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Phagocyte dysfunction in polymyalgia rheumatica and other age-related, chronic, inflammatory conditions

Lorena Álvarez-Rodríguez, Marcos López-Hoyos,† Jaime Calvo-Alén,‡ Elena Aurrecoechea,‡ Ignacio Villa,‡ and Víctor Manuel Martínez-Taboada*,1*

Divisions of *Rheumatology, and †Immunology, Hospital Universitario Marqués de Valdecilla, Instituto de Formación e Investigación Marqués de Valdecilla (IFIMAV), Facultad de Medicina, Universidad de Cantabria, Santander, Spain; and ‡Sección de Reumatología, Hospital de Sierrallana, Torrelavega, Cantabria, Spain

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ABSTRACT

This study was conducted to evaluate phagocyte function in patients with age-related chronic inflammatory conditions. It included 95 patients with PMR, 17 with GCA, 40 with EORA, and 25 age-matched HCs. Serum IL-8 was determined with a bead array. The chemotactic capacity, phagocytic ability, and oxidative burst activity of circulating leukocytes were determined with flow cytometry kits. Patients with active chronic inflammatory diseases showed a significant increase in circulating levels of IL-8 that remained elevated in patients with PMR or EORA, despite treatment. No correlation was found between circulating IL-8 and the migratoty capacity of neutrophils. Neutrophils from patients with active EORA without stimulus and after fMLP stimuli showed a higher capacity to migrate than those of the HCs (P=0.033). The phagocytic activity of granulocytes in the patients with GCA was significantly higher than in the HCs and the patients with PMR or EORA (P<0.05). The percentage and MFI of phagocytes that produce ROIs when stimulated with *Escherichia coli* was significantly reduced in neutrophils and monocytes from the patients with age-restricted inflammatory conditions. We concluded that the effector functions of phagocytes, determined to be chemotaxis, phagocytosis, and oxidative burst, are deregulated in age-restricted inflammatory disorders and may have a pathogenic role. *J. Leukoc. Biol.* 94: 1071–1078; 2013.

Introduction

Phagocytes (macrophages, monocytes, and neutrophils) perform various host-defense functions that rely on the phagocytic uptake of pathogens and drive the polarization of immune responses. Several factors contribute to the efficient function of the phagocytic system. These factors include the presence of an adequate number of monocytes and neutrophils in the peripheral blood, the ability to respond to signals from sites of inflammation, induction of migration to these sites, and the capacity to ingest and kill the invader microorganisms [1].

Even though phagocytic functions can be considered the most important event in the process of killing an invading microorganism, this process has not received enough attention in the elderly [2]. Although results have been conflicting, small differences in in vitro neutrophil functions have been found between young and elderly healthy subjects [2–4]. In fact, it appears that the frequently observed neutrophil dysfunction in the elderly is due to the effect of abnormal humoral components related to the aging process on otherwise normal neutrophils [4].

Little is known about the role of circulating phagocytes in the pathogenesis of age-restricted inflammatory conditions. Although most studies have focused on T-cell abnormalities, a separate line of evidence supports the possibility of an abnormal function of the innate immune system. Therefore, the study of age-restricted chronic inflammatory conditions provides a unique scenario for investigating the influence of aging on the development of disease and the changes induced in the innate immune system and phagocyte function.

PMR is a clinical syndrome characterized by pain and stiffness in the neck, shoulder, and pelvic girdle [5]. It is one of the most frequently seen inflammatory chronic conditions that exclusively affect elderly people [6, 7]. The pathologic substrate in PMR is synovitis characterized by vascular proliferation and infiltration of macrophages and lymphocytes [8]. However, the function of these infiltrating phagocytes remains

Abbreviations: CBA=cytometric bead array; CRP=C-reactive protein; CS=corticosteroid; CV=coefficient of variation; DHR=dihydrorhodamine; EORA=elderly-onset rheumatoid arthritis; FSC=forward scatter; FL1=fluorescence channel 1; GCA=giant cell arteritis; HC=healthy control; MFI=mean fluorescence intensity; PMR=polymyalgia rheumatica; ROIs=reactive oxygen intermediates

1. Correspondence: Rheumatology Division. Hospital Universitario Marqués de Valdecilla, Facultad de Medicina. Universidad de Cantabria, Avda. Valdecilla s/n. 39008. Santander, Spain. E-mail: vmartinezt@medynet.com

unclear. There is only one study in which neutrophil chemotaxis has been studied, in 8 patients with active PMR, and it was found to be normal [9].

PMR symptoms frequently affect elderly persons and may be the first manifestation of two other age-restricted, inflammatory, chronic conditions: GCA and EORA. GCA is a large- and medium-sized blood vessel systemic vasculitis characterized by the granulomatous involvement of the aorta and especially its cranial branches [10]. There are several facts that suggest that phagocytes play a role in the pathogenesis of GCA. First, monocytes are one of the main cell types in tissue infiltration, and they contribute to disease pathogenesis, with different functions, depending on their location [11]. Whereas tissue-infiltrating monocytes display various functions in the different layers of the artery, it has been suggested that circulating monocytes are mainly responsible for the production of circulating proinflammatory cytokines [10]. Second, although neutrophils are infrequently found in the inflamed temporal artery of patients with GCA [12], increased serum levels of neutrophil elastase have been detected in patients with active disease [13]. Third, Foell et al. [14] showed an increased expression of the S100 protein family by monocytes and neutrophils in temporal artery biopsies of patients with active GCA. Fourth, several genetic studies have identified genes that regulate the endothelial and phagocyte function associated with disease susceptibility [15–17].

Circulating phagocytic cells also participate in the initiation and progression of RA, although the exact mechanisms responsible for phagocyte accumulation in rheumatoid joints are not fully understood [18]. The function of these cells in RA is controversial: several studies have found no alteration in neutrophil function, and others have pointed out different abnormalities in migratory activity, phagocytosis, or oxidative burst [18–24]. Determining the influence of different therapies on phagocyte function has been the objective of several studies in patients with RA [21, 22, 25–29], although the impact of the disease itself on phagocyte function has rarely been addressed [20, 30].

Because of the increasing importance of innate immune function in the pathogenesis of aging and age-restricted inflammatory chronic conditions and because there are very few data showing the impact on phagocyte function in these disorders, we thought it important to delineate circulating phagocyte abnormalities that could further shed light on the pathogenesis of these disorders. In this study, we showed an increase in circulating IL-8 and the chemotactic activity of neutrophils and also an impaired respiratory burst in circulating phagocytes of patients with active disease.

MATERIALS AND METHODS

Study subjects

The study included 95 patients with PMR, 17 with GCA, 40 with EORA, and 25 age-matched healthy controls (HCs). The main demographic, clinical, and laboratory characteristics of the study population are shown in **Table 1**. All patients with GCA met the 1990 American College of Rheumatology classification criteria for the disease [31], and 75% had characteristic findings of arteritis in the temporal artery biopsy [32]. PMR was diagnosed according to the criteria proposed by Chuang et al. [5]. Patients with RA had to satisfy the ACR 1987 revised criteria for RA [33]. Those with RA were considered to have EORA if the age at symptoms onset was more than 60 years. The effect of CS treatment was assessed in 59 patients with PMR, 6 with GCA, and 20 with EORA in clinical remission after a mean (SD) treatment duration of 8.76 ± 6.75 wk. Seven patients (3 with GCA and 4 with PMR) in complete remission without CS therapy for at least 2 years were also studied. All the patients and control subjects gave written, informed consent, and the study was approved by the regional ethics committee.

Detection of soluble IL-8 in serum

The serum of each individual was isolated from 2.5 ml of blood obtained in tubes without additives and stored at −80°C until analysis. The quantitative determination of IL-8 in serum was performed with the CBA Human Inflammation kit (BD Biosciences, San Diego, CA) [34]. The fluorescence produced by CBA beads was measured on a FACSCalibur Flow Cytometer (BD Biosciences) and analyzed with BD CBA Analysis Software (BD Biosciences). The detection limit for IL-8 was 3.6 pg/ml.

Quantitative determination of the chemotaxis of neutrophilic granulocytes

The chemotaxis of neutrophils was determined with the Migratest kit (Orpegen Pharma, Heidelberg, Germany), according to the instructions of the manufacturer. In brief, leukocytes were isolated from heparinized whole blood by spontaneous sedimentation over leukocyte separation medium

TABLE 1. Demographic data and main clinical features of patients with PMR, GCA, or EORA

	Controls	GCA	PMR	EORA
Patients (*n*)	25	17	95	40
Age (mean ± SD)	68.8 ± 8.6	73.8 ± 8.3	73.3 ± 8.1	76.2 ± 6.6
Sex (% female)	80	52.9	67.7	62.5
Time to diagnosis (months)	–	2.5 ± 2.0	3.2 ± 2.6	3.2 ± 2.1
PMR symptoms (%)	–	31.3	100	76.9
Ischemic symptoms (%)	–	50.0	0	0
TAB (positive/done)	–	12/16	0/19	0/1
Pre-treatment ESR (mm/1 h)	–	78.5 ± 30.2	61.4 ± 30.0	68.9 ± 28.1
Pre-treatment CRP (mg/dL)	0.3 ± 0.2	10.4 ± 6.1	4.7 ± 5.9	6.0 ± 4.4
Rheumatoid factor positive (%)	–	–	–	17.5
Anti-CCP positive (%)	–	–	–	15.0

CCP=cyclic citrullinated peptide; ESR=erythrocyte sedimentation rate; TAB=temporal artery biopsy.

and placed on cell culture inserts with a pore size of 3.0 μm. Chemotaxis was conducted for 30 min at 37°C toward a gradient of fMLP in comparison with a control of incubation buffer. Afterward, the cells were stained for 10 min with an antibody reagent that also contained counting beads. Before flow cytometry, a special, vital DNA dye was added for 5 min on ice. The cells were analyzed with the 488 nm excitation light on a FACSCalibur with CellQuest Software (BD Biosciences). The following two parameters were analyzed for each sample: the number of cells and the mean value of the FSC signal. Because the shape change of the cells precedes cell migration, the change can be measured by analyzing the changes in the FSC signal. The intra-assay CVs were 9.1% and 1.9% for the number of cells and FSC signal, respectively.

Quantification of phagocytic activity of neutrophilic granulocytes and monocytesl

Quantitative determination of leukocyte phagocytosis was performed with the Phagotest kit (Orpegen Pharma), according to the manufacturer's instructions. This kit allows measuring of the overall percentage of monocytes and neutrophils that show phagocytosis in general (ingestion of one or more bacteria per cell) and the individual cellular phagocytic activity (number of bacteria per cell). Whole blood (100 μl) was mixed with 2×10^7 FITC-labeled and opsonized *E. coli* cells in a 37°C water bath for 10 min. As the negative control, whole blood and FITC-labeled *E. coli* were incubated at 0°C to reduce the phagocytic potential to a minimum. The reaction was stopped by placing the samples on ice. The fluorescence of the bacteria attached to the cell surface was quenched by using 100 ml of quenching solution. After 2 washes, the erythrocytes were lysed by adding lysing solution for 20 min at room temperature, after which DNA staining solution was added to stain the DNAs of the bacteria and the cells. The intra-assay CVs were 0.8% and 5% for the percentage of phagocytosing neutrophils and monocytes, respectively, and 5.1% and 6.6% for the mean intensity in FL1.

Quantification of the oxidative burst activity of monocytes and granulocytes

The production of reactive oxygen intermediates (ROIs) was determined with the Bursttest kit (Orpegen Pharma). Whole blood (100 μl) was mixed with 2×10^7 unlabeled *E. coli* cells (particulate stimulus), fMLP (low physiological stimulus), or PMA (high stimulus) at 0°C. Mixtures of heparinized whole blood and bacteria were incubated in a 37°C water bath for 10 min. As a control, whole blood was incubated with 2 ml of wash solution. DHR was added to the samples at 37°C, and incubation was continued for 10 min, to allow nonfluorescent DHR to convert to fluorescent rhodamine 123, after the production of ROIs. Lysing solution was added for 20 min at room temperature. After the cells were washed, DNA staining solution was added to stain the DNAs of the bacteria and the cells. The percentage of cells having produced ROIs was then analyzed, as well as the MFI (enzymatic activity). The intra-assay CVs were 0.9% and 2.6% for the percentage of oxidizing neutrophils and monocytes, respectively, and 3.2% and 8.5%, respectively, for the FL1.

Statistical analysis

Data were analyzed with SPSS 15.0 (Chicago, IL, USA). Data analyses were performed by applying nonparametric tests. The statistical comparisons of data between patients and controls were performed with the Mann-Whitney *U* test. Differences between pre- and posttreatment data were analyzed by the Wilcoxon signed-rank test. Relationships between various parameters were determined by simple correlation (Pearson or Spearman rank correlation coefficient). Results were considered to be significant at $P < 0.05$.

RESULTS

Patients with age-restricted inflammatory conditions had an increase in circulating IL-8 levels during the active phase of the disease

As shown in **Fig. 1**, the patients with active disease showed a significant increase in circulating levels of IL-8. Circulating IL-8 levels remained elevated in the patients with PMR and EORA, despite disease control with treatment, and also in cases of PMR after disease remission. However, in GCA, circulating IL-8 decreased after high-dose CS therapy and remained within the normal range in the patients in complete remission without treatment (Fig. 1). No correlation was found between circulating IL-8 and the migration capacity of innate immune cells or with the expression of activation or adhesion markers in the patients and the HCs (data not shown).

Patients with EORA had increased chemotactic activity

Ex vivo neutrophils of the patients with active EORA without stimulus showed a higher capacity (P=0.033) to migrate than those of the age-matched HCs (**Fig. 2**). After fMLP stimulus, the difference in chemotactic activity showed a similar picture (Fig. 2). As mentioned earlier, the shape change of the cells precedes the cell migration and can be measured by analyzing the changes in the FSC by flow cytometry. The mean FSC was also higher in the patients with active disease than in the HCs (data not shown), suggesting an increased capacity to migrate of circulating neutrophils in the patients with age-restricted inflammatory conditions, especially EORA.

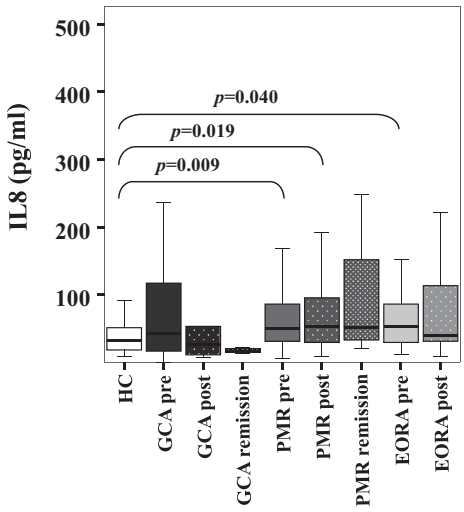

Figure 1. Circulating levels of IL-8 in patients with age-restricted conditions and active disease, clinical remission under treatment, and complete disease remission without treatment. Circulating levels of IL-8 were measured by CBA in 95 patients with PMR, 17 with GCA, 40 with EORA, and 25 age-matched HCs. The effect of CS treatment was assessed in one serum sample from 59 patients with PMR, 6 with GCA, and 20 with EORA in clinical remission after a mean (SD) treatment duration of 8.4 ± 8.2, 8.6 ± 5.8, and 8.2 ± 6.4 wk, respectively. Seven patients (3 with GCA and 4 with PMR) in complete remission without CS therapy for at least 2 years were also studied.

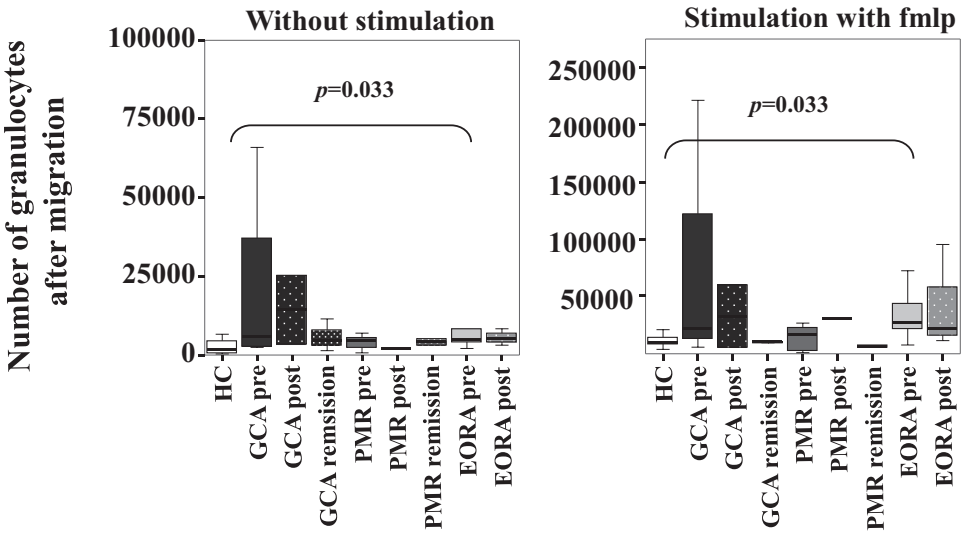

Figure 2. Patients with EORA have a significant increase in chemotactic activity. Chemotaxis of neutrophils was determined by flow cytometry in 16 patients with active (pre) PMR, 10 with GCA, 10 with EORA, and 17 age-matched HCs. The effect of CS treatment (post) was assessed in 7 patients with PMR, 3 with GCA, and 6 with EORA in clinical remission. Seven patients (3 with GCA and 4 with PMR) in complete remission without CS therapy for at least 2 years were also studied.

No significant changes in chemotactic activity were seen after clinical control of disease activity in these patients. The patients with PMR or GCA in complete remission without CS therapy had levels similar to those in the age-matched HCs (Fig. 2).

Cells in patients with GCA had increased phagocytic ability

The percentage of neutrophils and monocytes with phagocytic activity was very similar between the patients and the age-matched HCs (data not shown). As shown in **Fig. 3**, the phagocytic activity of granulocytes in the patients with GCA was significantly higher than in the HCs or the patients with PMR or EORA ($P<0.05$). However, no significant differences were found for the monocytes. Treatment with CSs induced a marginally significant increase, both in the proportion and MFI of monocytes from the patients with PMR (Fig. 3). The patients with PMR or GCA in complete remission without CS therapy again had values similar to those in the age-matched HCs.

Depressed oxidative burst in patients with age-restricted chronic inflammatory conditions

The percentage of phagocytes that produced ROIs in response to *E. coli* as a stimulus was significantly reduced in neutrophils but not in monocytes of the patients with age-restricted inflammatory conditions compared with the HCs (data not shown). These results were also confirmed with MFI expression on neutrophils and monocytes of the patients with active disease (**Fig. 4**). The same effect was found in the patients with active PMR after fMLP stimulation. The effect of treatment was variable within the different diseases, although high-dose CS therapy in GCA again recovered oxidative burst function. Patients with PMR in complete remission without CS therapy also recovered burst activity.

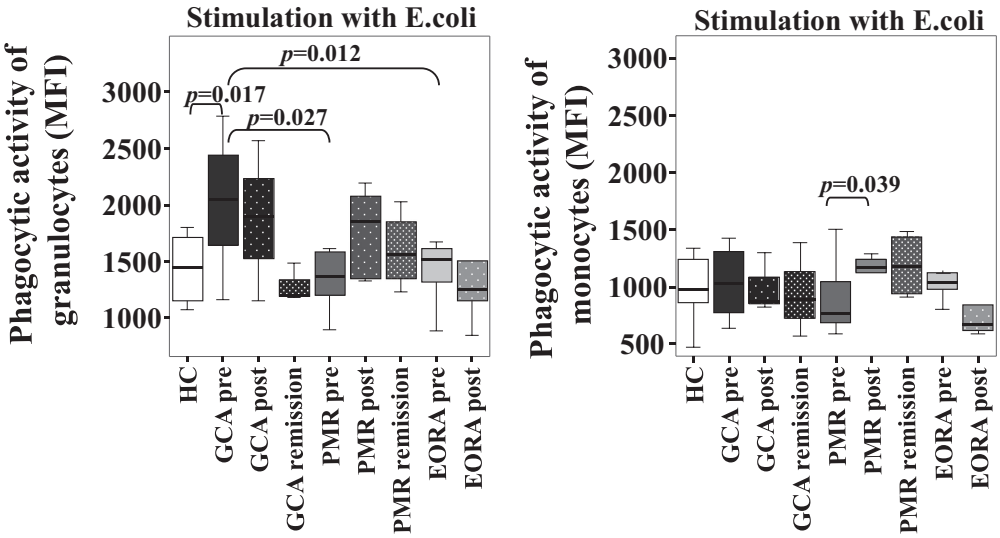

Figure 3. Patients with active GCA had a significant increase in phagocytic ability. Leukocyte phagocytosis was assessed by incubating whole blood with fluorescence-labeled *E. coli* and measuring the capacity (MFI) of each cell to phagocytose *E. coli* in 16 patients with active (pre) PMR, 10 with GCA, 10 with EORA, and 17 age-matched HCs. The effect of CS treatment (post) was assessed in 7 patients with PMR, 3 with GCA, and 6 with EORA in clinical remission. Seven patients (3 with GCA and 4 with PMR) in complete remission without CS therapy for at least 2 years were also studied.

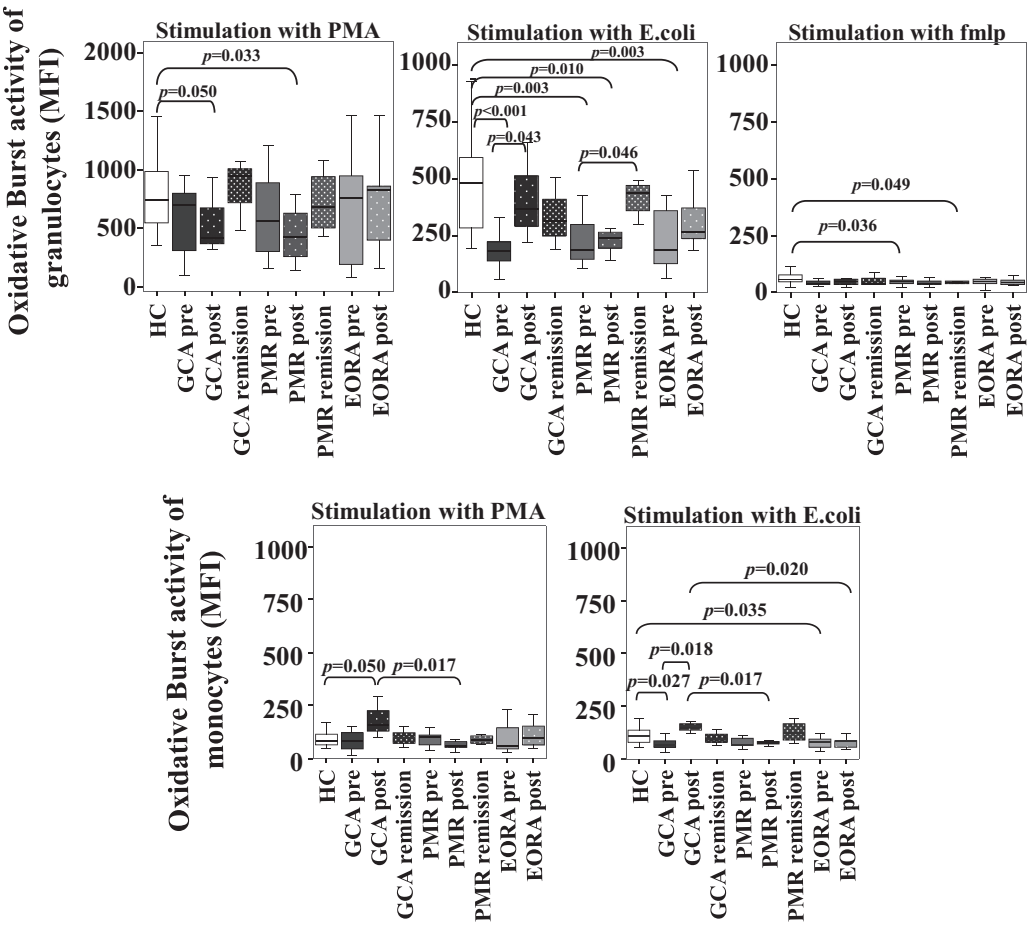

Figure 4. Depressed oxidative burst of peripheral blood granulocytes and monocytes. (A) Granulocytes and (B) monocytes of patients with age-restricted chronic inflammatory conditions. The percentage of granulocytes converting nonfluorescent dihydrorhodamine (DHR) to fluorescent rhodamine 123 on production of ROIs and the mean channel shift were assessed as a measure of oxidative burst in 16 patients with active (pre) PMR, 10 with GCA, 10 with EORA, and 17 age-matched HCs. The effect of CS treatment (post) was assessed in 7 patients with PMR, 3 with GCA and 6 with EORA in clinical remission. Seven patients (3 with GCA and 4 with PMR) in complete remission without CS therapy for at least 2 years were also studied.

Relationship between circulating leukocyte function and acute-phase reaction

To assess whether the abnormalities found in phagocyte function were related to the inflammatory process itself, we studied the correlation of chemotaxis, phagocytosis, and oxidative burst tests to CRP levels. As shown in **Fig. 5**, a significant inverse correlation of oxidative burst (*E. coli*) both in granulocytes and monocytes with CRP concentration was found in the patients with PMR. Although the results in GCA and EORA showed the same tendency, the differences were not statistically significant, probably owing to the small sample size. Furthermore, a negative correlation was also found in the phagocytic activity of granulocytes in the patients with GCA (P=0.021; r=−0.786) and a phagocytic tendency in monocytes from the patients with PMR (P=0.051; r=−0.530).

DISCUSSION

Although the pathogenesis of age-restricted inflammatory conditions remains unclear and they have traditionally been regarded as primary T-cell disorders, there is increasing evidence that innate immune cells, including phagocytes, play an important role.

Very little is known about the effect of aging on the chemokine system [35]. It has been reported that serum levels and T-cell production of IL-8 are increased in the elderly [35, 36]. It has been also suggested that circulating IL-8 levels are increased in different vasculitic diseases and may be a sensitive marker of disease activity [37] and play an important role in the pathogenesis of the diseases [37–42]. Circulating IL-8 levels have also been found to be increased and to correlate with disease activity in another large-vessel vasculitis, Takayasu's arteritis [38]. Circulating levels of IL-8 have also been studied in patients with PMR, but with conflicting results [43, 44]. Whereas some researchers have found no increase in IL-8 levels in patients with active PMR [43], others have found an increase in circulating and muscle IL-8 in patients with active PMR that normalized with prednisone treatment [44]. Furthermore, other chemokines, such as MCP-1 and RANTES, have been implicated in the pathogenesis of these disorders [43, 45, 46].

As IL-8 is one of the main chemokines involved in cell migration [47], circulating levels were studied in patients with active disease before CS therapy, patients in clinical remission with treatment, and in a small subset of patients with PMR or GCA in complete clinical remission and without any CS or immunosuppressive therapy. In the sera of the patients with these three inflammatory conditions, we found significantly raised levels of IL-8 compared with those in the sera of the HCs. No significant differences were found in circulating IL-8

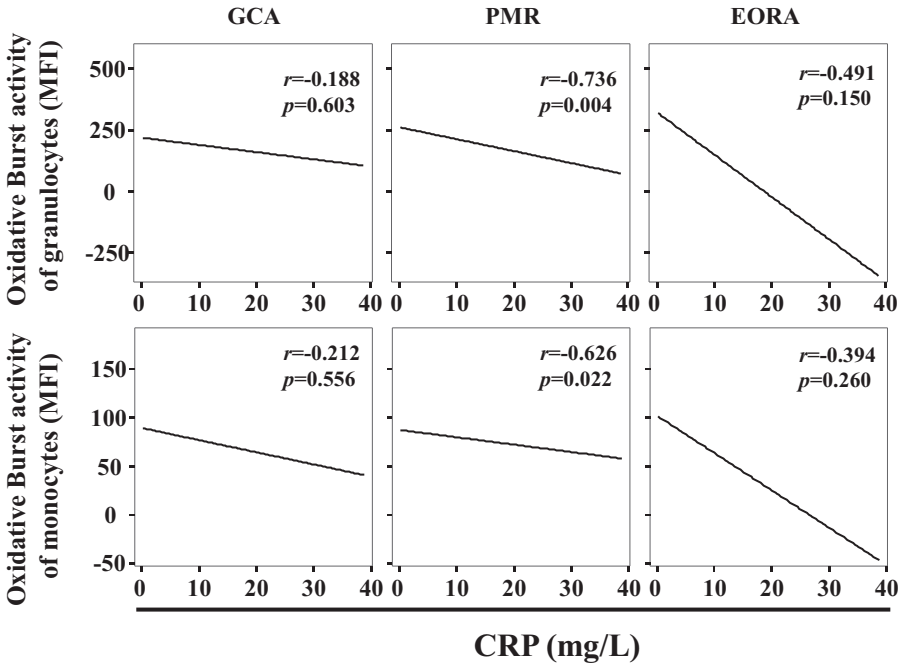

Figure 5. Correlation between burst oxidative and CRP in patients with age-restricted chronic inflammatory conditions.

in these age-restricted inflammatory diseases. Furthermore, we found no correlation between circulating IL-8 and the acute-phase response in these disorders or with the chemotactic activity. Although our results indicate that IL-8 may play a role in the inflammatory process in PMR, GCA, and EORA, the level of circulating IL-8 cannot distinguish between these 3 disorders, and the utility of measuring circulating IL-8 over time in these patients is still unknown. An important finding was that in the patients with GCA, but not in those with PMR or EORA, serum levels of IL-8 tended to decrease after CS therapy and disease remission. Although there is not a clear explanation, it is possible that the higher dose of CSs in GCA in comparison with those in PMR and EORA induces such a decrease [48, 49]. Furthermore, the GCA group was the smallest of the study, and the size of the sample could also be the reason for the lack of statistical significance in the changes observed in both active and controlled GCA.

In the current study, circulating phagocytes from patients with age-restricted inflammatory chronic conditions were characterized with respect to their function. Despite the relatively high prevalence of these disorders, an ex vivo characterization of these cells has rarely been performed [23]. In our study, whereas the patients with age-restricted inflammatory chronic conditions had intact chemotactic and phagocytic activity, oxidative burst capacity was significantly reduced.

Because a functional chemotactic defect diminishes the number of granulocytes that migrate from the circulation into the inflamed tissues, and therefore the amount of active cells at the sites of inflammation, we first studied the chemotactic function in patients with age-restricted inflammatory conditions. Our results suggest an increased migratory capacity of circulating neutrophils in such patients, although it was significantly demonstrated only in EORA. No significant changes in chemotactic activity were seen after short-term clinical control of disease activity with CS therapy in these patients. These results are in agreement with those in previous studies that showed increased migration of polymorphonuclear leukocytes in patients with early RA [20] and a minor effect of CSs on neutrophil activation [20, 50]. There are no published data on patients with EORA.

The role of neutrophils in the pathogenesis of GCA has often been neglected, although they are clearly among the first cells to invade inflammatory lesions [14]. Neutrophils may contribute to the inflammatory process in GCA by activating endothelial cells, secreting proinflammatory mediators, and producing ROIs. Proinflammatory S100 proteins are expressed in neutrophils and in recently recruited monocytes in inflamed tissue of patients with GCA. Furthermore, these proteins are also increased in the sera of patients with active disease, indicating release at the sites of inflammation [14]. The phagocytic activity of granulocytes in patients with GCA was significantly higher than in the HCs and the patients with PMR or EORA. Whether this is a characteristic of patients with GCA should be confirmed in larger studies. Furthermore, the relevance of this finding in the pathogenesis of GCA remains unclear.

The killing of microbes is a critical physiological function of phagocytes. These cells have different mechanisms for killing microorganisms, but the generation of ROIs and hypochlorous acid is still regarded as the critical killing mechanism for most invading pathogens [51]. Although a deficient respiratory burst activity in these patients would not necessarily have any impact on the ability of their leukocytes to kill microorganisms once they have been internalized [52], it may provide a different intracellular scenario for the appropriate processing and elimination of the foreign particles.

We have evaluated phagocytic ability by an in vitro flow cytometry method that uses whole blood and, as a consequence,

the method reflects intrinsic changes in monocytes and neutrophils that do not depend on changes in plasma components such as complement or immunoglobulins. TLRs can also be involved in the increased phagocytic ability of circulating neutrophils, as shown in our patients with GCA. In this regard, we have previously shown deregulation in the expression and function of TLRs in peripheral blood monocytes [53]. TLR7 expression is clearly increased in circulating monocytes of patients with active GCA, but its function is impaired, as determined by cytokine production. In our study, we partially showed functional defects in respiratory burst activation but not in *E. coli* phagocytosis of circulating monocytes of patients with GCA with active disease. Nor did we find any difference in the patients with active PMR or EORA. However, it is possible that there are other aspects of monocyte function, such as NO production [54], as previously demonstrated in GCA lesions. Moreover, increased production of inflammatory cytokines by tissue macrophages and circulating monocytes has been shown in GCA and PMR [53, 55]. We did not investigate these aspects. Although expression and function of TLRs on neutrophils has never been addressed in these inflammatory diseases, differential regulation by TLRs of phagocytosis by neutrophils [56, 57] has been shown. It is possible that, among other receptors, TLR expression and function in neutrophils is deregulated and has an effect on the effector functions analyzed in the present work. In addition, new immunomodulatory functions have been found for neutrophils [58, 59].

One further finding in the current study was the lack of influence of CS therapy in the effector functions of neutrophils and monocytes. Although CSs had a pleiotropic effect on immune response, those effects seemed to be more related to the inhibition of the ongoing T-cell response, once the disease had started.

In conclusion, the effector functions of neutrophils and monocytes, defined as phagocytosis and oxidative burst, and to a lesser extent, chemotaxis, were deregulated in age-restricted inflammatory disorders and may have a pathogenic role. This finding is even more important, because recent evidence suggests an immunomodulatory role for neutrophils in both acquired and innate immunity. The exact role of circulation and tissue infiltration in these disorders remains to be elucidated.

AUTHORSHIP

L.A.-R. contributed to sample collection and preparation, flow cytometry and culture studies, data and bioinformatics analyses, and manuscript preparation. M.L.-H. contributed to the study design, laboratory experiments data analyses, and manuscript preparation and editing. J.C.A. participated in the design of the study, analysis and discussion of results, and editing of the manuscript. E.A. participated in the collection of clinical data and discussion of results. I.V. participated in the design of the study, statistical analysis, and discussion of results. V.M.M.-T. contributed to the study's design, recruitment of patients, clinical assessments, data analysis, and writing and editing of the manuscript. All authors read and approved the final manuscript.

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KEY WORDS:
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