance their own clearance. To explore this possibility, we performed transient transfection assays with the *PPAR* reporter gene (AOx-PPRE$_3$-Luc). Treatment of transfected cells with apoptotic thymocytes increased luciferase reporter gene activity only in the presence of PPAR-δ (**Supplementary Fig. 4a**). Moreover, reporter gene assays with GAL4–PPAR-δ chimeric constructs confirmed that the ligand-binding domain of PPAR-δ is sufficient to transduce the transcriptional effects of apoptotic thymocytes (**Supplementary Fig. 4b**). Congruent with these data, apoptotic cell feeding induced expression of opsonins (*C1qb*, *Mfge8* and *Thbs1*), *Mertk*, *Tgfb1* (encoding transforming growth factor-β1 (TGF-β1)) and the PPAR-δ target gene *Plin2* (encoding perilipin-2) in thioglycollate-elicited wild-type

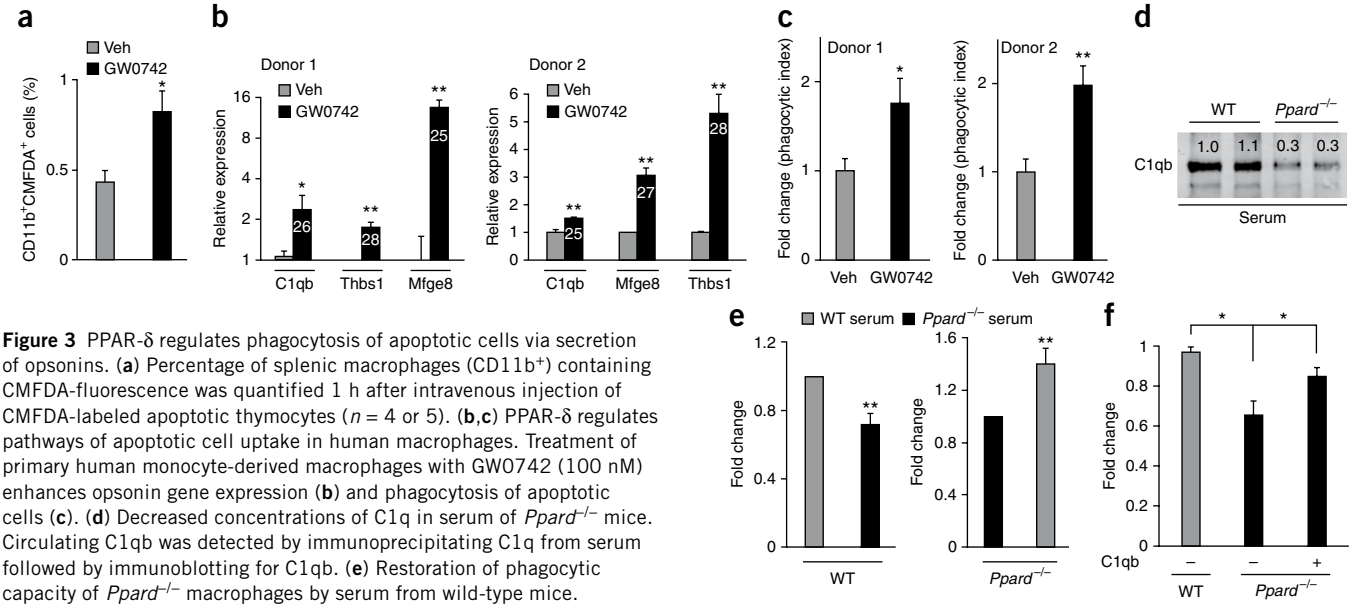

Figure 3 PPAR-δ regulates phagocytosis of apoptotic cells via secretion of opsonins. (**a**) Percentage of splenic macrophages (CD11b$^+$) containing CMFDA-fluorescence was quantified 1 h after intravenous injection of CMFDA-labeled apoptotic thymocytes ($n = 4$ or 5). (**b**,**c**) PPAR-δ regulates pathways of apoptotic cell uptake in human macrophages. Treatment of primary human monocyte-derived macrophages with GW0742 (100 nM) enhances opsonin gene expression (**b**) and phagocytosis of apoptotic cells (**c**). (**d**) Decreased concentrations of C1q in serum of *Ppard*$^{-/-}$ mice. Circulating C1qb was detected by immunoprecipitating C1q from serum followed by immunoblotting for C1qb. (**e**) Restoration of phagocytic capacity of *Ppard*$^{-/-}$ macrophages by serum from wild-type mice. (**f**) Enhancement of apoptotic cell uptake by purified human C1q in *Ppard*$^{-/-}$ macrophages. Data are presented as means ± s.e.m. $*P < 0.05$ and $**P < 0.01$. The cycle time for the highest expressing gene is indicated inside its corresponding bar.

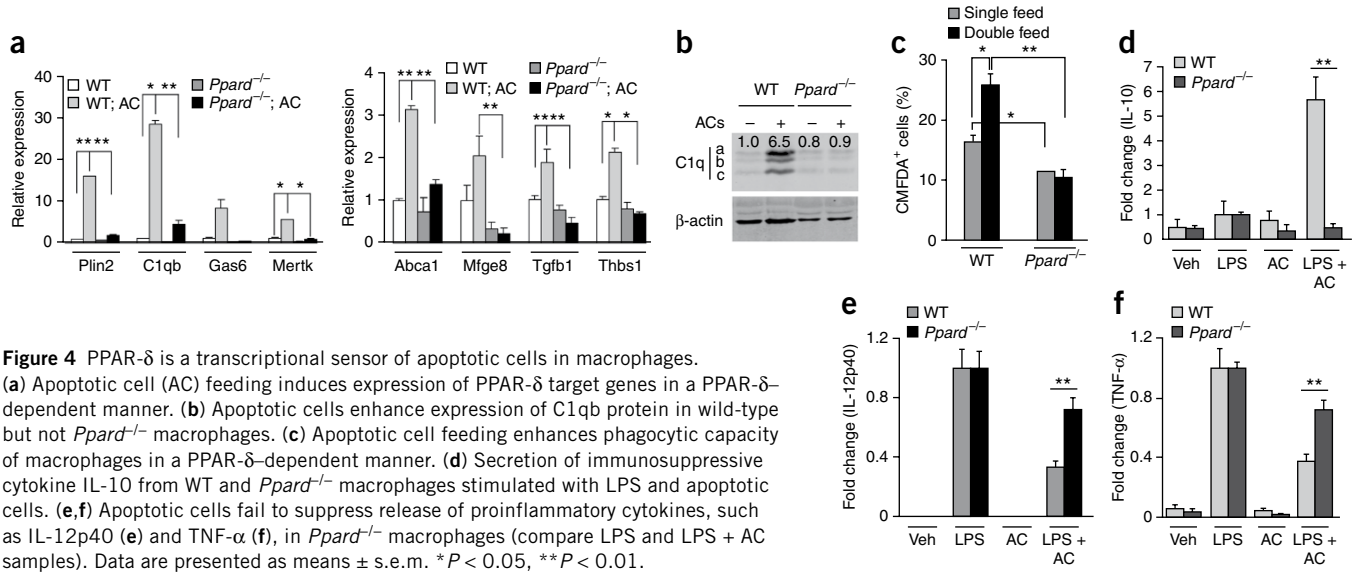

Figure 4 PPAR-δ is a transcriptional sensor of apoptotic cells in macrophages. (**a**) Apoptotic cell (AC) feeding induces expression of PPAR-δ target genes in a PPAR-δ–dependent manner. (**b**) Apoptotic cells enhance expression of C1qb protein in wild-type but not *Ppard*$^{-/-}$ macrophages. (**c**) Apoptotic cell feeding enhances phagocytic capacity of macrophages in a PPAR-δ–dependent manner. (**d**) Secretion of immunosuppressive cytokine IL-10 from WT and *Ppard*$^{-/-}$ macrophages stimulated with LPS and apoptotic cells. (**e**,**f**) Apoptotic cells fail to suppress release of proinflammatory cytokines, such as IL-12p40 (**e**) and TNF-α (**f**), in *Ppard*$^{-/-}$ macrophages (compare LPS and LPS + AC samples). Data are presented as means ± s.e.m. $^{*}P < 0.05$, $^{**}P < 0.01$.

macrophages (**Fig. 4a**), whereas the induction of all of these genes was diminished or absent in *Ppard*$^{-/-}$ macrophages (**Fig. 4a**). Moreover, challenge of wild-type but not *Ppard*$^{-/-}$ macrophages with apoptotic cells led to a ~650% increase in C1q protein abundance (C1qa, C1qb and C1qc) (**Fig. 4b**), leading us to postulate that apoptotic cells might enhance their own clearance via activation of PPAR-δ. To test this idea, we did a double-feeding experiment with BMDMs. Notably, priming of wild-type macrophages by apoptotic cells increased their phagocytic capacity by ~60%, whereas there was no significant difference in the phagocytic capacity of macrophages lacking PPAR-δ (**Fig. 4c**). Finally, this transcriptional activation of PPAR-δ by apoptotic cells was specific, because necrotic cells and opsonized sRBCs failed to activate PPAR-δ (**Supplementary Fig. 4c**,**d**).

Previous work has established that phagocytosis of apoptotic cells suppresses autoimmune responses by releasing immunosuppressive cytokines (interleukin-10 (IL-10) and TGF-β) and inhibiting production of proinflammatory cytokines (IL-12 and tumor necrosis factor-α (TNF-α))[3,26,27]. Because PPAR-δ functions as a sensor of apoptotic cells, we asked whether it might mediate this molecular switch in cytokine secretion. For these experiments, we stimulated thioglycollate-elicited wild-type and *Ppard*$^{-/-}$ macrophages with lipopolysaccharide (LPS) in the presence or absence of apoptotic cells, and cytokine secretion was monitored by ELISA. As expected, apoptotic cell feeding increased secretion of the anti-inflammatory cytokine IL-10 (**Fig. 4d**) while suppressing the release of the proinflammatory cytokines IL-12 and TNF-α in wild-type macrophages (**Fig. 4e**,**f**). Of note, this switch in cytokine secretion pattern was absent in *Ppard*$^{-/-}$ macrophages (**Fig. 4d–f**), suggesting that immunosuppressive effects of apoptotic cells require an intact PPAR-δ signaling pathway in macrophages.

Ppard$^{-/-}$ mice develop autoimmune disease

Defective clearance of apoptotic cells triggers immune response to self antigens, manifesting as lupus-like autoimmunity in mice[8,9,11,28]. Because we observed lower expression of opsonins and impaired clearance of apoptotic cells in *Ppard*$^{-/-}$ mice, we evaluated the onset of autoimmune disease in these mice. Notably, sera of *Ppard*$^{-/-}$ female mice ($n = 9$) had ~200–600% higher levels of autoantibodies, including those directed against nuclear antigens (antinuclear antibody, ANA; double-stranded DNA, dsDNA; single-stranded DNA, ssDNA) and cardiolipin, than age-matched wild-type controls ($n = 7$; **Fig. 5a–d**). Furthermore, these changes in autoantibody titers were comparable to those found in wild-type BALB/c mice treated with pristane, an established model for inducing lupus-like autoimmunity[29,30]. A hallmark of systemic lupus erythematosus is the deposition of autoantibodies as immune complexes in the kidney, leading to inflammation and destruction of the glomeruli[31]. Indeed, immunofluorescence staining revealed that the glomeruli of all *Ppard*$^{-/-}$ female mice ($n = 7$) had a ribbon-like pattern of IgG deposition, whereas the glomeruli of wild-type female mice were largely spared (**Fig. 5e**). Accordingly, increased urinary protein excretion, a sign of kidney dysfunction, was present in *Ppard*$^{-/-}$ female mice (**Fig. 5f**). Moreover, perivascular inflammation, a common histological finding in autoimmune kidney disease[32,33], was increased by ~600% in the kidneys of *Ppard*$^{-/-}$ female mice (**Supplementary Fig. 5a**,**b**). We obtained similar results in an independent cohort of wild-type and *Ppard*$^{-/-}$ female mice ($n = 8$ per genotype; **Supplementary Fig. 6**).

To determine whether the lupus-like autoimmunity in *Ppard*$^{-/-}$ female mice results from changes in lymphocyte subsets, we analyzed the spleens of 13-week-old female mice before they had any evidence of autoimmune disease. Notably, total numbers of B cells and CD4^{+} and CD8^{+} T cells were not different between wild-type and *Ppard*$^{-/-}$ female mice (**Supplementary Figs. 7a**, **8a** and **9b**). The numbers of regulatory, memory, naive and activated T cells and activated B cells were also similar amongst the genotypes (**Supplementary Fig. 7a**, **8a**,**b** and **9a**). Furthermore, spleen weights and splenic lymphocyte composition were similar in 14-month-old wild-type and *Ppard*$^{-/-}$ female mice (**Supplementary Fig. 7b**,**c**), suggesting that the autoimmunity in these mice is not due to aberrant proliferation or activation of lymphocytes.

To further explore this idea, we investigated whether mice deficient in *Ppard* specifically in their macrophages (Mac-*Ppard*$^{-/-}$ mice) showed similar defects in clearance of apoptotic cells. Genetic deletion of PPAR-δ in macrophages was sufficient to lower opsonin gene expression by 35–75% (**Fig. 6a**) and impair clearance of 5-chloromethylfluorescein diacetate (CMFDA)-labeled apoptotic thymocytes by ~350% in the spleen (**Fig. 6b**). Remarkably, this delay in clearance of apoptotic cells resulted in higher levels of autoantibodies

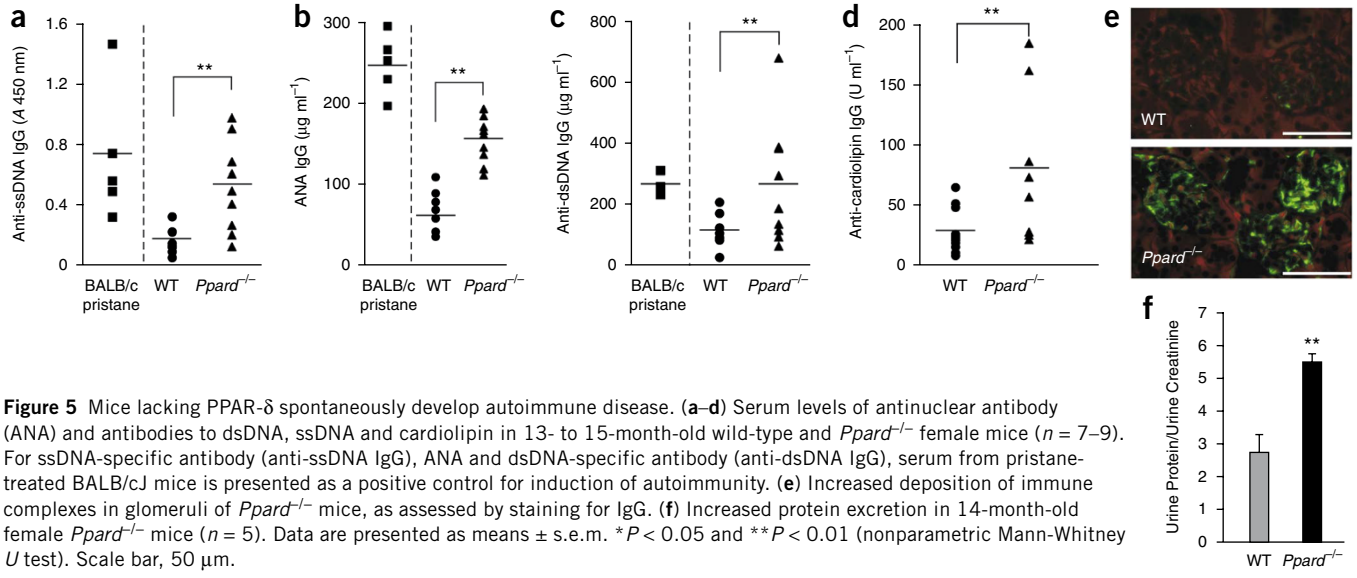

Figure 5 Mice lacking PPAR-δ spontaneously develop autoimmune disease. (**a**–**d**) Serum levels of antinuclear antibody (ANA) and antibodies to dsDNA, ssDNA and cardiolipin in 13- to 15-month-old wild-type and *Ppard*$^{-/-}$ female mice (n = 7–9). For ssDNA-specific antibody (anti-ssDNA IgG), ANA and dsDNA-specific antibody (anti-dsDNA IgG), serum from pristane-treated BALB/cJ mice is presented as a positive control for induction of autoimmunity. (**e**) Increased deposition of immune complexes in glomeruli of *Ppard*$^{-/-}$ mice, as assessed by staining for IgG. (**f**) Increased protein excretion in 14-month-old female *Ppard*$^{-/-}$ mice (n = 5). Data are presented as means ± s.e.m. $^{*}P < 0.05$ and $^{**}P < 0.01$ (nonparametric Mann-Whitney U test). Scale bar, 50 μm.

specific for ssDNA and cardiolipin in 3- to 6-month-old female Mac-*Ppard*$^{-/-}$ mice (**Fig. 6c–e**; n = 8 per genotype). Because Mac-*Ppard*$^{-/-}$ mice were generated on the C57BL/6J background[15], a strain known to be very resistant to spontaneous lupus-like autoimmune disease[34,35], we injected apoptotic thymocytes in a separate cohort of mice to potentiate autoimmunity[36,37]. Injection of syngeneic apoptotic cells increased titers dsDNA-specific and ssDNA-specific antibodies by ~200% (**Fig. 6f,g**) in Mac-*Ppard*$^{-/-}$ mice, resulting in increased perivascular inflammation and immune complex deposition in the kidney (**Fig. 6h,i**; n = 6 or 7 per genotype). Together, these data show that deletion of PPAR-δ in macrophages is sufficient to delay clearance of apoptotic cells, thereby providing the antigenic stimulus for breakdown of tolerance.

Systemic lupus erythematosus is a polygenic disease involving hits in multiple pathways, including B cell signaling and activation, apoptotic cell clearance and Toll-like receptor signaling[38,39]. As global or macrophage-specific deficiency of PPAR-δ primarily affects pathways of apoptotic cell clearance, we next investigated whether additional hits could potentiate autoimmunity in mice deficient in PPAR-δ. For these experiments, we gave cohorts of wild-type

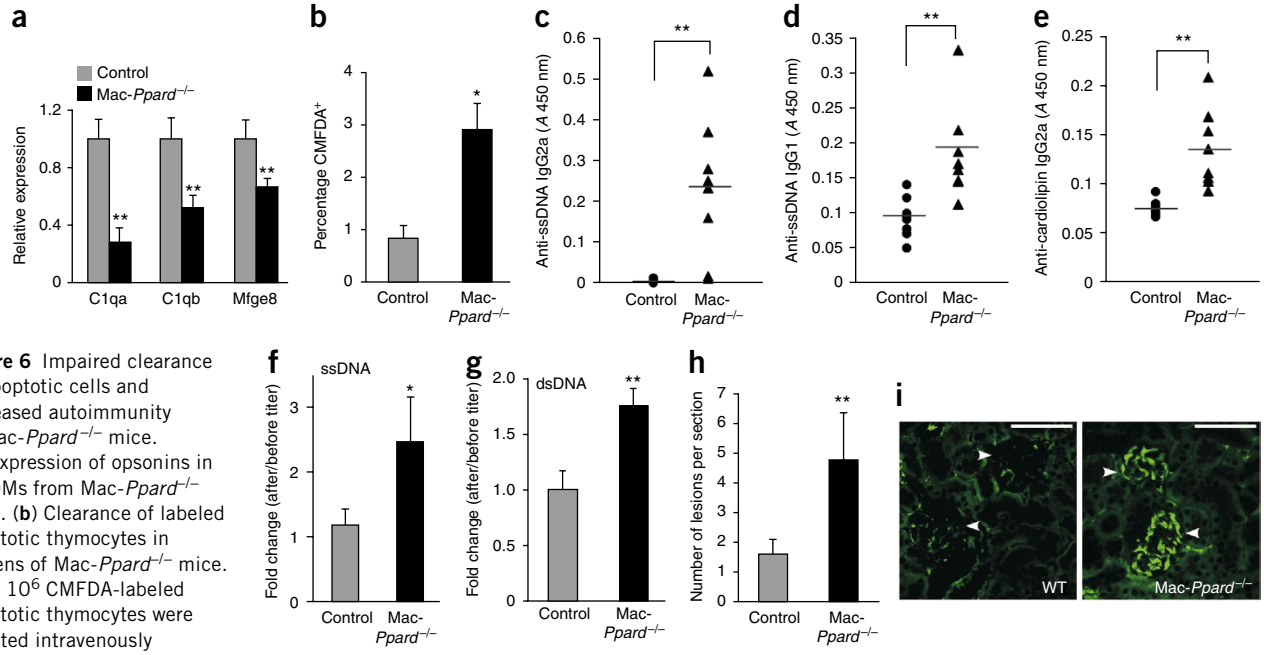

Figure 6 Impaired clearance of apoptotic cells and increased autoimmunity in Mac-*Ppard*$^{-/-}$ mice. (**a**) Expression of opsonins in BMDMs from Mac-*Ppard*$^{-/-}$ mice. (**b**) Clearance of labeled apoptotic thymocytes in spleens of Mac-*Ppard*$^{-/-}$ mice. 60 × 10^6 CMFDA-labeled apoptotic thymocytes were injected intravenously in control and Mac-*Ppard*$^{-/-}$ mice (n = 3), and the presence of CMFDA-labeled cells was quantified 4 h later in spleens. (**c**–**e**) Autoantibody production in young Mac-*Ppard*$^{-/-}$ female mice. Serum levels of ssDNA-specific antibodies (**c**,**d**) and cardiolipin-specific antibodies (**e**) in 3- to 6-month-old control and Mac-*Ppard*$^{-/-}$ mice (n = 8 per genotype). (**f**,**g**) Changes in titers of ssDNA (**f**) and dsDNA (**g**) autoantibodies in control and Mac-*Ppard*$^{-/-}$ mice injected with 1 × 10^7 syngenic apoptotic thymocytes for 4 weeks. (**h**,**i**) Histological and immunofluorescence analysis of kidneys performed 9 months after apoptotic cell injections (12-month-old mice) revealing increased perivascular inflammation (**h**) and immune complex deposition in the glomeruli (**i**). Scale bar, 50 μm. Data are presented as means ± s.e.m. $^{*}P < 0.05$ and $^{**}P < 0.01$.

($n = 6$–8) and *Ppard*$^{-/-}$ ($n = 8$–10) female mice a single injection of pristane, which induces lupus-like autoimmune disease by activating B cells[30]. As expected, wild-type female mice treated with pristane had higher levels of antibodies directed against nuclear and membranous antigens, including antibody to cardiolipin, antinuclear antibody, antibody to dsDNA and antibody to ssDNA, than their saline-injected littermates (**Supplementary Fig. 10a–d**). Notably, the concentrations of these antibodies in sera of pristane-treated *Ppard*$^{-/-}$ female mice were ~200–400% higher than in pristane-treated wild-type controls (**Supplementary Fig. 10a–d**), and the penetrance of pristane-induced autoimmunity was increased from 28% to 77% in pristane-treated *Ppard*$^{-/-}$ female mice (**Supplementary Fig. 10e**). In agreement with these results, massive deposits of complement-containing immune complexes were only seen in the glomeruli of pristane-injected *Ppard*$^{-/-}$ female mice (**Supplementary Fig. 10f**). Furthermore, histological analysis revealed that whereas glomeruli of wild-type female mice had mild to moderate mesangial hypercellularity (precursor lesions), severe endocapillary proliferative glomerulonephritis with necrosis and crescent formation (end-stage lesions) were the dominant pathologies present in the glomeruli of *Ppard*$^{-/-}$ female mice (**Supplementary Fig. 10g,h**). Together, these data show that genetic deficiency of PPAR-δ delays clearance of apoptotic cells and increases autoantibody production, leading to immune complex–mediated glomerulonephritis, features reminiscent of the human disease systemic lupus erythematosus.

DISCUSSION

During the past decade, numerous molecules responsible for recognition and uptake of apoptotic cells have been identified. Careful dissection of these pathways has revealed that prompt clearance of apoptotic cells by macrophages is essential for prevention of autoimmune disease. Notably, apoptotic cells are rapidly cleared by macrophages even during times of massive apoptosis[40,41], implying that phagocytic programs are dynamic, allowing macrophages to adapt to their changing microenvironment. However, the transcription factors that coordinate expression of phagocytic genes remain unknown. Our results show that the nuclear receptor PPAR-δ functions as a sensor of dying cells to orchestrate the phagocytic response when macrophages are confronted with apoptotic cells.

After engulfment, apoptotic bodies are rapidly broken down into their molecular constituents. This results in a marked increase in the cellular pools of oxidized fatty acids and sterols, the normal components of the cellular membranes of apoptotic cells. Since PPARs and liver X receptors are sensors of modified fatty acids and sterols, respectively[12,42], we and others have postulated that they might coordinate the transcriptional response of macrophages during apoptotic cell clearance[6,43]. Indeed, the evidence presented here provides strong support for a pivotal role of nuclear receptor signaling in the phagocytic response. Specifically, we demonstrate that apoptotic cell feeding potently induces and activates PPAR-δ, which then enhances the expression of opsonins, molecules that bridge apoptotic bodies to cell surface receptors on phagocytes. In addition, by sensing apoptotic cells, PPAR-δ functions as a molecular switch that discriminates between the proinflammatory and immunosuppressive actions of macrophages. Consequently, global or macrophage-specific deletion of PPAR-δ delays clearance of apoptotic cells, leading to increased production of autoantibodies and progressive lupus-like autoimmune disease.

The requirement for PPAR-δ in regulation of the expression of opsonin genes such as *C1qb* provides a direct link between this transcription factor, phagocytosis of apoptotic cells and development of autoimmune disease in mice and humans. Notably, loss of PPAR-δ signaling in the macrophage lowers expression of key opsonins, including *C1qa*, *C1qb*, *Mfge8* and *Thbs1*. Because genetic deletion of *C1qb* and *Mfge8* delays clearance of apoptotic debris by macrophages[11,28], the concomitant reduction of these opsonins provides a molecular explanation for the lower uptake of apoptotic cells by macrophages lacking PPAR-δ. Thus, we propose that the persistence of apoptotic debris and increased macrophage inflammatory responses to them in global or macrophage-specific *Ppard*$^{-/-}$ mice provides the antigenic stimulus that potentiates autoimmunity in these mice. Increased titers of autoantibodies and progressive renal pathology collectively suggest that PPAR-δ–deficient mice are a good model system for studying human systemic lupus erythematosus.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Accession codes. The microarray data has been deposited in the Gene Expression Omnibus under accession number GSE17890.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

L.M, J.I.O. and C.R.M. were involved in project planning, experimental work and data analysis; J.E.H., R.R.R.-G., J.W.M., A.R.E., S.E.D, J.U.H.A., Y.P.S.G. and K.D.N. performed experimental work; S.A.M. and L.S. were involved in project planning; and A.C. was involved in project planning, data analysis and manuscript preparation.

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ONLINE METHODS

Mice. We used *Ppard*$^{-/-}$ mice, backcrossed onto the 129/SvJ strain for eight generations, for these studies[44]. We used 129/SvJ mice (The Jackson Laboratories) as wild-type controls. We allowed for spontaneous development of autoimmunity and its potentiation by systemically administering apoptotic cells to littermate control (*Ppard*$^{flox/flox}$) and macrophage-specific *Ppard*$^{-/-}$ (Mac-*Ppard*$^{-/-}$; *Ppard*$^{flox/flox}$; LysM^{Cre}) female mice on the C57BL/6J background[15]. We backcrossed these mice onto the C57BL/6J strain for more than ten generations, ensuring that their genetic makeup is primarily (99.9%) derived from the C57BL/6J strain. We prepared BMDMs from wild-type and *Ppard*$^{-/-}$ mice as previously described[14]. We prepared apoptotic targets by culturing thymocytes from 4- to 8-week-old wild-type mice in serum-free RPMI medium for 16–18 h or by treatment with dexamethasone (10 μM) for 3 h[9,45]. Before being fed to macrophages, we labeled apoptotic thymocytes by incubation with 2 μM CMFDA (Molecular Probes) for 30 min.

***In vitro* apoptotic cell uptake and clearance.** For uptake assays, we added apoptotic thymocytes to wild-type and *Ppard*$^{-/-}$ BMDMs at a 5:1 thymocyte: macrophage ratio for 15, 30 or 60 min. We determined apoptotic cell uptake and clearance by flow cytometry, which employed doublet discrimination to distinguish internalized from externally bound apoptotic cells. We calculated phagocytic index by manually counting >200 cells per sample and applying the following formula: [2(number of macrophages containing one apoptotic cell) + (number of macrophages containing two apoptotic cells) + 3(number of macrophages containing three apoptotic cells) + 4(number of macrophages containing more than four apoptotic cells)] / total number of macrophages counted. For studies with opsonized cells, we opsonized apoptotic CMFDA-labeled thymocytes with CD3-specific antibody (0.5 μg per 1×10^6 cells; Pharmingen) for 30 min at 37 °C. We resuspended sheep red blood cells (Colorado Scientific) in PBS (1×10^6 per μl), and opsonized with rabbit antibody to sheep red blood cells (1 in 200) for 1 h at 25 °C. We removed unbound antibody by PBS washes. We generated necrotic targets by incubation at 56 °C for 10 min. We confirmed cellular necrosis by Trypan blue staining. For double feeding experiments, we treated day 7 BMDMs, plated in six-well plates, with vehicle or apoptotic thymocytes (1:1) for 24 h. One day later, we rechallenged macrophages with CMFDA-labeled apoptotic thymocytes and quantified phagocytosis as described above. We performed rescue experiments with BMDMs of both genotypes plated in DMEM (1 g l^{-1}) supplemented with macrophage colony–stimulating factor (10 ng ml^{-1}; PeproTech) and 10% sera of wild-type or *Ppard*$^{-/-}$ mice. We quantified phagocytosis of labeled apoptotic cells 1 d later. For rescue experiments with recombinant C1q, we plated BMDMs in DMEM (1g l^{-1}) supplemented with 1% FBS and macrophage colony–stimulating factor (10 ng ml^{-1}). We added purified human C1q (4 μl of 1.1 mg ml^{-1}; Sigma) to medium (400 μl) 1 h before feeding of CMFDA-labeled apoptotic thymocytes (1:2 ratio). We quantified phagocytosis as described above.

***In vivo* apoptotic cell uptake and clearance.** For splenic uptake and clearance assays, we injected 6×10^7 CMFDA-labeled apoptotic thymocytes intravenously into 8- to 12-week-old wild-type, *Ppard*$^{-/-}$, control or Mac-*Ppard*$^{-/-}$ mice. We killed the mice at the time points indicated in **Figures 1e**, **1f**, **3a** and **6b**, and we analyzed single-cell splenocyte suspensions by flow cytometry[9,10]. For splenic macrophage uptake, we positively selected CD11b$^+$ splenocytes with magnetic beads coated with antibodies to CD11b (Miltenyi) before flow cytometric analysis. We performed peritoneal uptake and clearance assays similarly. Briefly, we injected labeled apoptotic thymocytes into the peritoneal cavity of naive mice; 6 h later, we retrieved the population by peritoneal lavage and analyzed by flow cytometry.

***In vivo* models of autoimmunity.** We induced autoimmunity in female wild-type and *Ppard*$^{-/-}$ mice with a single intraperitoneal injection of 1 ml pristane (Sigma) at 6 months of age, and we killed the mice at 11 months of age[30]. We assessed spontaneous autoimmunity in unmanipulated female mice at 3–6 months (Mac-*Ppard*$^{-/-}$) or 13–15 months (*Ppard*$^{-/-}$) of age. We performed provocative challenge with exogenously administered apoptotic cells in control and Mac-*Ppard*$^{-/-}$ female mice (n = 6 or 7 per genotype), as previously described[36]. Briefly, we injected 1×10^7 apoptotic thymocytes via tail vein into 12- to 15-week-old female mice weekly for 4 weeks. We collected serum before initiation of injections and 2 d after the last injection and analyzed for autoantibody production.

Statistical analyses. Continuous data are presented as means ± s.e.m., and we calculated P values with the two-tailed Student's t test for two samples of unequal variance. Ordinal data are presented as proportion, and we calculated P values with the nonparametric Mann-Whitney U test. Statistical significance is indicated by a single asterisk ($P < 0.05$) or two asterisks ($P < 0.01$).

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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