Elevated CD16 Expression by Monocytes From Patients With Tumor Necrosis Factor Receptor–Associated Periodic Syndrome

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Objective. Tumor necrosis factor receptor–associated periodic syndrome (TRAPS) is an inherited autosomal-dominant autoinflammatory condition caused by mutations in the ectodomain of the 55-kd tumor necrosis factor (TNF) receptor superfamily 1A. Proinflammatory blood monocytes with the phenotype CD14+,CD16+,HLA–DR++ are a major source of TNF, and the number of such monocytes is increased during infection and inflammation. The aim of this study was to investigate whether the expression of circulating CD16+ monocytes is affected in patients with TRAPS.

Methods. Peripheral blood obtained from patients with TRAPS and healthy control subjects was stained with monoclonal antibodies to detect CD14+,CD16+,HLA–DR+ monocytes and CD14+,CD16+ monocytes, using flow cytometry. Lipopolysaccharide-induced TNF production was measured by intracellular cytokine staining. Activation-induced shedding of CD16 was investigated by treating blood samples with phorbol myristate acetate.

Results. The level of CD16 expression by CD14+, CD16+ monocytes, but not their absolute number, was significantly elevated in patients with TRAPS, even though the patients were not experiencing clinically overt episodes of autoinflammation at the time of sampling. These findings are similar to those for the C-reactive protein levels and erythrocyte sedimentation rates in the same patients. The enhanced level of CD16 expression by monocytes from patients with TRAPS was not attributable to a defect in activation-induced shedding of CD16. The CD14+,CD16+ monocytes were the predominant source of TNF in both patients and healthy control subjects.

Conclusion. The level of CD16 expression by monocytes was elevated in patients with TRAPS, as a feature of the underlying constitutive inflammation status.

Tumor necrosis factor receptor–associated periodic syndrome (TRAPS; MIM 142680) is an inherited autosomal-dominant autoinflammatory condition caused by mutations in the ectodomain of the 55-kd tumor necrosis factor receptor (TNFR) superfamily 1A (TNFRSF1A; also known as TNFR1, TNFR p55, and CD120a) (1). More than 40 different pathogenic mutations in the TNFRSF1A gene have been associated with TRAPS (see http://fmf.igh.cnrs.fr/infevers/). This periodic fever syndrome is characterized by recurrent fever and a variety of tissue manifestations, including abdominal pain, myalgia, and erythematous skin rashes. Amyloidosis is the most serious complication of TRAPS and occurs in ~15% of patients. Levels of interleukin-6 tend to be particularly elevated in the circulation, consistent with raised levels of C-reactive protein (CRP) (2). Immunohistologic studies have indicated a specific involvement of monocytes and macrophages in the inflammatory tissue changes in TRAPS: monocytic fasciitis is associated with the myalgia in TRAPS (3), and monocytes are a prominent feature of the skin lesions that frequently overlie areas of muscle involvement (4).

A subset of mature blood monocytes is distinguished by the phenotype CD14+,CD16+,HLA–DR++, compared with the majority of monocytes,
which are CD14+CD16− (5). CD16+ monocytes are recognized as being proinflammatory, because they are a major source of TNF (6), and their numbers are increased during infection and in inflammatory diseases such as rheumatoid arthritis (RA) (7–9). We therefore investigated circulating CD16+ monocytes in patients with TRAPS to determine whether these monocytes are also affected in this autoinflammatory condition.

**PATIENTS AND METHODS**

**Patients.** The patients with TRAPS who were investigated (7 men and 4 women, median age 39 years [range 21–60 years]) were members of the prototype familial Hibernian fever cohort, all of whom possess a C33Y mutation in **TNFRSF1A** (1). Peripheral blood samples were collected in EDTA for immunofluorescence phenotyping of monocytes. Blood samples were obtained simultaneously for determination of the CRP level and the erythrocyte sedimentation rate (ESR) by routine clinical laboratory methods. None of the patients were experiencing clinically overt episodes of autoinflammation at the time that blood samples were obtained. Most of the patients were being treated with prednisolone and/or etanercept, and certain patients were also receiving other drugs. Unrelated healthy control subjects (4 men and 4 women, median age 39 years [range 28–58 years]) also provided peripheral blood samples in EDTA. The participants gave informed consent, and the study was approved by the University of Nottingham Medical School Research Ethics Committee and by the University of Nottingham Medical School Research Ethics Committee.

**Immunofluorescence staining and flow cytometry.** The analysis of CD14+CD16− monocytes and CD14+,CD16+ monocytes was based on previously published methods (6,7), with the modification of incorporating staining for HLA–DR (10) and adding Flow-Count beads (Beckman Coulter) for determining absolute cell numbers in a single-platform assay. Briefly, 5 µl of anti-CD14–fluorescein isothiocyanate (FITC), 5 µl of anti–HLA–DR–PC5, and 10 µl of anti-CD16–phycoerythrin (PE) monoclonal antibodies (or IgG1–PE or IgG2a–FITC isotype control antibodies in place of anti-CD16–PE or anti-CD14–FITC, respectively) (all from Beckman Coulter) were added to 100 µl of peripheral whole blood collected in EDTA. The blood was incubated for 15 minutes at room temperature in the dark. Five hundred microliters of OptiLyse C (Beckman Coulter) was added, and the samples were mixed and then incubated in the dark for 10 minutes at room temperature. Next, 500 µl of phosphate buffered saline (PBS) was added to each sample and mixed. Flow cytometry data were collected within 24 hours of sample preparation, on an EPICS XL flow cytometer (Beckman Coulter). Prior to data collection, 100 µl of Flow-Count beads was added to each sample. Statistical comparisons between groups were made by nonparametric Mann-Whitney test, using GraphPad Prism software (San Diego, CA). P values less than 0.05 were considered significant.

**Analysis of CD16 shedding.** Peripheral whole blood was collected into lithium heparin and centrifuged at 600g for 8 minutes. The plasma was removed and the cells washed twice in Hanks’ balanced salt solution (HBSS) containing 1% fetal calf serum and centrifuged. Cells were then diluted with a volume of HBSS equal to the volume occupied by the cells and mixed thoroughly by inversion. Cells were stimulated with phorbol myristate acetate (PMA; Sigma, Poole, UK) at a concentration of 10 ng/ml for 15 minutes at 37°C, with periodic mixing. Duplicate cells not treated with PMA were incubated on ice. The cells were then washed with HBSS. Cells were then washed with PBS containing 0.5% bovine serum albumin and 0.1% sodium azide (PBA), centrifuged, and resuspended in PBA to a volume of 1 ml. Immunofluorescence staining and flow cytometry were then performed as described above. Statistical comparisons between patients and controls were made by unpaired t-test.

**Analysis of TNF production.** Peripheral whole blood collected in EDTA was incubated with lipopolysaccharide (LPS) (Sigma) and protein transport inhibitor (GolgiPlug; Becton Dickinson, Cowley, UK) for 5 hours at 37°C in 5% CO2. Erythrocytes were then lysed (PharmLyse; Becton Dickinson), and the leukocytes were washed with PBA and incubated with anti–HLA–DR–PC5 and anti-CD14–FITC for 15 minutes at room temperature in the dark. The cells were washed with PBA and fixed and permeabilized (Fix/Perm; Becton Dickinson) for 20 minutes in the dark. The cells were then washed for 10 minutes (Perm/Wash; Becton Dickinson) and incubated with anti-TNF–PE or IgG1–PE (Beckman Coulter) for 30 minutes at room temperature in the dark. The cells were finally washed and resuspended in 1% formaldehyde fixative. Flow cytometric analysis was performed as described above, with the monocyte population defined by forward and side scatter. On the plot of CD14 versus HLA–DR staining, the CD14++ population and the CD14+,HLA–DR+++ population were gated separately, and the mean fluorescence intensity (MFI) of TNF staining in each population was determined, as previously described (6). Statistical comparisons between stimulated and unstimulated cells were made by paired t-test.

**RESULTS**

**Analysis of CD16 expression by monocytes from patients with TRAPS.** Peripheral blood leukocytes from TRAPS patients with the C33Y mutation and from unrelated healthy control subjects were stained with fluorochrome-labeled monoclonal antibodies specific for CD14, HLA–DR, and CD16 (or IgG1–PE isotype control in place of anti-CD16–PE), as described in Patients and Methods, and analyzed by flow cytometry, gating on monocytes and the upper portion of the lymphocytes in the light scatter histogram (7,8,10). Within this population, cells that expressed CD14 and high levels of HLA–DR were gated and further analyzed for the expression of CD16 relative to that of CD14.
(Figure 1). Separate gates were created around the CD14+,CD16− and the CD14+,CD16+ populations (Figures 1A and C). The position of CD14−,CD16+ cells on the scatter plots was confirmed by staining with IgG2a–FITC isotype control antibody in place of anti-CD14–FITC, and the CD14−,CD16+ cells were excluded from the CD14+,CD16+ gate (Figures 1A and C). Comparison of the CD16 signal in a healthy control subject (Figure 1A) and in a patient with TRAPS (Figure 1C) demonstrated a higher level of CD16 expression in TRAPS (i.e., the CD14+,CD16+ events were nearer to the top of the gate in Figure 1C compared with Figure 1A). The IgG1–PE isotype control antibody staining confirmed the specificity of the CD16 staining (Figures 1B and D).

Figure 2 summarizes these data for all of the patients with TRAPS and all of the healthy control subjects. All of the blood samples from patients with TRAPS were obtained when the patients were not experiencing a clinically overt episode of autoinflammation. In cases in which individual participants provided ≥2 blood samples at different times, the mean values from that patient are presented. Figure 2A shows the amount or level of surface CD16 expression per monocyte, as defined by the MFI of anti-CD16–PE–specific staining; this was calculated by determining the anti-CD16–PE MFI for the flow cytometry events in the CD14+,CD16+ gate exemplified in Figures 1A and C, and subtracting the MFI for the events in the gates exemplified in Figures 1B and D (staining by the IgG1–PE isotype control) to correct for nonspecific staining. The level of CD16 expression per monocyte was significantly higher in the patients with TRAPS (median 383.7, interquartile range [IQR] 298.9–619.7) than in control subjects (median 175.0, IQR 146.3–209.2) (P = 0.0015). There was little or no expression of
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CD16 by the CD14++ monocytes (compare the PE signal for the CD14++ cells in Figures 1A and B, or in Figures 1C and D).

Figure 2B shows that there was no difference in the absolute numbers of CD14+,CD16+ monocytes in the circulation of the patients with TRAPS (median 39 cells/µl, IQR 21–57 cells/µl) compared with healthy control subjects (median 39 cells/µl, IQR 31–45 cells/µl) (P = 0.8688). There was also no significant difference in the absolute numbers of CD14++,CD16− monocytes in patients with TRAPS (median 609 cells/µl, IQR 446–787 cells/µl) and control subjects (median 479 cells/µl, IQR 378–551 cells/µl) (P = 0.1267) (Figure 2C).

The percentage of CD14+,CD16+ monocytes among all monocytes was calculated as the number of flow cytometry events in the CD14+,CD16+ gate divided by the number of events in this gate plus the number in the CD14++,CD16− gate (see Figures 1A and C). The proportions of monocytes that expressed CD16 (Figure 2D) in patients (median 5.8%, IQR 4.4–10.7%) and control subjects (median 7.4%, IQR 5.9–8.5%) were not significantly different (P = 0.5358).

The finding that the level of CD16 expression was significantly raised in patients, even though the patients were not overtly unwell at the time of sampling, is consistent with the CRP levels and ESRs that were determined at the same time. The median CRP level was 27 mg/liter (IQR 10–45 mg/liter), with 7 of 9 patients for whom data were available having CRP levels above the upper limit of normal (10 mg/liter). The median ESR was 22 mm/hour (IQR 12.5–48 mm/hour), with 5 of 9 patients having ESRs above the upper limit of normal (20 mm/hour). However, neither the CRP levels nor the ESRs were significantly correlated with the monocyte parameters discussed above.

Shedding of CD16 by monocytes. The most probable explanation for the elevated levels of surface expression of CD16 by CD14+,CD16+ monocytes from patients with TRAPS described above (Figure 2A) is enhanced production or translocation to the cell surface of CD16. However, it is known that the activation of neutrophils induces CD16 cleavage by metalloproteinases (1), and activation of CD14+,CD16+ monocytes down-regulates surface expression of CD16 (6). Thus, it
is possible that defective cleavage of cell surface CD16 in patients with TRAPS could account for the elevated levels of CD16 expression. In order to test this possibility, we determined the MFI of CD16 expression by monocytes from patients with TRAPS and control subjects, with and without treatment with 10 ng/ml PMA for 15 minutes. The reductions in the MFI of CD16 expression by monocytes following PMA treatment were 50%, 58%, 61%, and 75%, respectively, in 4 patients with TRAPS (mean ± SD 61 ± 10%) and 15%, 54%, 62%, and 63%, respectively, in 4 control subjects (mean ± SD 49 ± 23%) (P = 0.3554). Thus, there was no apparent defect in CD16 cleavage in monocytes from these TRAPS patients with the C33Y mutation.

**TNF production by monocytes.** CD16+,HLA–DR++ monocytes are known to be a major source of TNF (6), and this was also found to be the case in patients with TRAPS. TNF production was determined by intracellular cytokine staining following LPS stimulation of peripheral blood leukocytes; the pro-inflammatory monocytes were defined as the CD14+,HLA–DR++ population, because their CD16 expression is down-regulated upon activation (6). For cells from 5 patients with TRAPS, the MFI of TNF staining was significantly higher in CD14+,HLA–DR++ cells (mean ± SD 903.7 ± 215.9) than in CD14++ cells (171.9 ± 82.0) (P = 0.0003). This was similar to cells from 3 healthy control subjects, for whom the corresponding values were 761.6 ± 221.6 for CD14+,HLA–DR++ cells and 171.8 ± 61.1 for CD14++ cells (P = 0.0254). In the absence of stimulation (with LPS), TNF production was negligible in either cell population for both patients and control subjects.

**DISCUSSION**

The main findings in this study were that the level of CD16 expression by CD14+,CD16+ peripheral blood monocytes was significantly elevated in patients with TRAPS (Figure 2A), and that these cells are a major source of TNF. Increased CD16 expression by monocytes has been reported in RA (9,11), which is consistent with the similarities observed between the inflammatory features of RA and TRAPS. In RA, however, the level of monocyte CD16 expression was shown to be increased only during active (as opposed to quiescent) disease (11). In contrast, the elevated CD16 expression by monocytes in TRAPS that we observed occurred in the absence of clinically overt episodes of inflammation, indicating that CD16 expression by monocytes in TRAPS is increased as a feature of the underlying constitutive inflammatory status that is also characterized by elevated CRP levels and ESRs (ref. 2 and present report). Consistent with this comparison of RA and TRAPS, we previously reported both similarities and differences in the circulating cytokine profiles in patients with these conditions (2).

It is possible that monocyte CD16 expression may be even more elevated during an episode of autoinflammation in TRAPS, but none of the patients was having such an episode at the times of sampling, and advances in the clinical management of these patients have reduced the opportunities to assess this. However, in a preliminary study of 4 of the patients with TRAPS in whom it was possible to compare samples obtained when they were well and samples obtained during episodes of disease, 3 of the 4 patients had higher levels of CD16 expression by CD14+,CD16+ monocytes during such episodes. It was unavoidable that most of the patients with TRAPS were receiving various antiinflammatory drugs at the time we obtained blood samples, including prednisolone (used in 4 of the patients), which has been reported to induce depletion of CD16+ monocytes (12). Indeed, these 4 patients had lower numbers of CD14+,CD16+ monocytes than most of the other patients. Thus, the treatment being received by the patients may also have reduced the numbers of CD14+,CD16+ monocytes, and tapering of prednisolone may lead to an increase in the number of these cells, with increased risk of autoinflammation.

Our finding that monocytes from TRAPS patients with the C33Y mutation were not defective in the shedding of CD16 when activated with PMA is consistent with the previous observation that neutrophils from TRAPS patients with the C52F mutation shed CD16 normally when stimulated with PMA (1).

An increase in the MFI of CD16 monocytes has been reported during infection (8) as well as during active RA (11), but our observation of enhanced CD16 expression without increased numbers of circulating CD14+,CD16+ cells in patients with TRAPS was unexpected. In this regard, it may be relevant that CD16+ monocytes show enhanced adherence to vascular endothelium due to high adhesion molecule expression (13), and tissue infiltration by monocytes is a feature of TRAPS (3,4). Thus, the apparently normal numbers of circulating CD14+,CD16+ monocytes in patients with TRAPS may be attributable to increased adhesion and extravasation of these cells. Consistent with this, there was a trend toward an inverse correlation between the level of CD16 expression by monocytes and the numbers of circulating CD16+ monocytes in the patients. For
example, the 2 patients shown in Figure 2B to have the highest numbers of circulating CD14+CD16+ monocytes (181 cells/µl and 104 cells/µl, respectively) actually had the lowest MFI values for CD16 expression by monocytes (260 and 241, respectively) (Figure 2A). It is also possible, as noted above, that the antiinflammatory treatments that the patients were receiving had an effect on the number of CD14+,CD16+ monocytes.

Several models for the pathophysiology of TRAPS have been proposed, and these models are not mutually exclusive. Low circulating levels of soluble TNFRSF1A occur in patients with TRAPS, and defective shedding of leukocyte surface TNFRSF1A has been demonstrated in some patients with TRAPS, which may lead to a deficiency in TNF neutralization (1). Defective apoptosis has been observed in cells from patients with TRAPS (14,15). In a transfection system, we demonstrated normal signaling activity but reduced cell surface expression and TNF binding by TRAPS-associated mutant forms of TNFRSF1A and postulated gain-of-function proinflammatory consequences due to TNFRSF1A misfolding, aggregation, and ligand-independent signaling (16,17). Other investigators also recently published data consistent with this model (18,19). In addition, we recently demonstrated that neutrophils from patients with TRAPS show cytoplasmic retention of mutant, but not wild-type, TNFRSF1A, consistent with the above-mentioned model systems (20).

It remains to be determined how the constitutive increase in CD16 expression by monocytes reported here might relate to these models of TRAPS pathophysiology and its relevance to episodes of autoinflammation. However, the capacity for high TNF production by CD14+,CD16+ monocytes from patients with TRAPS is consistent with a role in this autoinflammatory syndrome. Furthermore, because ligation of CD16 on monocytes is known to induce cytokine production (21–23), the elevated expression of CD16 by monocytes from patients with TRAPS may enhance their inflammatory potential in the presence of antibody-opsonized antigens (e.g., during infection).

Extrinsic factors known to increase the proportion of monocytes expressing CD16 and enhance the levels of CD16 expression include interleukin-10, macrophage colony-stimulating factor, and transforming growth factor β1 (9). However, it will be important to determine whether the increase in CD16 expression by monocytes from patients with TRAPS is attributable to the effects of extrinsic factors or is an intrinsic property of monocytes expressing mutant forms of TNFRSF1A. Conversely, the relative expression of mutant and wild-type TNFRSF1A by CD14+,CD16+ and CD14+++,CD16− monocytes is an important issue for investigation.

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AUTHOR CONTRIBUTIONS

Dr. Todd 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.

Study design. Todd, Ziegler-Heitbrock, Ghaemmaghami, Powell, Tighe.

Acquisition of data. Radford.

Analysis and interpretation of data. Todd, Radford, Ziegler-Heitbrock, Ghaemmaghami, Powell, Tighe.


Statistical analysis. Todd.

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