

Letter to the Editor

Impaired efferocytosis in human chronic granulomatous disease is reversed by pioglitazone treatment*To the Editor:*

Patients with chronic granulomatous disease (CGD) suffer from infections and exaggerated inflammation.¹ Defects in *efferocytosis*, the process of clearing dying cells by macrophages, may contribute to the heightened, prolonged, and granulomatous inflammation in this disease. Impaired efferocytic capability has been shown for murine CGD macrophages *in vitro* and *in vivo*,^{2,3} as well as human gp91^{phox}-deficient PLB-985 cells, and in 1 study, monocyte-derived macrophages from patients with CGD.⁴ Efferocytosis generally leads to active suppression of inflammatory mediator production by macrophages, and this too is reportedly deficient in CGD.^{2,5,6}

Peroxisome proliferator-activated receptor γ (PPAR γ), a nuclear receptor activated by oxidized lipids, is a driver of metabolic programming of macrophages and enhances their ability to take up and digest dying cells.² PPAR γ is ordinarily expressed at low levels in circulating monocytes, but its expression and activation are upregulated once monocytes transit into oxidant-rich inflamed tissues and begin to differentiate into macrophages. PPAR γ expression and activation are delayed and deficient in inflammatory macrophages derived from immigrant monocytes during inflammation in the murine model of X-linked CGD, but are restored by treatment of the animals with the PPAR γ agonist pioglitazone, a drug approved for type 2 diabetes.² Treatment of mice with CGD with pioglitazone also restored the efferocytic capability of inflammatory macrophages and suppressed excessive inflammatory mediator production.²

In normal phagocytes, PPAR γ activation links nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity with enhanced mitochondrial reactive oxygen species (ROS) production.⁷ Accordingly, the lack of this upstream signaling by the NADPH oxidase and PPAR γ in CGD results in deficient mitochondrial ROS production in CGD phagocytes. Notably, mitochondrial ROS production was restored in murine and human CGD monocytes treated with pioglitazone, *in vivo* and *ex vivo*, respectively.⁷

We hypothesized that human CGD blood monocytes, precursors of inflammatory macrophages, would show impaired efferocytosis in comparison to monocytes from normal human subjects and that treatment with pioglitazone would restore CGD monocyte efferocytic capability. We investigated monocytes rather than monocyte-derived macrophages because preliminary experiments indicated that prolonged culturing required for derivation minimized differences between CGD and normal macrophages, and likely explains disparate reports of their efferocytic capability.^{4,5} Monocytes were isolated from PBMCs obtained from patients with CGD and healthy normal volunteers at the National Institutes of Health Clinical Center (see the Methods section and Table E1 in this article's Online Repository at www.jacionline.org). In a first set of experiments, freshly isolated monocytes were "fed" fluorescently labeled apoptotic Jurkat cells or carboxylated beads, mimicking apoptotic cells. Efferocytosis was then quantified either by flow cytometry as

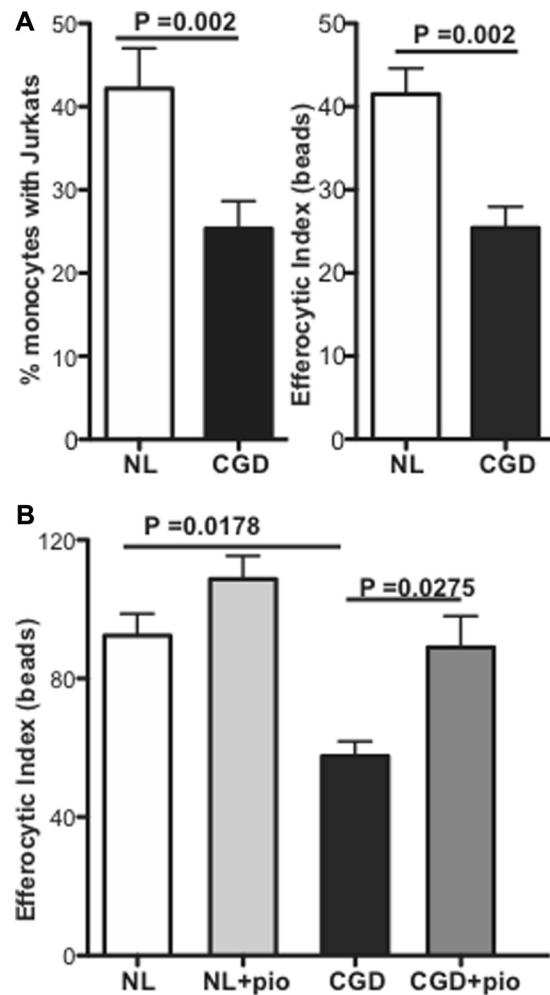


FIG 1. Impaired efferocytosis by CGD monocytes is restored by pioglitazone treatment *ex vivo*. **A**, Freshly isolated monocytes from patients with CGD and normal (NL) subjects were "fed" fluorescently labeled apoptotic Jurkat cells (left) or carboxylated beads (right) (N = 9 subjects/group). **B**, Overnight-shipped CGD and normal monocytes, plated and treated for 24 hours with pioglitazone or vehicle, were then "fed" carboxylated beads as in Fig 1, A (N = 5 subjects/group). *pio*, Pioglitazone.

the percentage of monocytes taking up Jurkat cells or by visual inspection of internalized beads and expressed as an efferocytic index (Fig 1, A; see Fig E1). As shown in Fig 1, A, freshly isolated monocytes from subjects with CGD were significantly impaired in the ability to take up both apoptotic Jurkat cells and carboxylated beads in comparison to monocytes from normal healthy volunteers. Monocytes were provided with killed *Candida* to measure generalized phagocytic ability. No impairment was seen in the uptake of killed *Candida* by CGD monocytes (Fig E1, B). These data are in keeping with murine CGD macrophage data and limited data in human CGD macrophages.^{2,4}

In further experiments, PBMCs from patients with CGD and healthy subjects (Table E1) were shipped overnight from the National Institutes of Health to Denver where monocytes were isolated by plating and then cultured overnight with pioglitazone

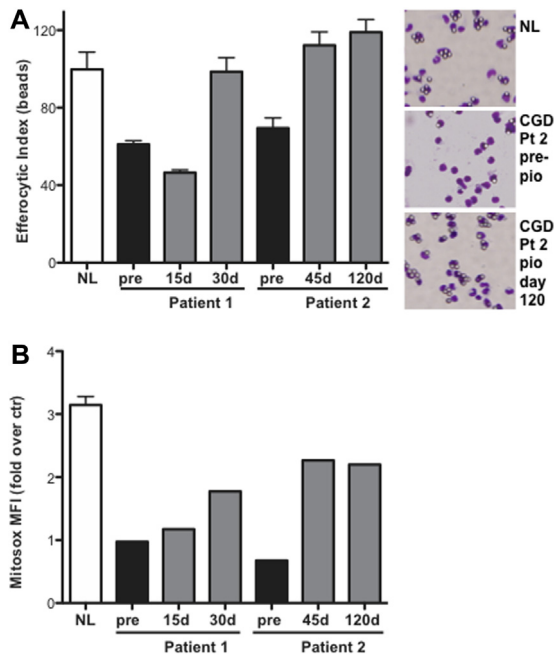


FIG 2. Efferocytosis is restored and mitochondrial oxidant production enhanced in monocytes isolated from patients with CGD during treatment with pioglitazone. **A**, Efferocytosis by CGD monocytes appears to be restored (compared with normal monocytes) after treatment with pioglitazone for at least 30 days in 2 subjects with CGD. Patient data are shown as mean $\pm$ range for duplicate wells at each time point. **B**, Mitochondrial oxidant production by stimulated CGD monocytes was enhanced during treatment with pioglitazone. ctr, Control; MFI, mean fluorescence index; NL, normal; pio, pioglitazone; pre, before; Pt, patient.

(10 μ M). Culturing in this manner with pioglitazone mimics *in vivo* pioglitazone treatment of CGD mice as measured by restoration of stimulated mitochondrial ROS production.⁷ As hypothesized, overnight culture of CGD monocytes with pioglitazone significantly enhanced the efferocytic capability, restoring it to levels seen for healthy control monocytes (Fig 1, B). Pioglitazone treatment of normal monocytes had minimal effect on efferocytic capability, and also little effect on mitochondrial ROS production.⁷

Monocytes from 2 patients with CGD (gp91^{phox} and p47^{phox} deficient, respectively) who were treated with pioglitazone (15 mg/d) for severe inflammatory bowel disease⁸ were also available for investigation. Blood from both patients was obtained before pioglitazone treatment and at 2 time points during treatment and shipped overnight along with healthy control monocytes to Denver for investigation. Monocytes were plated, rested for 2 hours, and then investigated for efferocytic capability. At baseline, monocytes from patient 1 with CGD showed an efferocytic capability of approximately 60% of that for healthy control monocytes (Fig 2, A). Although no enhancement was noted at 15 days of pioglitazone treatment, after 30 days of treatment, efferocytosis by monocytes was enhanced and comparable to that in healthy controls. For patient 2 with CGD, baseline efferocytosis before pioglitazone treatment was similarly diminished as compared to that in normal monocytes. Efferocytosis was enhanced by day 45 and day 120 of pioglitazone treatment. As predicted, stimulated production of mitochondrial ROS was also enhanced in monocytes from these

patients after their treatment with pioglitazone (Fig 2, B) and mirrored monocyte efferocytic capability.

PPAR γ agonists such as pioglitazone given over weeks alter cellular metabolism of liver and muscle cells as well as inflammatory macrophages.⁹ In addition, PPAR γ agonists have many anti-inflammatory effects including suppression of T_H1- and T_H17-induced inflammation, inhibition of nuclear factor kappa b, and upregulation of IL-10.^{9,10} As such, PPAR γ agonists are being trialed for a number of inflammatory diseases (www.clinicaltrials.gov). We have shown that PPAR γ activation occurs downstream of the activated NADPH oxidase and is lacking in CGD phagocytes, but can be restored by pioglitazone.^{2,7} PPAR γ agonism restores efferocytosis by macrophages in murine CGD, and, as shown here, in human CGD monocytes as well. This was demonstrated after both *ex vivo* treatment of human CGD monocytes and treatment of 2 patients with CGD with pioglitazone. As in our earlier studies, PPAR γ agonism was also accompanied by enhanced phagocyte mitochondrial ROS production.⁷ Whether mitochondrial ROS contributes to efferocytic capability is an area for future study. Whether pioglitazone will decrease inflammation in CGD and whether pioglitazone-enhanced mitochondrial ROS will bolster host defense in human CGD, as it did in murine models,⁷ are key unanswered questions. If human data are similar to preclinical models, pioglitazone, an on-the-shelf therapy, may ameliorate both immunodeficiency and inflammatory aspects of CGD.

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METHODS

Subjects

Blood was obtained by venipuncture from healthy donors and patients with CGD (Table E1) seen at the National Institutes of Health Clinical Center (Bethesda, Md) after informed consent was obtained. All study procedures were approved by the National Institutes of Health Institutional Review Board.

Monocyte isolation and culture

Blood was drawn into cell preparation tubes (Becton Dickinson, Franklin Lakes, NJ) and centrifuged to obtain the PBMC layer. For the first set of experiments (see Fig 1, A), monocytes were isolated from PBMCs by negative selection using magnetic beads (Miltenyi Biotec, Auburn, Calif) and allowed to adhere in X-vivo^{E1} for 2 hours. Nonadherent cells were removed by washing thrice with X-vivo^{E1} and then the monocytes were plated in X-vivo^{E1} with 10% pooled human serum to perform uptake assays as detailed below. For the second set of experiments (see Fig 1, B), centrifuged cell preparation tubes were shipped overnight to Denver, Colorado, where the PBMC layer was washed thrice with PBS and monocytes were isolated by adherence in 24-well plates. The cells were then cultured overnight in X-vivo^{E1} with 10% pooled human serum, with and without 10 μ M pioglitazone or vehicle, and efferocytosis investigated as below.

Apoptotic Jurkat cells

Cultured Jurkat cells were stained with Cell Proliferation Dye eFluor 450 (1 μ M) for 10 minutes, washed, subjected to ultraviolet light for 10 minutes, and then cultured overnight in RPMI with 10% FBS. Ninety-five percent of the cells were apoptotic by Annexin V/propidium iodide staining.

Efferocytosis assays

Efferocytosis of Jurkat cells. Freshly isolated monocytes (0.5×10^6 per well in a 24-well plate) were plated in X-vivo^{E1} with 10% pooled human serum and fed fluorescently labeled apoptotic Jurkat cells at a ratio of 1 monocyte to 4 Jurkat cells for 2 hours. Cells were then washed thrice with PBS, removed from the wells using cold PBS, and stained with CD14 (fluorescein isothiocyanate) and CD16 (phycoerythrin) for 1 hour in PBS (5% human serum) on ice. The cells were then washed and monocytes were analyzed for uptake of fluorescent Jurkat cells using flow cytometry (LSR II; BD, Franklin Lakes, NJ).

Efferocytosis of beads. Monocytes (0.3×10^6 per well on a glass coverslip in a 24-well plate), either freshly isolated and adhered or cultured overnight, were fed 5 μ m carboxylated beads (Bangs Laboratories, Fishers, Ind) at a ratio of 2 beads per monocyte for 1 hour, washed with PBS vigorously to remove free beads, and stained using a modified Wright Giemsa (Fisher Scientific, Pittsburgh, Pa).^{E2} Efferocytosis of beads was determined by visual

inspection of duplicate wells (Fig E1). A minimum of 200 monocytes were counted per well, and the efferocytic index was calculated by multiplying the percentage of macrophages that had phagocytosed beads by the average number of beads per macrophage.^{E2}

Phagocytosis assay

Freshly isolated monocytes (0.3×10^6 per well on a glass coverslip in a 24-well plate) were fed heat-killed *Candida* at a ratio of 2 cells per monocyte for 1 hour, washed with PBS, and stained using a modified Wright Giemsa. Phagocytosis of *Candida* was determined by means of visual inspection. A minimum of 200 monocytes were counted, and the phagocytic index was calculated by multiplying the percentage of macrophages that had phagocytosed *Candida* by the average number of *Candida* per macrophage.

MitoSOX assay for mitochondrial oxidant production

Upon arrival in Denver, PBMCs containing approximately 10^6 monocytes were stained with CD14 (Pacific Blue) and CD 16 (allophycocyanin-cyanine 7; APC-Cy7) in PBS (1% human serum) for 1 hour on ice. Cells were then washed, resuspended in Dulbecco modified Eagle medium (10% FBS), and stained with 5 μ M MitoSOX (Life Technologies, Grand Island, NY) for 10 minutes at 37°C in a shaking water bath.^{E3} Half the cells received 200 ng/mL phorbol 12-myristate 13-acetate and were incubated for an additional 30 minutes. The cells were then washed twice with PBS, resuspended in ice-cold PBS, and kept on ice until analysis by flow cytometry.

Statistics

All data were displayed as means $\pm$ SE except where otherwise noted. Statistical analysis and *P* values were determined using a paired *t* test. A *P* level of less than .05 was considered significant.

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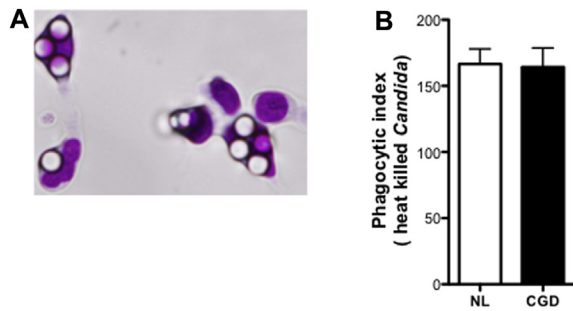


FIG E1. A, Carboxylated beads taken up by monocytes in efferocytosis assays (magnification 40×). **B,** Phagocytosis of heat-killed *Candida* by normal (NL) and CGD monocytes is not different.

TABLE E1. Demographic data for subjects with CGD

Patients	Mutation	Age (y)	Sex	Race/ethnicity
Experiment 1				
CGD1	gp91	21	M	Asian
CGD2	gp91	28	M	Black
CGD3	gp91	29	M	White
CGD4	p22	36	F	Unknown/Hispanic
CGD5	p47	47	M	Black
CGD6	gp91	23	M	White
CGD7	gp91	26	M	White
CGD8	gp91	31	M	White
CGD9	gp91	29	M	White
Experiment 2				
CGD10	gp91	31	M	White
CGD11	p47	37	M	White
CGD12	gp91	6	M	Black
CGD13	p47	30	M	White/Hispanic
CGD14	p47	36	M	White

F, Female; *M*, male.