Modulatory Effects of CD14+CD16++ Monocytes on CD14++CD16− Monocytes

A Possible Explanation of Monocyte Alterations in Systemic Lupus Erythematosus

C. Burbano, G. Vasquez, and M. Rojas

Objective. **In various chronic inflammatory processes, both the proportion and numbers of monocyte subsets are altered. In systemic lupus erythematosus (SLE), this has not been clearly determined. The monocyte subpopulations in patients with SLE, patients with other autoimmune diseases, and healthy controls were evaluated. The effects of nonclassic monocytes and apoptotic cells (ACs) on the differentiation and function of CD14++CD16− monocytes were also studied.**

Methods. **Monocyte subpopulations derived from the blood samples of SLE patients (n = 88), patients with other autoimmune diseases (n = 37), and healthy control subjects (n = 61) were separated by fluorescence-activated cell sorting. To evaluate the effect of CD14+CD16++ monocytes and ACs on the differentiation of CD14++CD16− monocytes, we developed a coculture model of highly purified sorted monocyte subpopulations, which were reconstituted with defined proportions of CD14++CD16− and CD14+CD16++ monocytes in the presence or absence of ACs. After differentiation into macrophages, CD3+ lymphocytes were added, and the proliferating cells and CD3+IFNγ+ cells were evaluated. A cytokine bead array panel was used to test the coculture supernatants.**

Results. **There was a reduction in CD14+CD16++ monocytes in patients with active SLE. Monocytes from SLE patients had decreased expression of HLA–DR and decreased ability to bind and phagocytize ACs. In healthy controls, but not SLE patients, treatment with macrophages derived from CD14+CD16++ monocytes reduced T cell proliferation and proliferating CD3+IFNγ+ cells and increased the accumulation of tumor necrosis factor α, interleukin-10 (IL-10), and IL-1β.**

Conclusion. **Our findings show that CD14+CD16++ monocytes, a population that is reduced and nonfunctional in SLE patients, have modulatory effects on CD14++CD16− monocytes and T cells.**

Different pathologic conditions show altered ratios of circulating CD14++CD16− and CD14+CD16+ monocytes. Increased numbers of CD16+ monocytes have been reported to be correlated with disease severity and poor prognosis (1). However, during episodes of hypoxia, such as those caused by myocardial infarction and stroke, an increase in the number of CD14++CD16−, but not CD16+, monocytes has been correlated with disease severity and poor prognosis (1,2). The distribution of monocyte subsets in SLE patients is a subject of controversy (3–5). An increase in CD16+ monocytes in these patients has been shown, albeit not significantly increased (4), whereas other studies have found no differences from healthy controls (5).

CD16+ monocytes highly express CX3CR1 and migrate in response to fractalkine (6,7). They are recruited to the areas of tissue damage and inflammation, and they are considered proinflammatory (8). After Toll-like receptor 4 stimulation, they produce more tumor necrosis factor α (TNFα) but less interleukin-10 (IL-10) compared with CD14++CD16− monocytes (9). Changes in the expression of CD14 and CD16 occur very

C. Burbano, MSc, G. Vasquez, MD, PhD, M. Rojas, PhD: University of Antioquia, Medellín, Antioquia, Colombia.

Address correspondence to M. Rojas, PhD, Unidad de Citometría de Flujo, Sede de Investigación Universitaria, Universidad de Antioquia, Calle 70 No. 52-21, Medellín, Antioquia 050031, Colombia. E-mail: mrojaslop@hotmail.com.

quickly and do not enable the tracking of subsets in vitro. Functional and morphologic heterogeneity and the complexity of the techniques used to sort monocyte subsets make their study an unsettled issue.

Under physiologic conditions, apoptotic cells (ACs) must be removed quickly to avoid the release of their intracellular contents, limiting the inflammatory response that induces tolerance to self antigens and preventing the oxidative changes in the extracellular milieu (10). It was hypothesized that these events are truncated in systemic lupus erythematosus (SLE) and that the contact with dead cells may promote the secretion of inflammatory cytokines in these patients (11). Although ACs are recognized by different phagocytes (10), monocytes and macrophages have been shown to play a determinant role, in contrast to other phagocytic cells, such as dendritic cells and polymorphonuclear cells (12,13). The imbalance in the proportions of CD14++ CD16− and CD14+CD16+ monocytes in different pathologic conditions involving chronic inflammation processes, such as in SLE, may affect the course of different functions of these cells, including differentiation and activation, with subsequent impact on the adaptive immune response.

In the present study, we examined 3 monocyte subsets in patients with SLE, patients with other autoimmune diseases, and healthy controls. Our results demonstrate that in patients with active SLE, there was a reduction in CD14+CD16++ monocytes. In both CD14++CD16− and CD14++CD16+ monocytes from these patients, there was decreased expression of HLA–DR and a decreased ability to bind and phagocytize ACs. To evaluate the effect of CD14+CD16++ monocytes and ACs on the differentiation of CD14++ CD16− monocytes, we developed a model in which highly purified sorted monocyte subpopulations from healthy controls and SLE patients were cocultured with defined proportions of CD14++CD16− and CD14+CD16++ monocytes in the presence or absence of ACs. After their differentiation to macrophage-derived monocytes (MDMs), CD3+ lymphocytes were added, and proliferating cells and CD3+IFNγ+ cells were then evaluated. CD14+CD16++ MDMs isolated from healthy controls, but not from SLE patients, reduced T cell proliferation and the percentage of CD3+IFNγ+ proliferating cells and increased the accumulation of the TNFα, IL-10, and IL-1β cytokines. Taken together, these findings suggest a modulatory effect of CD14+CD16++ monocytes on their CD14++CD16− counterparts. This ability was not observed in CD14+CD16++ monocytes from SLE patients.

PATIENTS AND METHODS

Reagents and antibodies. RPMI 1640 GlutaMax medium, phosphate buffered saline (PBS), and fetal bovine serum (FBS) were purchased from Gibco BRL. Paraformaldehyde was purchased from Fisher Scientific. Histopaque 1077, trypan blue, anhydrous DMSO, phytohemagglutinin leukoagglutinin (PHA-L), and bovine serum albumin (BSA) were obtained from Sigma-Aldrich. Penicillin and streptomycin were purchased from Cambrex-BioWhittaker. Carboxyfluorescein succinimidyl ester (CFSE) and 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) were obtained from Invitrogen. Propidium iodide (PI), apoptosis detection annexin V/7-aminoactinomycin D (7-AAD), and a human inflammatory Cytometric Bead Array were purchased from BD PharMingen. OptiLyse and absolute counting beads were purchased from Beckman Coulter. Rosette Sep Human Monocyte Enrichment Cocktail was obtained from StemCell Technologies.

CyChrome-labeled monoclonal anti-human CD14 (clone MφP9), fluorescein isothiocyanate (FITC)–labeled CD16 (clone 3G8), phycoerythrin (PE)–Cy7–labeled CD45 (clone HI30), FITC-labeled CD80 (clone MOPC-21), PE–Cy5–labeled CD86 (clone IT2, 2), PE-labeled HLA–DR (clone G46-6), PerCP-labeled CD3 (clone SK7), FITC-labeled CD19 (clone HIB19), PE-labeled CD56 (clone B159), allophycocyanin (APC)–labeled interferon-γ (IFNγ) (clone B27), APC-labeled CD36 (clone CB38), PE-labeled CD4 (clone RPA-T4), APC-labeled isotype IgG1κ (clone G155-178), PE-labeled IgG2aκ (clone X39), FITC-labeled IgG1κ (clone MOPC-1), PE–Cy5–labeled IgG2bκ (clone 27-35), FITC-labeled IgG1κ (clone X40), and APC-labeled IgMκ (clone 6155-228) were obtained from BD Biosciences PharMingen. PE-labeled MY4 (clone 322A1) was obtained from Beckman Coulter, PE-labeled immune receptor expressed by myeloid cells 2 (IREM-2) (CD300e clone UP-H2) from BioLegend, V450-labeled CD33 (clone WM53) from BD Biosciences, and eFluor 450–labeled CD8 (clone OKT8) from eBioscience.

Patients and controls. Eighty-four patients with SLE, which was diagnosed according to the 1982 criteria of the American College of Rheumatology (14), were recruited at the Hospital Universitario San Vicente Fundacion, at the Rheumatology Service of the Universidad de Antioquia Medellín. The patients were classified according to the Systemic Lupus Erythematosus Activity Index (SLEDAI) as having either inactive SLE (SLEDAI ≤4) or active SLE (SLEDAI >4) (15). As controls, we included 61 healthy subjects as well as 37 patients with other autoimmune diseases who had received treatment similar to that of the SLE patients (Table 1). All subjects reported being negative for human immunodeficiency virus infection and had no evidence of other infections. The healthy controls had no history of cancer, diabetes mellitus, rheumatoid arthritis (RA), SLE, or other autoimmune diseases, and none of them were receiving immunosuppressive therapy.

All patients and healthy controls gave informed consent for study. This study was approved by the Ethics Committee of the Universidad de Antioquia Medical Research Institute.

Immunophenotyping of monocyte subsets. Twenty-five microliters of EDTA-anticoagulated peripheral blood was stained with 5 μl of PerCP-labeled anti-CD14, FITC-labeled anti-CD16, and PE-labeled anti–HLA–DR or the isotype

Table 1. Demographic and clinical characteristics of the study patients and controls*

	Healthy controls (n = 61)	Patients		
		Inactive SLE (SLEDAI ≤4) (n = 34)	Active SLE (SLEDAI >4) (n = 54)	Other autoimmune diseases (n = 37)
Age, median (IQR) years	29 (24–40)	35 (24–47)	26 (22–37)	44 (28–55)†
No. female/male	54/7	32/2	50/4	29/8
SLEDAI, median (IQR)	–	1 (0–3)	12 (8–20)	–
Medications, no. (%)				
Prednisolone	–	31 (91)	52 (96)	31 (84)
Chloroquine	–	20 (59)	27 (50)	13 (35)
Azathioprine	–	2 (6)	4 (7)	1 (3)
Cyclophosphamide	–	5 (15)	8 (15)	2 (5)
Methotrexate	–	2 (6)	2 (4)	5 (14)
Mycophenolate	–	9 (26)	11 (20)	3 (8)
Other	–	10 (29)	15 (28)	13 (35)

* Patients with systemic lupus erythematosus (SLE) were diagnosed according to the criteria of the American College of Rheumatology. The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) was used to define disease activity. Patients with other autoimmune diseases had rheumatoid arthritis (n = 15), Sjögren's syndrome (n = 5), systemic sclerosis (n = 3), vasculitis (n = 3), psoriatic arthritis (n = 2), granulomatosis with polyangiitis (Wegener's) (n = 2), dermatomyositis (n = 2), antiphospholipid syndrome (n = 1), myopathy (n = 1), polyneuropathy (n = 1), juvenile idiopathic arthritis (n = 1), or mixed connective tissue disease (n = 1). IQR = interquartile range.
† $P < 0.05$ versus the healthy control group and versus the active SLE group, by Kruskal-Wallis test with Dunn's post hoc test.

controls. In some samples, anti–IREM-2 (16,17), CD33, and CD45 were used to confirm monocytes (data available upon request from the corresponding author). Samples were mixed and incubated for 20 minutes at room temperature in the dark. Red cells were lysed with 250 μl of OptiLyse and analyzed using a FACSCanto II (Becton Dickinson). Leukocyte counting was performed on 10 μl of peripheral blood plus 210 μl of 0.03% (volume/volume) acetic acid in a Neubauer chamber under an optical microscope. The results of manual counting were compared with the results using absolute counting beads (data available upon request from the corresponding author).

Isolation of peripheral blood mononuclear cells (PBMCs) and monolayer culture of CD14+ phagocytes. Monolayers were obtained as previously reported (18), and adherent mononuclear phagocyte cells were incubated in an atmosphere of 5% CO_2 for 24 hours at 37°C in 500 μl of RPMI 1640 plus 10% heat-inactivated autologous serum. The remaining cells were mainly CD3+, CD19+, and CD56+, which each represented <5%. Cell viability, as determined by trypan blue exclusion, was >98%.

Preparation of ACs from Jurkat cells. Jurkat cells (ATCC TIB-152) were cultured at 1×10^6 cells/ml in tissue culture flasks containing RPMI 1640 supplemented with 5% heat-inactivated FBS, 100 μg/ml of streptomycin, and 100 IU/ml of penicillin at 37°C in an atmosphere of 5% CO_2 and allowed to overgrow for more than 120 hours to obtain dead cells. The cells were stained with $DIOC_6$ (20 nM) and 1 μg/ml of PI to detect cells with mitochondrial and membrane damage. Then, $DIOC_6^{low}$ and PI− cells were electromagnetically sorted using a MoFlo XDP (Beckman Coulter). The purity and efficiency were always ≥98% and ≥95%, respectively. The ACs were frozen in 10% DMSO in heat-inactivated FBS and stored in liquid nitrogen until they were used in the experiments. The ACs were annexin V+ and 7-AAD−.

Uptake of ACs and expression of HLA–DR, CD80, and CD86 after exposure to ACs. To evaluate the binding and uptake of ACs by CD14+ phagocytes, monolayers from 24-hour cultures were left unexposed or were exposed to $DIOC_6$-labeled ACs at a ratio of 1:2 (phagocytes to ACs) per well. This ratio was maintained for all experiments that used ACs. The cells were centrifuged at 400g for 1 minute and incubated for 1 hour at 37°C in an atmosphere of 5% CO_2. Next, the unbound ACs were removed by washing with prewarmed PBS supplemented with 0.5% heat-inactivated FBS, and the phagocytes were collected by mechanical scraping. The binding and uptake of ACs was estimated by fluorescence-activated cell sorting (FACS). To estimate the percentage of cells with internalized ACs, the samples were acquired again on the FACS in the presence of 0.01% v/v trypan blue to quench the fluorescence of bound and noninternalized ACs (19).

In parallel cultures, after 24 hours in the presence or absence of ACs, the expression of HLA–DR, CD80, and CD86 in adherent mononuclear phagocytes was evaluated by FACS.

Purification of CD14++CD16− and CD14+CD16++ monocytes. Monocyte enrichment was performed using commercial tetrameric antibody complexes according to the manufacturer's instructions. Enriched monocytes (purity ≥65%) were then labeled using the following antibodies: PE–Cy5–labeled anti-CD45, PerCP-labeled anti–HLA–DR, PE-labeled anti-MY4, and FITC-labeled anti-CD16. The CD45+ and HLA–DR+ CD14++CD16− monocytes and the CD45+ and HLA–DR++ CD14+ CD16++ monocytes were electromagnetically sorted using MoFlo XDP. The monocyte subsets had purities of 93−98%. The recovery efficiencies were ≥90% for CD14++CD16− monocytes and ≥70% for CD14+CD16++ monocytes.

Coculture of monocyte subsets. CD14++CD16− and CD14+CD16++ monocytes were cocultured based on previ-
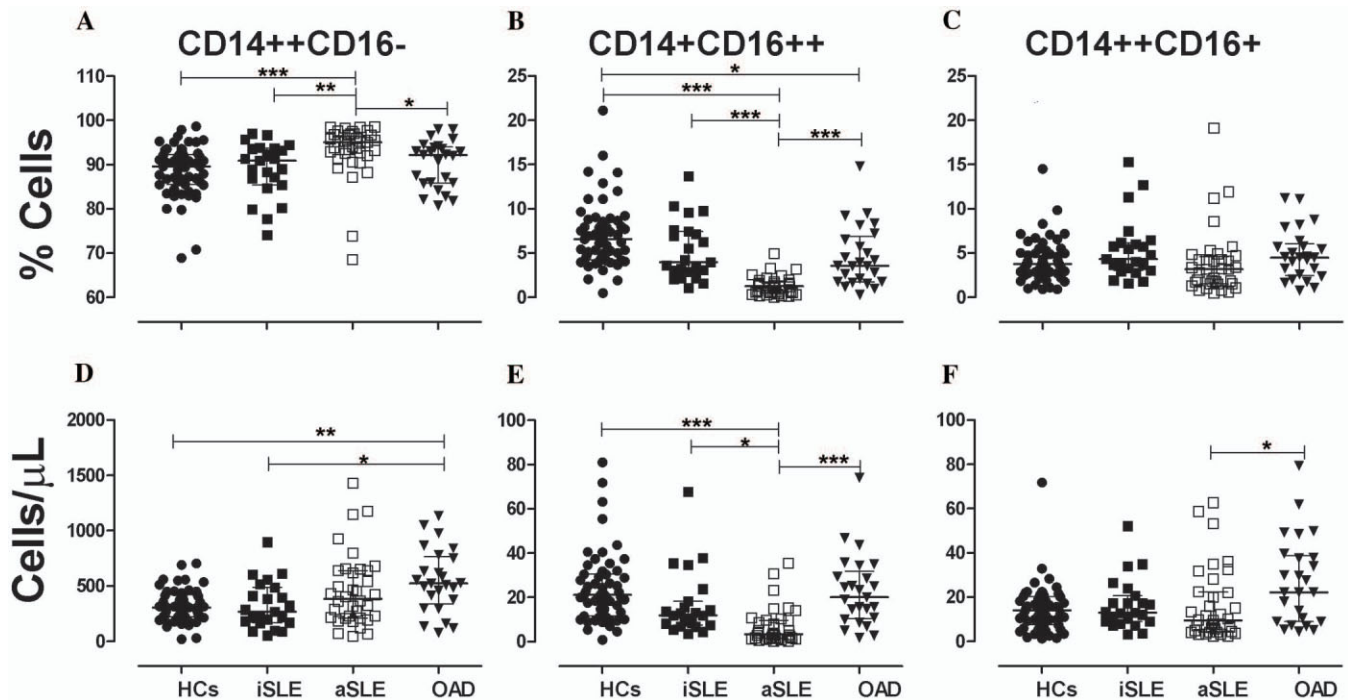
Figure 1. Monocyte subsets in the patient and control groups. Monocytes were defined by fluorescence-activated cell sorter analysis based on the surface expression of CD14, CD16, and HLA–DR, as previously reported (24,47). Shown are the percentages (**A–C**) and absolute numbers (**D–F**) of classic (CD14++CD16−) (**A** and **D**), nonclassic (CD14+CD16++) (**B** and **E**), and intermediate (CD14++CD16+) (**C** and **F**) monocytes in the healthy controls (HCs; n = 55), patients with inactive systemic lupus erythematosus (iSLE; n = 23), patients with active SLE (aSLE; n = 36), and patients with other autoimmune diseases (OAD; n = 27). Each symbol represents an individual subject; horizontal lines with error bars show the mean and interquartile range. ∗ = $P \leq 0.05$; ∗∗ = $P \leq 0.01$; ∗∗∗ = $P \leq 0.001$ by Kruskal-Wallis test with Dunn's post hoc test.

ously reported ratios (6:4 ratio of CD14++CD16− cells to CD14+CD16++ cells) (2,18,20–22). The final number of cells per well was 1.2×10^5, using 96-well plates. The cells were incubated for 4 hours in 200 μl of RPMI 1640 with 0.5% inactivated autologous serum plus 100 μg/ml of streptomycin and 100 IU/ml of penicillin to allow their adhesion. The cells were then washed and incubated overnight in 200 μl of RPMI 1640 with 10% inactivated autologous serum. At this time, ACs were added at a 2:1 ratio (ACs to monocytes), and the cells were incubated for 120 hours to complete the differentiation of monocytes into CD14++CD16− MDMs and CD14+ CD16++ MDMs. Cytokines (IL-12p70, TNFα, IL-10, IL-6, IL-8, and IL-1β) in 50 μl of culture supernatants were analyzed using a Cytometric Bead Array and FACS. The concentrations (in pg/ml) were calculated using commercial kit standards.

Coculture of MDM with autologous CD3+ lymphocytes. To evaluate the effects of the CD14++CD16− MDMs and CD14+CD16++ MDMs plus ACs on lymphocyte proliferation and the proportion of IFNγ-producing cells, freshly isolated autologous CD3+ cells (purity >95% and efficiency >90%) were electromagnetically sorted, labeled with CFSE, and added to the cocultures at 120 hours of differentiation. Cocultures with CD3+ cells were left unstimulated or were stimulated with 10 μg/ml of PHA-L and incubated for an additional 96 hours (23). Four hours before the end of the culture, the cells were treated with 1 μg/ml of brefeldin A. The

cells were then recovered by washing with PBS, stained for 30 minutes with PE-labeled anti-CD4 and eFluor 450–labeled anti-CD8, fixed with 2% paraformaldehyde, and permeabilized with 0.5% Tween 20 and 0.2% BSA for 30 minutes. The lymphocytes were intracellularly stained for 1 hour with APC-labeled anti-IFNγ or an isotype control and analyzed by FACS.

Statistical analysis. Analyses were conducted using GraphPad Prism software version 5.0. Comparisons among groups were performed using the Kruskal-Wallis test with Dunn's post hoc test. Data are presented as the median and interquartile range. When necessary, two-way analysis of variance (ANOVA) with the Bonferroni post hoc test was performed. Wilcoxon's signed rank test was used to compare the effects in paired experiments. *P* values less than or equal to 0.05 were considered statistically significant. The analysis of proliferating CD4+ and CD8+ cells was performed using FlowJo software version 7.6.5 (Tree Star).

RESULTS

Demographic and clinical characteristics of the patients and controls. The demographic characteristics of the patients and healthy controls and the SLEDAI of the patients with inactive SLE and patients with active
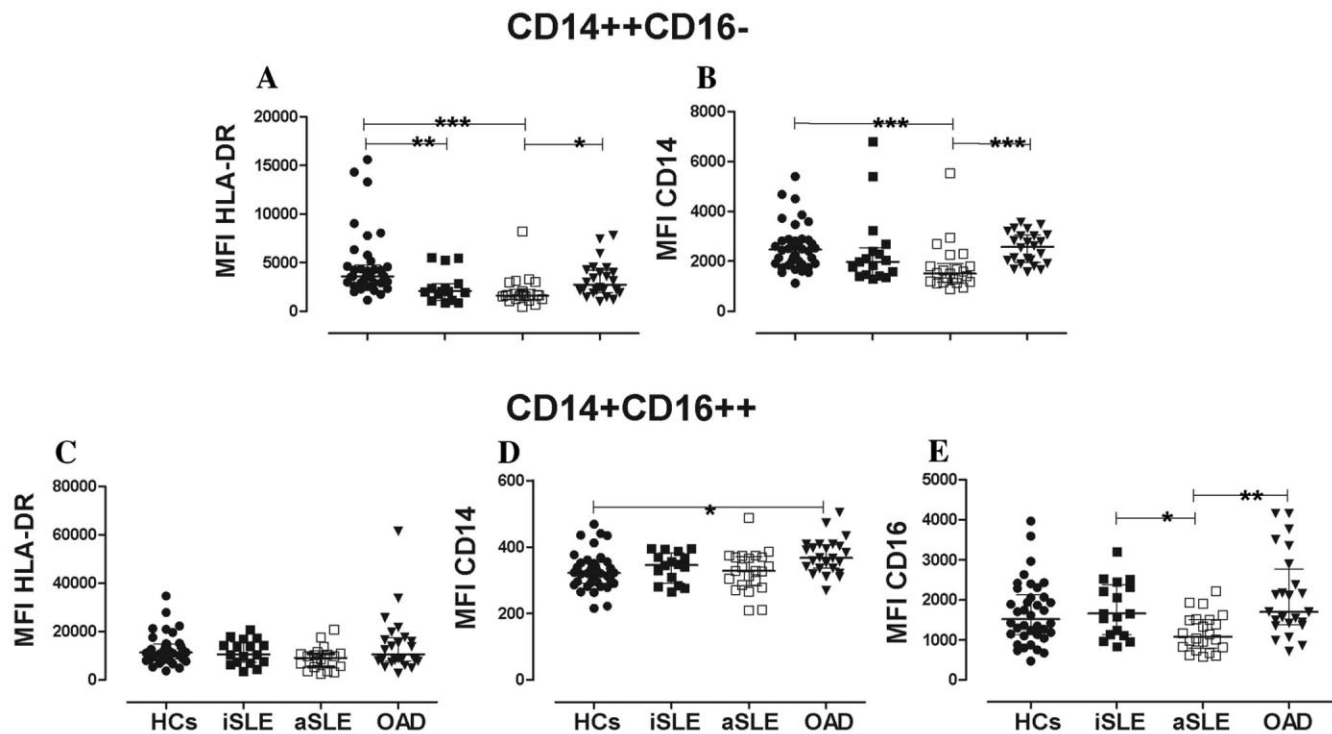
Figure 2. Expression of HLA–DR, CD14, and CD16 by monocyte subsets from the patient and control groups. HLA–DR and CD14 were analyzed in the classic monocyte (**A** and **B**) and nonclassic monocyte (**C** and **D**) populations from the healthy controls (HCs; n = 38), patients with inactive systemic lupus erythematosus (iSLE; n = 17), patients with active SLE (aSLE; n = 22), and patients with other autoimmune diseases (OAD; n = 26). CD16 fluorescence was analyzed in nonclassic monocytes (**E**) from the same study groups. Results are expressed as the mean fluorescence intensity (MFI). Each symbol represents an individual subject; horizontal lines with error bars show the mean and interquartile range. ∗ = $P \leq 0.05$; ∗∗ = $P \leq 0.01$; ∗∗∗ = $P \leq 0.001$ by Kruskal-Wallis test with Dunn's post hoc test.

SLE are displayed in Table 1. The age range was similar in the patients and healthy controls; however, the median age of the patients with other autoimmune diseases was significantly higher than that of the patients with active SLE and the healthy controls. In all groups, the majority of subjects were women. Medications taken were comparable among the patient groups.

Monocyte subsets. Monocyte subpopulations were defined as 3 subsets based on previous reports (20,24): CD14++CD16− (classic), CD14+CD16++ (nonclassic), and CD14++CD16+ (intermediate) monocytes. Patients with active SLE had a higher percentage of CD14++CD16− monocytes (Figure 1A) and a decrease in CD14+CD16++ monocytes (Figure 1B) as compared with the inactive SLE, other autoimmune diseases, and healthy control groups. In terms of absolute numbers (Figures 1D–F), only the patients with other autoimmune diseases had significantly higher numbers of CD14++CD16− monocytes (Figure 1D) compared with the inactive SLE and healthy control

groups, and the patients with active SLE had lower numbers of CD14+CD16++ monocytes compared with the other groups (Figure 1E).

There were no differences in the percentages or numbers of CD14++CD16+ monocytes between the inactive and active SLE groups compared with the healthy control group (Figures 1C and F). Only the patients with other autoimmune diseases had higher numbers as compared with the active SLE group (Figure 1F). Notably, the absolute number of leukocytes was similar between the inactive and active SLE groups and was similar to the healthy control group. Only the patients with other autoimmune diseases showed a greater number of total leukocytes ($P < 0.01$) (data not shown).

CD14++CD16− monocytes from patients with active SLE expressed less HLA–DR and CD14 per cell compared with those from the healthy controls and patients with other autoimmune diseases (Figures 2A and B). In the patients with inactive SLE, there was

significantly lower expression of HLA–DR compared with the healthy controls, and the expression of CD14 was lower compared with that of the healthy controls, but this difference was not significant.

The CD14+CD16++ monocytes had a similar expression of HLA–DR per cell in all study groups (Figure 2C). The expression of CD14 was higher in the CD14+CD16++ monocytes from the patients with other autoimmune diseases than in the healthy controls (Figure 2D), and the expression of CD16 was significantly lower in the cells from patients with active SLE than in those from the inactive SLE and other autoimmune diseases groups (Figure 2E).

Interaction of monocytes with ACs. Patients with SLE have been reported to have an impaired ability to remove ACs (25), and the interaction with ACs may affect monocyte differentiation and activation. Thus, CD14+ phagocytes from the healthy controls, SLE patients, and patients with other autoimmune diseases were exposed for 1 hour to ACs derived from Jurkat T lymphocytes to evaluate their binding and internalization and for 24 hours to evaluate the expression of CD80, CD86, and HLA–DR. As shown in Figure 3, although phagocytes from SLE patients had a reduced capacity to bind (Figure 3A) and take up (Figure 3B) ACs as compared with those from the healthy controls and patients with other autoimmune diseases, both were dramatically decreased with increasing disease activity, since patients with active SLE displayed the lowest uptake and binding of ACs.

Although healthy control phagocytes exposed to ACs significantly increased their expression of CD80 per cell in all individuals ($P \leq 0.001$) (Figure 3C), this treatment did not increase the expression of this molecule in phagocytes from SLE patients or patients with other autoimmune diseases. Phagocytes from the SLE patients and patients with other autoimmune diseases expressed lower levels of CD86 than did those from the healthy controls, but treatment with ACs had no significant effect on CD86 expression (Figure 3D).

To establish whether there was a differential change in the regulation of CD86 and CD80 in response to the ACs, we compared the CD86-to-CD80 ratio. In phagocytes from the healthy controls, exposure to ACs significantly reduced this ratio ($P \leq 0.001$) (Figure 3E), whereas in phagocytes from the SLE and other autoimmune diseases groups, there were no changes. These differences were not significant between the active and inactive SLE groups.

Treatment with ACs had no significant effect on the surface expression of HLA–DR among the study
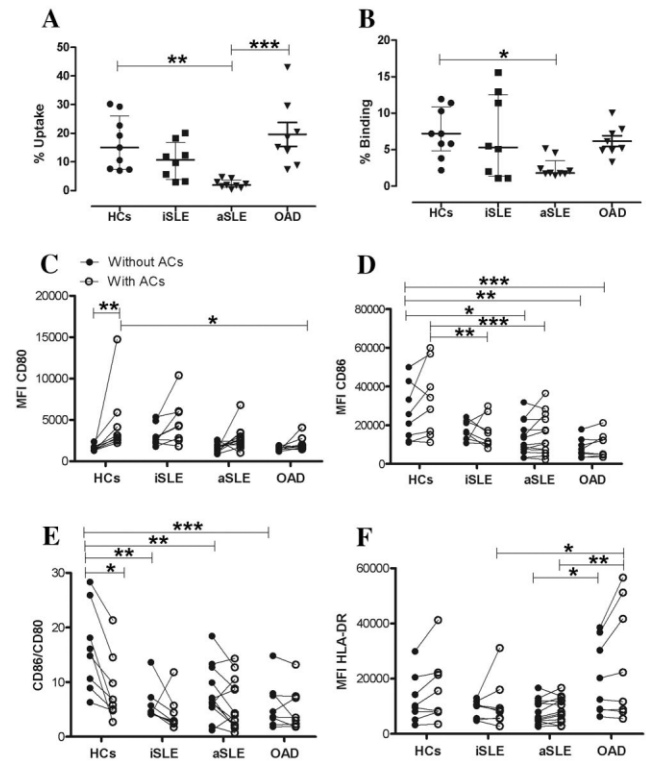
Figure 3. Impaired interactions of CD14+ phagocytes from systemic lupus erythematosus (SLE) patients with apoptotic cells (ACs). Jurkat cells were overgrown, stained with 3,3′-dihexyloxacarbocyanine iodide (DiOC$_6$) and propidium iodide (PI), and electromagnetically sorted to obtain DiOC$_6$^{low} cells without cell membrane damage (PI−) (data not shown). After 24 hours in culture, ACs were added, and the cells were incubated for 1 hour. The percentage of uptake (**A**) and binding (**B**) of ACs by CD14+ phagocytes from the healthy controls (HCs; n = 9), patients with inactive SLE (iSLE; n = 8), patients with active SLE (aSLE; n = 9), and patients with other autoimmune diseases (OAD; n = 8) were determined. CD80 expression (**C**), CD86 expression (**D**), the CD86-to-CD80 ratio (**E**), and HLA–DR expression (**F**) per cell in the healthy controls (n = 8) and in the patients with inactive SLE (n = 8), active SLE (n = 13), and other autoimmune diseases (n = 8) were evaluated in CD14+ phagocytes that had been incubated for 24 hours followed by an additional 24 hours in the presence (solid circles) or absence (open circles) of ACs. Cells were then analyzed by fluorescence-activated cell sorting. Each symbol represents an individual subject; horizontal lines with error bars in **A** and **B** show the mean and interquartile range. ∗ = $P \leq 0.05$; ∗∗ = $P \leq 0.01$; ∗∗∗ = $P \leq 0.001$ by Kruskal-Wallis test with Dunn's post hoc test in **A** and **B**, by two-way analysis of variance with the Bonferroni post hoc test in **C–F**, and by Wilcoxon's signed rank test for the comparison of AC treatment versus non–AC treatment in **C–F**. MFI = mean fluorescence intensity.

groups. However, phagocytes from patients with other autoimmune diseases expressed higher levels of HLA–DR in both the absence and presence of ACs, and this difference was significant compared with the healthy controls ($P \leq 0.05$) (Figure 3F).
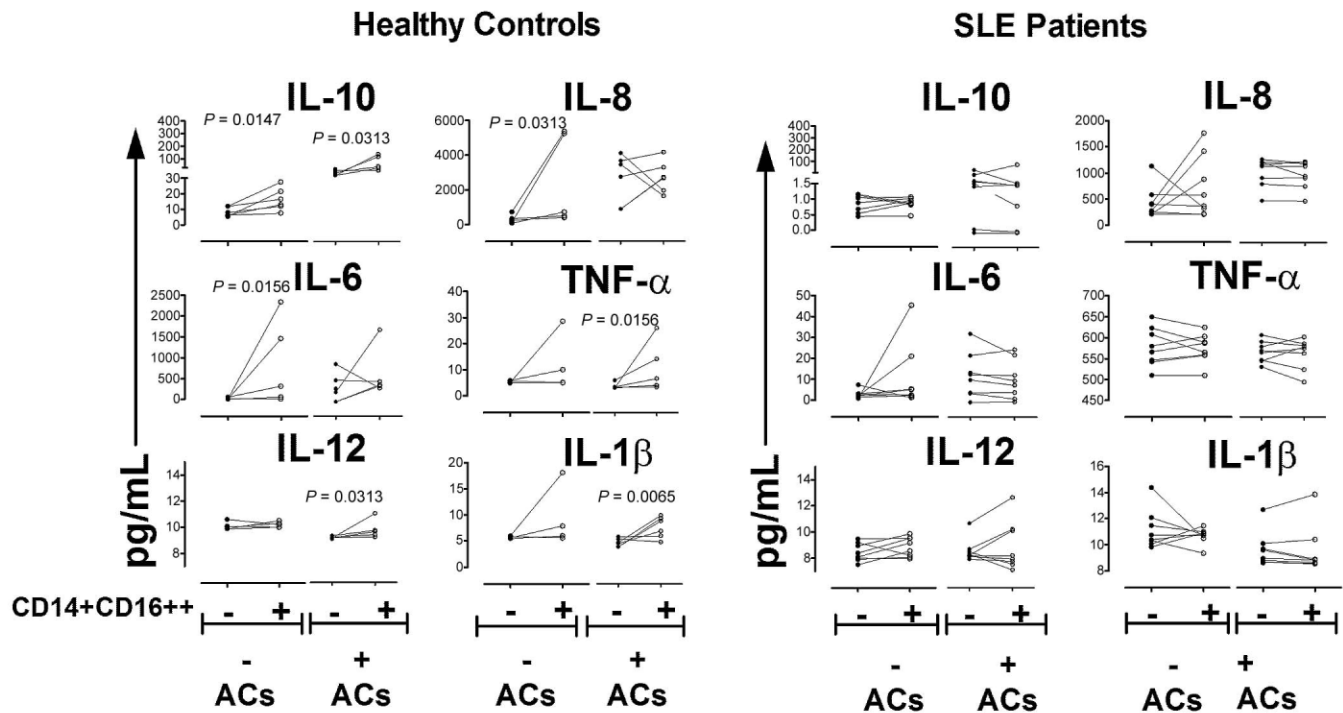
Figure 4. Levels of cytokines released into culture supernatants from monocyte-derived macrophages (MDMs) obtained from healthy controls and patients with systemic lupus erythematosus (SLE), as measured by Cytometric Bead Array assay. Levels of interleukin-10 (IL-10), IL-6, IL-12p70, IL-8, tumor necrosis factor α (TNFα), and IL-1β were evaluated at 120 hours of coculture of CD14++CD16− MDMs in the presence or absence of CD14+CD16++ MDMs that were left untreated (−) or were treated (+) with apoptotic cells (ACs) (before the addition of autologous CD3+ T cells). The patients and healthy controls were young women of the same age range. The healthy control (n = 5), inactive SLE (n = 2), active SLE (n = 6), and the combined inactive and active SLE groups had similar cytokine profiles. P values compare the presence versus absence of CD14+CD16++ MDMs, as determined by Wilcoxon's signed rank test.

Negligible effect of CD14+CD16++ MDMs alone on cytokine accumulation. As patients with SLE have an impaired proportion of CD14+CD16++ and CD14++CD16− monocytes, we postulated that this imbalance could affect the course of their differentiation and their adaptive immune response. To test this hypothesis, CD14++CD16− and CD14+CD16++ monocytes were purified from the peripheral blood of healthy controls and SLE patients. The CD14++CD16− monocytes were then differentiated into macrophages for 120 hours in the presence or absence of CD14+CD16++ monocytes and ACs. Next, we assessed the accumulation of cytokines in the culture supernatants (Figure 4). In healthy controls, in the absence of ACs, the presence of CD14+CD16++ MDMs significantly increased IL-10, IL-6, and IL-8 expression (Figure 4). There was a nonsignificant trend toward increases in the TNFα and IL-1β levels, and the accumulation of IL-12p70 was not modified (Figure 4).

In supernatants from CD14++CD16− MDMs obtained from healthy controls, treatment with ACs increased IL-10 ($P < 0.0156$), IL-6 ($P < 0.0313$), and IL-8 ($P < 0.0156$) expression as compared with supernatants from CD14++CD16− MDMs alone (Figure 4). In supernatants from CD14++CD16− MDMs plus CD14+CD16++ MDMs and ACs, there was a significant increase in IL-10 ($P < 0.0313$) compared with supernatants from CD14++CD16− MDMs plus CD14+CD16++ MDMs differentiated in the absence of ACs (Figure 4). The most noteworthy finding was that cells from SLE patients were dependent upon the presence of ACs; however, the presence of CD14+CD16++ MDMs did not significantly affect cytokine accumulation (Figure 4).

Alterations in the T cell response induced by CD14+CD16++ MDMs in the presence or absence of ACs. To determine the possible effects of the cocultures of MDM on the T cell response, highly purified CD3+ lymphocytes were stained with CFSE to assess cell division and the frequency of IFNγ+ cells (Figure 5A).
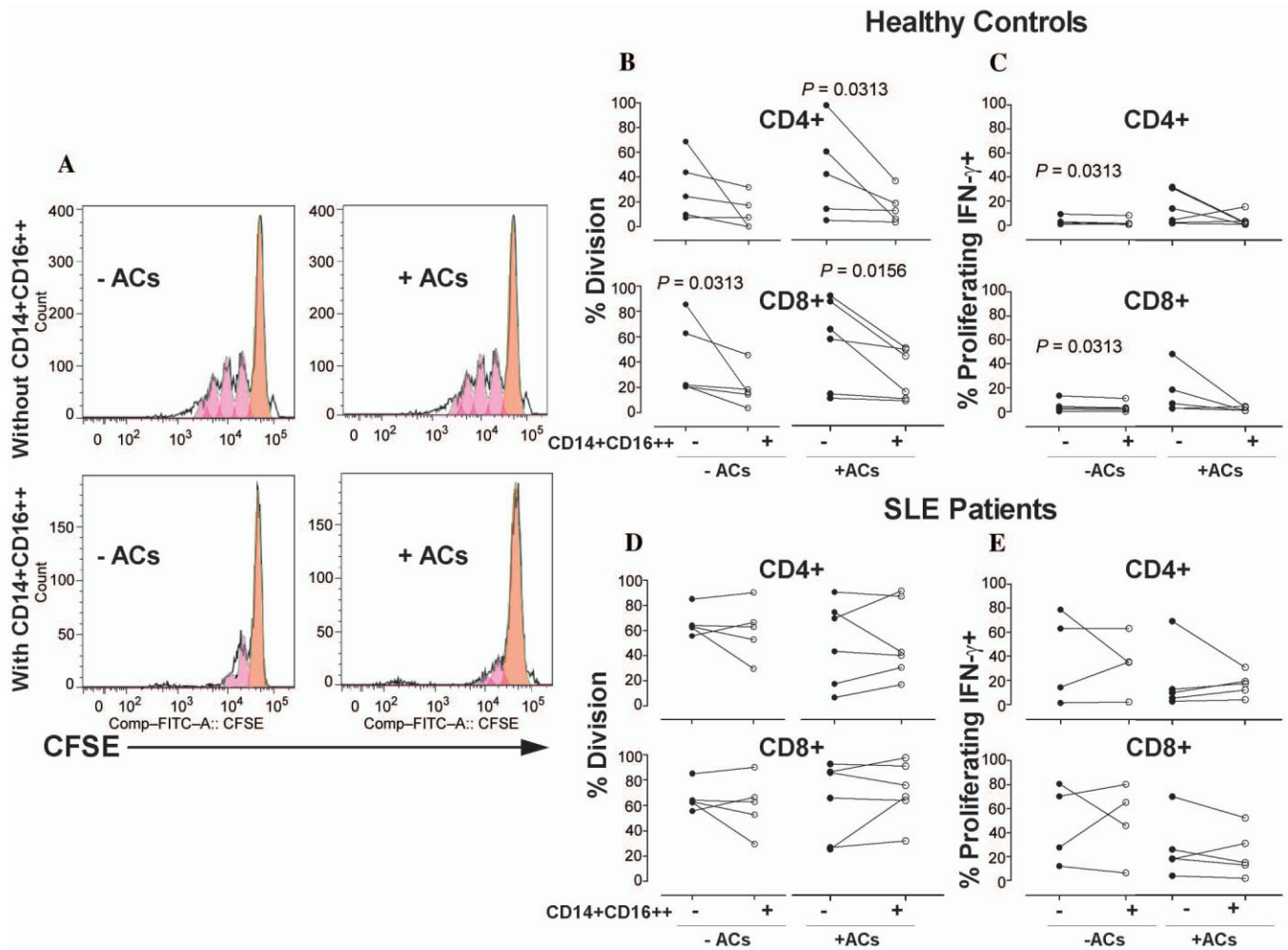
Figure 5. Reduced T cell proliferation and proportion of proliferating interferon-γ (IFNγ)–positive T cells by CD14+CD16++ macrophage-derived monocytes (MDMs) independently of the presence of apoptotic cells (ACs). **A,** Representative histograms showing carboxyfluorescein succinimidyl ester (CFSE) dilution of CD4+ lymphocytes cocultured with CD14++CD16− MDMs that had been differentiated in the absence (top) or presence (bottom) of CD14+CD16++ MDMs. Cocultured cells were also cultured in the absence or presence of ACs and stimulated with phytohemagglutinin leukoagglutinin. **B–E,** Percentage of cell division (**B** and **D**) and proliferating IFNγ+ (**C** and **E**) in CD4+ and CD8+ T cells from healthy controls and patients with systemic lupus erythematosus (SLE). The patients and healthy controls were young women of the same age range. The healthy control (n = 5), inactive SLE (n = 2), active SLE (n = 4), and the combined inactive and active SLE groups had similar T cell proliferation and proportion of proliferating IFNγ+ T cell profiles. Results are representative of 5 independent experiments. *P* values compare the presence versus absence of CD14+CD16++ MDMs, as determined by Wilcoxon's signed rank test.

Although the presence of ACs did not affect the percentage of dividing cells (Figure 5B), the presence of CD14+CD16++ MDMs in the cocultures reduced the percentage of dividing cells in both CD4+ and CD8+ lymphocytes in all 5 individuals tested. The simultaneous presence of CD14+CD16++ MDMs and ACs also reduced the rates of division in both CD4+ and CD8+ lymphocytes (*P* = 0.0156 for each comparison; data not shown) and the percentage of dividing cells in both CD4+ (*P* = 0.0313) and CD8+ (*P* = 0.0156) lympho-

cytes (Figure 5B). In contrast, in only 1 of the 4 SLE patients tested did the CD14+CD16++ MDMs reduce T cell proliferation (Figure 5D), which highlights the dysfunction of this subset in SLE patients.

Notably, in healthy controls the presence of CD14+CD16++ MDMs, independently of the presence of ACs, had no effect on the frequency of quiescent CD4+IFNγ+ and CD8+IFNγ+ lymphocytes (data not shown). In the presence of CD14+CD16++ MDMs but the absence of ACs, a lower percentage of proliferating

CD4+IFNγ+ and CD8+IFNγ+ lymphocytes was observed (Figure 5C). In the presence of ACs, the effect on CD14+CD16++ MDMs showed a clear trend, albeit not sufficient to reduce the percentage of CD4+IFNγ+ and CD8+IFNγ+ lymphocytes. The presence of CD14+ CD16++ MDMs from SLE patients did not significantly change the proportions of IFNγ+ lymphocytes.

DISCUSSION

Herein, we describe our finding that CD14+ CD16++ monocytes are decreased in patients with active SLE and display alterations in the molecules associated with their functions. Similar proportions of monocyte subsets in both SLE patients and healthy controls have been previously reported (4,5,26). In contrast, one study demonstrated that patients with inactive SLE without immunosuppressive therapy had increased numbers and percentages of CD14+CD16+ monocytes as compared with patients with active SLE and healthy controls. In patients with active SLE, this subpopulation was also found to be decreased (27). In our study, although glucocorticoid treatment was similar among the patient groups, the patients with active SLE received the highest dose of prednisolone (1 mg/kg of body weight) compared with the other patients receiving similar treatment who were receiving low doses of steroids <7.5 mg/day. However, it is worth noting that the total numbers of leukocytes and monocytes in patients with active SLE were comparable with those in the healthy controls.

Altered proportions of monocyte subsets could be explained by in vivo cytokine and hormone environments in SLE (28,29); alternatively, the conversion of CD14++CD16− monocytes into CD14+CD16++ monocytes may occur. In addition, CD14+CD16++ may be recruited to sites of inflammation, which would explain their lower numbers in the peripheral circulation (6). In patients with RA, CX3CL1 attracts CD16+ monocytes to the synovial tissue (30), and a reduction of circulating CD14+CD16++ monocytes has been associated with lupus nephritis (31). Of the patients in our study with active SLE, ~37% had renal symptoms, ~32% had articular symptoms, and the remaining patients had skin or nervous system involvement. Migration of CD14+CD16++ monocytes could therefore not be excluded.

The reduction of HLA–DR and CD14 expression on CD14+ phagocytes from patients with active SLE could be explained by several observations: HLA–DR expression is down-modulated by IL-10, SLE patients have high plasma levels of IL-10 compared with healthy controls (32), and CD14++CD16− monocytes express high levels of the receptor for this cytokine (33,34). CD14 is down-regulated by glucocorticoid treatment (27) and the cytokine environment and was found to be decreased in classic monocytes from patients with active SLE. CD16 expression in nonclassic and intermediate monocytes from patients with active SLE was low compared with that in monocytes from patients with other autoimmune diseases. Additionally, it has been well established that CD16 participates in the removal of immune complexes, favoring tissue damage (35). In monocytes from SLE patients, several alterations in Fc receptor expression have been reported (31,36). The decreased expression of CD16 and CD32 in monocytes from SLE patients has been correlated with disease activity and kidney damage (37).

ACs are considered an important source of immune complexes (38). We observed that monocytes from SLE patients had a reduced ability to bind and internalize ACs compared with those from healthy controls and patients with other autoimmune diseases. Independent of the type of ACs and serum source used, a reduction in the capacity to remove ACs by monocytes, macrophages, and neutrophils from SLE patients has been reported (39). The deficient removal of ACs results in their persistence in the extracellular milieu, with further oxidative changes, the formation of neoantigens (40), and autoimmunity.

Notably, CD14+ phagocytes from the SLE and other autoimmune diseases groups did not up-regulate CD80 after interacting with ACs, and no changes in the CD86-to-CD80 ratios were seen, as was observed in cells from the healthy controls. CD86 is considered constitutive (41), and CD80 is considered inducible; CD80 has a higher affinity for CTLA-4 than CD28 (41). The lack of CD80 up-regulation and the higher stimulation of CD86 have been associated with enhanced T cell activation, and blockade of CD86 was shown to reduce anti-DNA antibodies in SLE patients (42). Our results are consistent with other studies demonstrating that monocytes from SLE patients do not up-regulate CD80 in the presence of ACs (43), a critical event for T cell activation.

To study some of the effects of CD14+CD16++ monocytes in coculture with CD14++CD16− monocytes and ACs, an in vitro model was developed. We first examined the coculture effect on the accumulation of cytokines and then on IFNγ+ and proliferating lymphocytes. Previous reports indicated that the removal of ACs by monocytes is primarily a noninflammatory process (10,44). We demonstrated that ACs increased the

production of IL-10, IL-6, and IL-8 by CD14++CD16−
MDMs. In the presence of CD14+CD16++ MDMs,
there was an increase in IL-10, TNFα, and IL-1β levels
and induction of IL-12p70 as compared with unstimu-
lated CD14++CD16− MDMs. Several studies have
shown that, at least in vitro, the phagocytosis of ACs by
monocytes and macrophages induces the production of
IL-8, IL-1β (45), TNFα, CCL3 (46), IL-6, CCL22, and
CCL4 (25).

We cannot rule out the presence of alarm signals
that could be released from ACs and interact with the
components of innate immunity. In this regard, the use
of other AC sources, including microvesicles, to stimu-
late monocytes may be more appropriate. However, low
numbers of available cells of the different monocyte
subsets became a serious limitation.

Although the influence of cytokines in our system
was not assessed, as blockade assays would have been
required, our data clearly indicate that the presence of
CD14+CD16++ monocytes affects events such as dif-
ferentiation of CD14++CD16− monocytes, cytokine
production, and T cell responses. However, cocultures
performed with monocytes from SLE patients allow us
to unambiguously affirm that in addition to the surface
alterations, these cells are dysfunctional and might lead
to uncontrolled expansion of T cells. In addition,
whether cytokines are related to a decrease in lympho-
cyte proliferation remains unclear. Preliminary evidence
from our laboratory indicated that these effects could be
said to be due to the presence of nonclassic monocytes
involving early events, such as the decreased expression
of CD80 and HLA–DR on CD14++CD16− MDMs
(data not shown), but this possibility requires additional
experimental evidence.

Interestingly, the frequencies of proliferating
CD4+IFNγ+ and CD8+IFNγ+ cells were lower in the
presence of CD14+CD16++ MDMs from healthy con-
trols, but this effect was not observed in nonproliferating
CD4+IFNγ+ and CD8+IFNγ+ cells. And CD14+
CD16++ MDMs from SLE patients were unable to
induce this regulatory effect. These observations suggest
that CD14+CD16++ MDMs may affect events follow-
ing T cell activation. In addition, when a reduction in T
cell proliferation was observed, irrespective of the pres-
ence or absence of ACs, there was an increase in IL-10,
TNFα, and IL-1β in cocultures of CD14+CD16++
MDMs and CD14++CD16− MDMs. IL-10 levels also
increased in the cultures in the absence of CD14+
CD16++ MDMs, without any effect on the prolifera-
tion of CD3+ T lymphocytes.

In summary, coculture with CD14+CD16++
MDMs from healthy controls, but not from SLE pa-

tients, reduced the proliferation and number of prolif-
erating CD3+IFNγ+ cells and increased the accumula-
tion of TNFα, IL-10, and IL-1β. Based on this in vitro
model with cells from healthy controls, we propose that
CD14+CD16++ monocytes have a regulatory effect on
the expansion of T cells. It is tempting to speculate that
the effects in SLE could be explained by the absence of
CD14++CD16− monocytes, which seem to have the
ability to limit the expansion of the self-reactive clones
activated in response to self antigens. The reduction and
dysfunction of CD14+CD16++ monocytes or their
tissue recruitment in SLE patients observed in this study
plus the lack of changes in the CD86-to-CD80 ratio in
response to ACs and their effects on T cell function may
explain several monocyte alterations previously de-
scribed in SLE and the high expression of IREM-2
(16,17) compared with classic monocytes.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it
critically for important intellectual content, and all authors approved
the final version to be published. Dr. Rojas had full access to all of the
data in the study and takes responsibility for the integrity of the data
and the accuracy of the data analysis.
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