Mechanism of glucocorticoid-induced depletion of human $CD14^+CD16^+$ monocytes

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Abstract: Healthy donors infused with high doses of glucocorticoids [GCs; methyl-prednisolone (MP); 500 mg/day for 3 days] suffer a selective depletion of the $CD14^+CD16^+$ monocytes such that these cells are reduced by 95% on day 5. In vitro studies revealed that at 11 h of culture in the presence of 10^{-5} M MP, no depletion was observed as yet, but a reduction by 80% was seen after 24 h. In dose-response analysis, MP still led to a 50% reduction of $CD14^+CD16^+$ monocytes at 10^{-7} M. Depletion could not be overcome by addition of the cytokines interleukin-1β or macrophage-colony stimulating factor, and it was independent of CD95. Depletion was, however, inhibited by the caspase 3,8 blocker z-Val-Ala-Asp, suggesting that cell death occurs in a caspase-dependent manner. Furthermore, blockade of depletion by RU-486 indicates that the intracellular GC receptor (GCR) is involved. Measurement of GCR by flow cytometry revealed a 50% higher level of expression in the $CD14^+CD16^+$ monocytes. Our studies show a selective depletion of $CD14^+CD16^+$ monocytes by GC treatment in vivo and in vitro, an effect to which the modestly increased level of GCR may contribute. ***J. Leukoc. Biol.*** **74: 33–39; 2003.**

Key Words: *glucocorticoids · monocyte · macrophage · apoptosis*

INTRODUCTION

The human monocyte/macrophage system derives from myelomonocytic precursors in bone marrow. These cells give rise to monoblasts, which further mature to monocytes, which are then released into blood where they remain for a few days to migrate into tissue and to develop into the different types of macrophages.

In blood, when using CD14 and CD16 monoclonal antibodies (mAb), two types of monocytes can be defined: the classical monocytes, which are strongly CD14-positive but negative for CD16 ($CD14^{++}$ monocytes), and the weakly CD14-positive monocytes, which coexpress CD16 ($CD14^+CD16^+$ monocytes). The $CD14^+CD16^+$ monocytes appear to be more mature, they readily express the proinflammatory tumor necrosis factor (TNF), and they fail to produce significant amounts of the anti-inflammatory interleukin (IL)-10 [1]. Based on this pattern of cytokine expression, we have termed these cells proinflammatory monocytes. Consistent with this concept, the $CD14^+CD16^+$ monocytes are expanded in various inflammatory diseases such as sepsis and systemic inflammatory response syndrome [2].

Conversely, anti-inflammatory therapy with glucocorticoids (GCs) has been shown previously to deplete the proinflammatory $CD14^+CD16^+$ monocytes [3]. The mechanism of depletion remained, however, elusive.

GCs are very effective anti-inflammatory and immunosuppressive compounds, which are widely used in clinical medicine [4]. They act via an intracellular receptor that dimerizes after ligand binding, followed by binding to recognition sequences in promoters of various genes. This leads to enhanced or reduced gene expression depending on the promoter context [5].

Reduced expression of genes such as TNF and IL-2 may explain in part the immunosuppressive activity of GCs. In addition, in rodent models, GCs have been shown to induce apoptosis in T lymphocytes, while human T cells are rather resistant to GC-induced apoptosis [6]. Other cells such as the eosinophils appear to undergo apoptosis after GC treatment [7], and for human monocytes, apoptosis has been demonstrated after prolonged culture. This latter, in vitro effect appears to be indirect and a result of the suppression of the production of cytokines, which are required for monocyte survival [8].

The depletion of $CD14^+CD16^+$ monocytes, as described in patients with multiple sclerosis, is quite selective, as the classical $CD14^{++}$ monocytes are not depleted but rather increase in number [3]. Hence, there has to be a selective mechanism of depletion that affects only the $CD14^+CD16^+$ monocytes.

In the present report, we have studied this selective mechanism by analyzing the action of high-dose GCs in healthy donors in vivo and in vitro. We show herein that depletion involves caspase activation leading to apoptosis and that higher

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levels of GC receptor (GCR) expression in the $CD14^+CD16^+$ monocytes may explain the selective depletion of these cells by GC therapy.

The depletion of the proinflammatory $CD14^+CD16^+$ monocytes may be an important mechanism of GC action in various inflammatory diseases including autoimmune diseases.

MATERIALS AND METHODS

Infusion of methyl-prednisolone (MP)

Healthy male volunteers were subjected to a complete medical examination, and their blood was tested for a broad panel of parameters covering hematology, liver and kidney function, and glucose levels to exclude major illnesses. Volunteers with evidence of infection, diabetes, or mental illness were excluded. After written, informed consent, 500 mg MP (Urbason solubile, Hoechst-Marion-Roussel, Bad Soden, Germany) dissolved in 50 ml Ringer solution was infused daily for 3 consecutive days over a period of 15 min into a cubital vein. Blood was drawn daily over a 5-day period. The Ethics Committee of the University of Munich Medical Faculty (Germany) approved the study, and DBV-Winterthur Insurance (Hamburg, Germany) provided insurance to cover any damage caused by the study.

Cells and culture

For in vitro culture, peripheral blood mononuclear cells (PBMCs) were prepared from heparinized blood obtained from healthy volunteers by density gradient separation. Cells were adjusted to 10^6 per ml in RPMI 1640 with 10% fetal calf serum (FCS) and were incubated in 15 ml polypropylene tubes (#188261, Cellstar, Frickenhausen, Germany) at 37°C for different periods of time with or without MP (#M-0639, Sigma, Munich, Germany) at doses of 10^{-5}–10^{-8} M. RU-486 (Mifepristone, #M-8046, Sigma, München, Germany) and z-Val-Ala-Asp-fluoromethylketone (z-VAD-FMK; #P-416, Biomol, Hamburg, Germany) were used as inhibitors of MP-mediated depletion. Recombinant IL-1β (#20-01BS) was purchased from Peprotech Ltd. (London, UK), and Genetics Institute (Cambridge, MA) generously provided recombinant macrophage-colony stimulating factor (M-CSF).

The biological activity of IL-1β was demonstrated by testing its effect on dexamethasone-induced cell death of classical $CD14^{++}$ monocytes after 3 days of culture. Percent annexin V-positive cells was 29.6 ± 12.9% in control cultures, 59.6 ± 18.6% with dexamethasone at 5×10^{-6} M, and 13.8 ± 3.9% with dexamethasone plus IL-1β at 50 ng/ml (100 U/ml; n=4, $P<0.05$). M-CSF biological activity was tested by serum deprivation using the Mono Mac 6 cell line. Cells cultured with 10% FCS were 10.0 ± 0.2% annexin V-positive; after 3 days of culture with 0.5% FCS, they were 19.2 ± 1.5%-positive, and with M-CSF at 100 ng/ml, they were back to 10.9 ± 2.6% (n=3, $P<0.05$). Hence, both cytokines were biologically active and were capable of increasing monocyte survival.

Monocyte staining and fluorescein-activated cell sorter (FACS) analysis

Whole-blood staining was done on 200 μl samples of EDTA–blood, and PBMCs were stained at 10^6 cells/ml in 50 μl volumes. Samples were incubated with directly conjugated mAb at saturating concentrations for 20 min on ice. The antibodies used were anti-CD14-fluorescein isothiocyanate (FITC; clone My-4, #6603511, Coulter, Krefeld, Germany), anti-CD16-phycoerythrin (PE; Leu11c, clone B73.1, #347617, BD, Heidelberg, Germany), anti-CD33-PE (clone W135, #30945X, BD), and anti-major histocompatibility complex (MHC) class II (DR, DP, DQ, clone I3, #6603 24, Coulter). For three-color immunofluorescence staining, anti-CD95-PE (clone DX2, #33455, BD) was combined with anti-CD14-FITC and anti-CD16-PE CyChrome5 (PC5; 3G8, #2642, Coulter). For analysis of GC effects on human leukocyte antigen (HLA)-DR expression, anti-CD14-FITC and anti-CD16-PE were combined with anti-HLA-DR-PC5 (Immu357, #2659, Coulter). FACS analysis was done on a FACScan (BD) by gating in the light-scatter histogram on monocytes plus the upper portion of lymphocytes and recording fluorescence signals of at least 7500 cells in the monocyte gate. Absolute numbers were calculated based on the percentage of cells among all leukocytes, and the leukocyte count was determined in a Celldyn 3200 hematology analyzer (Abbott Laboratories, Abbott Park, IL).

Three-color immunofluorescence analysis of intracellular GCR expression

Cells were first surface-stained with anti-CD14-PC5 (RMO52, #2640, Coulter) and anti-CD-16-PE. After washing, they were kept for 20 min at room temperature (RT) in 200 μl vol Cytofix/Cytoperm (#2090KZ, BD). They were then washed and resuspended in Perm/Wash buffer (#2097, BD) for 20 min at RT and finally stained with the anti-GCR mAb 5E4-FITC at 10 μg/ml [9]. As a negative control, cells were incubated with the anti-GCR antibody in the presence of a tenfold molar excess of the APTEK-26 antigenic peptide [9]. Cells were then washed and resuspended in 0.5% paraformaldehyde. FITC staining was analyzed for $CD14^{++}$ compared with $CD14^+CD16^+$ monocytes. Results were expressed as specific median fluorescence intensity (sMed), i.e., Med for anti-GCR-stained cells minus Med for cells stained for anti-GCR plus blocking peptide.

CD95 blockade

PBMCs were preincubated for 1 h with the CD95-blocking antibody ZB4 at an effective concentration of 500 ng/ml [10]. Without washing, MP at 10^{-6} M was added, and cells were analyzed after 24 h. The blocking activity of the antibody at 500 ng/ml was demonstrated in U-937 cells, which undergo apoptosis after serum deprivation. Cells grown in 10% FCS show 1.9 ± 0.1% annexin V-positive cells, and cells cultured for 48 h without serum were 19.4 ± 5.8%-positive. Incubation of the cells with ZB4 reduced the percentage to 6.5 ± 1.6% (n=3, $P<0.05$).

Statistics

Student's *t*-tests or Wilcoxon-ranking tests were used for statistical analysis.

RESULTS

Effect of high-dose MP on blood monocytes in vivo

Healthy volunteers were infused for 3 consecutive days with 500 mg MP, and monocyte subpopulations were determined by flow cytometry on a daily basis for 5 days. As shown in **Figure 1**, the classical $CD14^{++}$ monocytes showed a slight increase

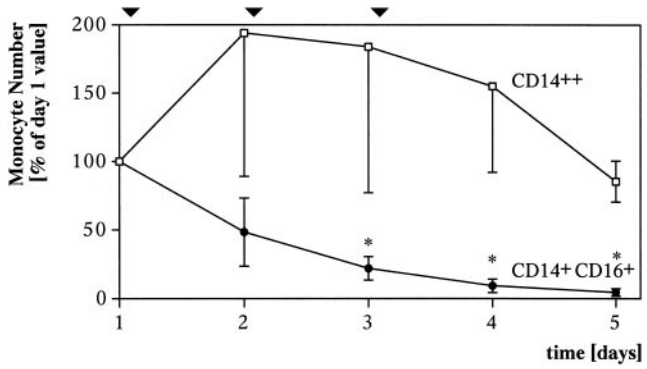

Fig. 1. Time course of monocyte population numbers during in vivo high-dose GC treatment. Five healthy donors received 15-min infusions of 500 mg MP on days 1, 2, and 3 (▼), and monocyte populations were studied for 5 days (□, $CD14^{++}$ monocytes; ●, $CD14^+CD16^+$ cells). The numbers of $CD14^{++}$ (473±437 cells/μl) and $CD14^+CD16^+$ (35±28 cells/μl) monocytes were equated with 100%. Significance at $P < 0.05$ compared with the pretreatment value.

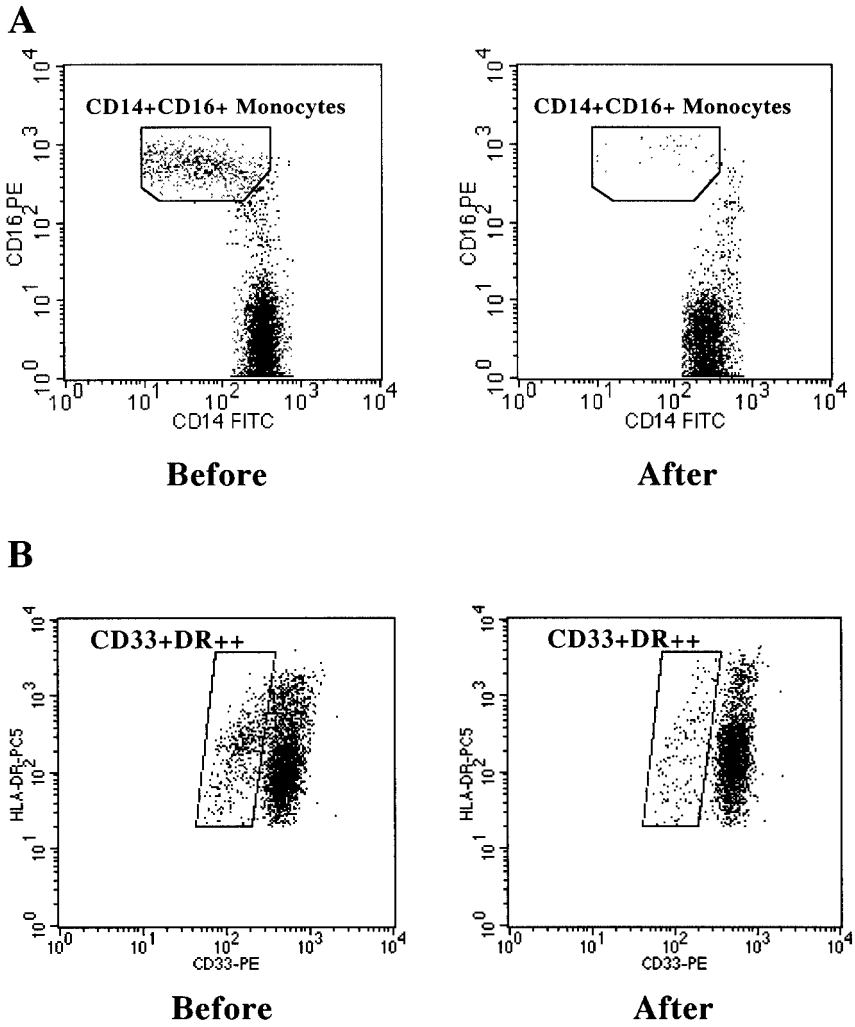

Fig. 2. Monocyte populations before and after in vivo high-dose GC treatment. (A) A healthy donor was studied before and after three daily treatments with a 15-min infusion of 500 mg MP. On day 1, before (left) and on day 5, i.e., 48 h after the third infusion (right), cells were stained for CD14 and CD16 and analyzed by FACS. In this example, the number of $CD14^+CD16^+$ monocytes was reduced from 27 cells/μl to 2 cells/μl. One of five experiments. (B) Another volunteer's cells were studied before and after MP treatment using CD33 and HLA-DR antibodies. In this example, the number of $CD33^+DR^{++}$ cells was reduced from 20.9 cells/μl to 3.3 cells/μl. One of three experiments.

in numbers over time and returned to the starting level on day 5. By contrast, the number of $CD14^+CD16^+$ monocytes continuously decreased, leading to a 50% reduction on day 2 and a 95% reduction on day 5 (Fig. 1). A typical example of the CD14/CD16 staining, demonstrating a near complete depletion of the $CD14^+CD16^+$ monocytes on day 5, is shown in **Figure 2A**.

To exclude that the disappearance of the $CD14^+CD16^+$ monocytes is merely a result of the disappearance of these cell-surface markers, we analyzed the monocyte subpopulations with a second set of markers. As a low level of CD33 and a high level of HLA-DR also characterize the $CD14^+CD16^+$ monocytes, we determined the $CD33^{++}DR^+$ and the $CD33^+DR^{++}$ populations are, by and large, identical to the $CD14^{++}$ and the $CD14^+CD16^+$ monocytes, respectively. Here, high-dose MP depleted the $CD33^+DR^{++}$ monocytes, and the $CD33^{++}DR^+$ monocytes were unaffected (Fig. 2B). In an average of three experiments, the $CD33^+DR^{++}$ monocytes were depleted from 8.4 ± 2.1% to 4.3 ± 1.6%. These data indicate that in vivo treatment with high-dose MP, in fact, depletes the $CD14^+CD16^+$ monocyte subpopulation.

Effect of MP on blood monocytes in vitro

To analyze the mechanism of $CD14^+CD16^+$ monocyte depletion, we incubated PBMCs for 24 h in vitro with MP at 10^{-5} M. Such treatment led to some induction of CD16 expression on the $CD14^{++}$ monocytes, but it left their numbers unaffected. In contrast, the $CD14^+CD16^+$ monocytes were almost completely depleted (**Fig. 3**). In an average of five experiments, they were reduced from 7.2 ± 2.1% to 1.2 ± 0.9% ($P<0.05$), which reflects a depletion down to 17% of the starting level (time, 0 h). In dose-response analysis, no significant change was detected in the percentage of $CD14^+CD16^+$ monocytes

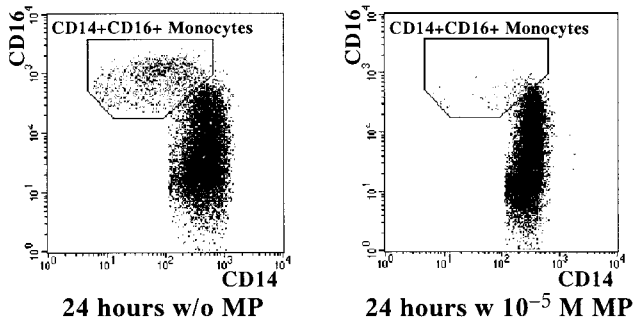

Fig. 3. Effect of high-dose GC treatment on monocyte populations in vitro. PBMCs were cultured for 24 h in the absence or presence of MP at 10^{-5} M, and monocyte subsets were determined by two-color flow cytometry.

with 10^{-8} M MP, but at concentrations of 10^{-7} M and 10^{-6} M, these cells were decreased by factors 2 and 3, respectively (**Fig. 4A**). The most effective depletion of these cells down to 1.4 ± 0.8% (fivefold reduction) was achieved at the highest concentration of 10^{-5} M MP.

Looking at the kinetics of this depletion (Fig. 4B), no effect was detectable after 6 or 11 h. However, after 24 h, the $CD14^+CD16^+$ cells were depleted. Again, we could demonstrate that after 24 h, MP depleted the $CD33^+DR^{++}$ but not the $CD33^{++}DR^+$ monocytes at 10^{-5} M. The numbers of $CD33^+DR^{++}$ monocytes decreased from 11.9 ± 1.4% to 5.3 ± 1.9%, confirming also that in vitro, the cells do not just down-regulate some cell-surface receptors but really disappear.

Effect of the cytokines IL-1β and M-CSF on MP-mediated $CD14^+CD16^+$ monocyte depletion

GC might act by suppressing production of cytokines that are required for survival of $CD14^+CD16^+$ monocytes. We therefore tried to overcome the depletion of these cells by admixing high doses of the cytokines IL-1β and M-CSF to 24-h cultures of PBMCs. Although in control cultures, the $CD14^+CD16^+$ monocytes were depleted by GC down to 32.3 ± 11.6%, the depletion was down to 34.0 ± 7.6% in the presence of M-CSF at 100 ng/ml and down to 26.3 ± 8.1% in the presence of IL-1 at 100 U/ml. Hence, addition of these cytokines does not overcome the depletion of $CD14^+CD16^+$ monocytes by GCs.

Role of CD95 in MP-mediated $CD14^+CD16^+$ monocyte depletion

The depletion of the $CD14^+CD16^+$ monocytes by GC might involve ligation of CD95 on monocytes. We therefore asked whether CD95 expression is higher on this monocyte population as compared with the classical $CD14^{++}$ monocytes. Three-color immunofluorescence analysis revealed that the $CD14^{++}$ monocytes expressed CD95 with a specific median of 165.1 ± 8.3 channels, and the $CD14^+CD16^+$ monocytes showed a significantly lower level of expression with a sMed of 73.3 ± 4.8 channels ($P<0.05$). When cultured for 8 h with or without MP, there was no change in the expression level of CD95 on these monocyte populations (data not shown).

Although the $CD14^+CD16^+$ monocytes showed a twofold lower expression level of CD95, we still asked whether the CD95 receptor might be involved in the selective depletion of these cells. For this, we cultured PBMCs for 24 h with or without a CD95-blocking antibody. MP depleted $CD14^+CD16^+$ monocytes in the presence of the anti-CD95 antibody at 10^{-6} M from 13.7 ± 2.8% to 7.7 ± 2.5%. This is very similar to the depletion in cultures treated with MP alone (8.4±2.3%). Hence, CD95 does not appear to be involved in the selective depletion of the $CD14^+CD16^+$ monocytes by GCs.

Inhibition of MP-mediated $CD14^+CD16^+$ monocyte depletion by a caspase inhibitor

Induction of apoptosis may operate via activation of the caspase cascade. To test involvement of caspases, PBMCs were cultured with MP at 10^{-6} M with or without z-VAD-FMK. In these experiments, treatment of PBMCs with MP led to a depletion of $CD14^+CD16^+$ monocytes from 8.1 ± 1.4% to 3.7 ± 0.7% (**Fig. 5**). z-VAD-FMK at 10 μM could in fact partially prevent the depletion of the $CD14^+CD16^+$ monocytes, such that their percentage was increased from 3.7 ± 0.7% to 6.7 ± 2.0% (Fig. 5).

Inhibition of MP-mediated $CD14^+CD16^+$ monocyte depletion by GCR blockade

Next, we asked whether this effect of GCs was mediated by a postulated cell-surface receptor or via the nuclear steroid receptor. Addition of the steroid receptor blocker RU-486 at

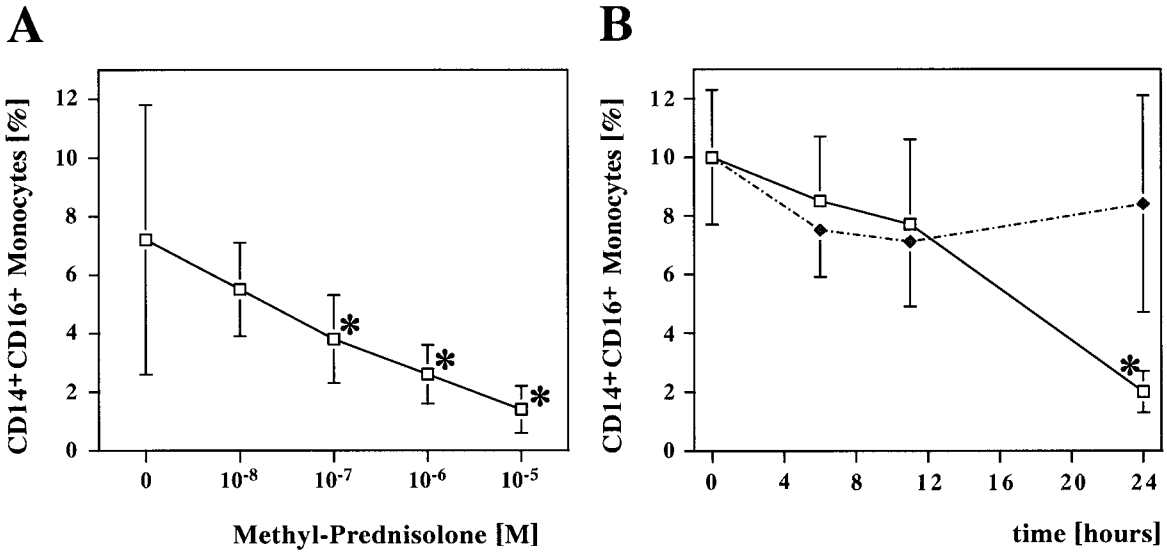

Fig. 4. Dose and time dependence of in vitro depletion of proinflammatory monocytes by GC. (A) Dose dependence: PBMCs were treated with or without different concentrations of MP for 24 h, and the percentages of the $CD14^+CD16^+$ monocytes were determined. Given is the average ± SD from five donors. *, Significance at $P < 0.05$. (B) Time dependence: PBMCs were treated without or with MP at 10^{-5} M for up to 24 h, and the $CD14^+CD16^+$ monocytes were determined (□, treated cells; ◆, untreated cells). Given is the average ± SD from five donors. *, Significance at $P < 0.05$.

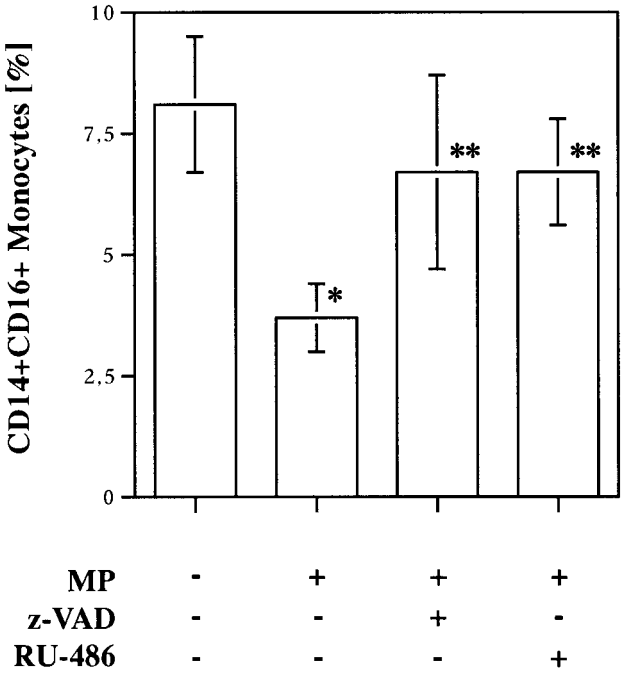

Fig. 5. Blockade of MP induced $CD14^+CD16^+$ monocyte depletion. PBMCs were cultured for 24 h without or with MP at 10^{-6} M in the presence of the caspase inhibitor z-VAD-FMK at 10 μM or the steroid receptor inhibitor RU-486 at 3 μM. After 24 h, $CD14^+CD16^+$ monocyte numbers were determined. Given is the average ± SD from five donors; *, significant differences between untreated and MP-treated cultures at $P < 0.05$. **, Significant differences between MP-treated and MP/z-VAD-FMK- or MP/RU-486-treated cultures at $P < 0.05$.

3×10^{-6} M partially prevented the depletion by MP at 10^{-6} M. Although the $CD14^+CD16^+$ monocyte percentage decreased from 8.1 ± 1.4% to 3.7 ± 0.7% with MP alone, the addition of RU-486 to the MP culture significantly inhibited the depletion (6.7±1.1% with RU-486; Fig. 5). At the same time, the MP-induced down-regulation of HLA-DR expression was completely inhibited. The sMed fluorescence intensity (analyzed on the classical monocytes) was 173.5 ± 57.9 channels for untreated samples, 78.0 ± 21.6 channels for cells treated with MP alone, and 148.5 ± 29.0 channels for cells treated with MP plus RU-486 ($P<0.05$ for the reduction by MP as compared with untreated and $P<0.05$ for MP plus RU-486 vs. MP alone). The data indicate that the decrease of MHC class II expression and the depletion of $CD14^+CD16^+$ monocytes operate via the classical nuclear GCR.

Expression of GCR in monocyte subpopulations

We then asked whether the selective depletion of $CD14^+CD16^+$ monocytes by GC could be explained by differences in the expression of the GCR in the two monocyte populations. For this, PBMCs were stained for CD14 and CD16 followed by permeabilization and incubation with the anti-GCR antibody 5E4. As a negative control, 5E4 was admixed with a 100-fold molar excess of the APTEK26 peptide, which is the target peptide for the antibody. As shown in the example in **Figure 6**, the $CD14^{++}$ monocytes expressed GCR (258.5 channels), but the level of expression was clearly higher in the $CD14^+CD16^+$ monocytes (314.8 channels). This becomes obvious when looking at the wider gap between the two histograms in the $CD14^+CD16^+$ monocytes panel. In an average of five donors, the classical $CD14^{++}$ monocytes expressed GCR at a level of 226.7 ± 65.1 channels, whereas the expression level was 268.1 ± 69.0 channels for the $CD14^+CD16^+$ monocytes ($P<0.05$; Wilcoxon test). The average 42 channels–sMed fluorescence intensity reflects a higher level of GCR expression in the $CD14^+CD16^+$ monocytes compared with the classical monocytes. These data suggest that the $CD14^+CD16^+$ monocytes may be more susceptible to the apoptotic effects of GC as

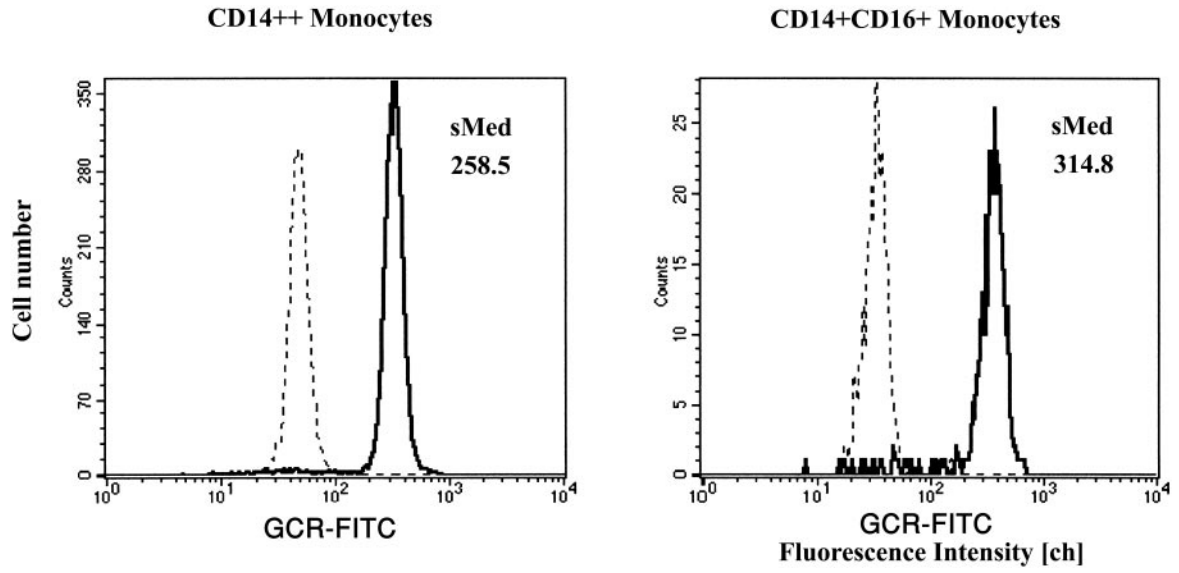

Fig. 6. Expression of GCRs by monocyte subpopulations. PBMCs were cell-surface stained for CD14 and CD16, permeabilized, and stained with a directly conjugated, anti-GCR antibody. As negative control, cells were reacted with the anti-GCR antibody in the presence of a tenfold molar excess of the antigenic peptide. Given is the intracellular staining intensity for the $CD14^{++}$ monocytes (left) and the $CD14^+CD16^+$ monocytes (right). Anti-GCR antibody (solid line); anti-GCR antibody plus blocking peptide (broken line); one of five experiments. ch, Channels.

a result of a higher level of GCR expression in comparison with the $CD14^{++}$ monocytes.

DISCUSSION

In our initial studies in multiple sclerosis patients treated with high-dose GC, we noted a selective depletion of the $CD14^{+}CD16^{+}$ monocytes, but the mechanism of this depletion remained unclear [3]. We have now analyzed this mechanism in healthy volunteers to avoid interference from an underlying disease. Also, this allowed for more controlled GC application at the same time of day (8 AM). Similar to the patient study, we see a 50% decrease of the $CD14^{+}CD16^{+}$ monocytes after 24 h and an almost complete elimination of these cells on day 5. To exclude that the disappearance of the $CD14^{+}CD16^{+}$ monocytes is merely the result of a disappearance of the CD14 and CD16 cell-surface antigens, we studied monocyte populations based on their differential expression of CD33 and HLA-DR antigens [11]. Cells with low expression of CD33 and high expression of HLA-DR ($CD33^{+}DR^{++}$ monocytes) are by and large identical with the $CD14^{+}CD16^{+}$ monocytes. These cells were in fact also depleted by GC therapy in healthy donors. The depletion of $CD33^{+}DR^{++}$ cells is, however, not as complete as depletion of $CD14^{+}CD16^{+}$ cells, as the $CD33^{+}DR^{++}$ population contains additional cells that are CD14-low or CD14-high [12]. These additional cells appear not to be depleted by GCs.

When looking at hourly intervals after GC infusion, there is a rapid depletion from peripheral blood of the $CD14^{+}CD16^{+}$ and the $CD14^{++}$ monocytes (data not shown). However, after 24 h, the $CD14^{++}$ monocytes reach a slightly higher number compared with the starting value, whereas the $CD14^{+}CD16^{+}$ monocytes remain at reduced levels. The rapid disappearance and reappearance of these cells very likely reflect a redistribution of leukocytes in and out of another compartment such as the marginal pool [6]. Such mechanisms of redistribution are difficult to analyze in man, and they appear to be different from the long-term depletion that occurs in the case of the $CD14^{+}CD16^{+}$ monocytes. We therefore have focused in our studies on the selective, long-term depletion of the $CD14^{+}CD16^{+}$ monocytes.

In in vitro studies, we observed a strong depletion of these cells after 24 h at 10^{-5} M MP. This dose is quite high but still reflects a concentration that is reached in vivo: After infusion of 500 mg MP and homogenous distribution in the extracellular volume of a 75-kg individual, a value of 8.8×10^{-5} M can be calculated. In vivo values will decrease with catabolism and excretion, resulting in plasma levels that are down to 10% after 12 h [13]. By contrast, there is little decrease during in vitro culture [14]. This may explain the more pronounced depletion of the $CD14^{+}CD16^{+}$ monocytes after 24 h in vitro. The in vitro depletion by GC does not require these excessive doses, as there is still a significant depletion at 100-fold lower concentrations, i.e., 10^{-7} M (Fig. 4A). Also, GC therapy with lower doses, such as prednisolone at 40 mg/day, may deplete the $CD14^{+}CD16^{+}$ monocytes in vivo (unpublished), but no systematic, dose-effect analysis has been performed as yet.

The in vitro time-course analysis revealed that the depletion of the $CD14^{+}CD16^{+}$ monocytes did not occur within hours, as at 11 h of culture, no depletion was evident as yet (Fig. 4B). After 24 h, however, there was an effective depletion. Schmidt et al. [8] also studied the effects of GC treatment on monocytes in vitro. They did not address the $CD14^{+}CD16^{+}$ monocytes but studied the classical $CD14^{++}$ monocytes. In their study, they observed an effect after 2 days with an optimal depletion of the classical monocytes after 3 days. This effect was shown to be indirect, as it involved suppression of the expression of cytokines such as IL-1β, and the depletion could be overcome by addition of exogenous IL-1β [8]. Based on these studies, we have selected IL-1β for our analysis, and in addition, we have tested M-CSF, as this cytokine has been reported to improve monocyte survival [15]. Both of these failed to overcome GC-induced cell death, suggesting that blockade of their production is not the relevant mechanism of action in the depletion of $CD14^{+}CD16^{+}$ monocytes. We cannot, however, exclude an effect of GC on other cytokines still to be identified.

We envisaged that the depletion of the $CD14^{+}CD16^{+}$ monocytes might occur by apoptosis and asked whether the CD95 receptor might be involved in this process. Spontaneous apoptosis of monocytes has been shown to involve the CD95/CD95L system [16], and IL-10-induced apoptosis of monocytes after 2–3 days of culture was also demonstrated to be dependent on this system [17]. In the case of GC-induced apoptosis of $CD14^{+}CD16^{+}$ monocytes, the lower level of CD95 on these cells argued against this possibility, and blockade of the CD95 receptor did not prevent depletion. At this point, we cannot exclude that ligation of another receptor related to CD95 [18] might be involved in the process of GC-mediated depletion. Still, the molecular mechanism of GC action in apoptosis remains elusive. Studies in leukemic leukocytes (reviewed in Greenstein et al. [19]) have demonstrated an involvement of caspases, Bcl-2, and mitochondria [20–25]. In addition, a GC-induced blockade of nuclear factor-κB activity may lead to reduced expression of antiapoptotic genes [26, 27]. Our experiments with inhibition of caspases by z-VAD can in fact demonstrate an involvement of caspases in the depletion of the $CD14^{+}CD16^{+}$ monocytes, similar to what has been shown for apoptosis of leukemic cells.

The question then arises: Why is GC-induced apoptosis selective for the $CD14^{+}CD16^{+}$ monocytes? One possibility is that there may be a higher level of the GCR protein in the $CD14^{+}CD16^{+}$ cells. In fact, intracellular staining with a mAb demonstrates a 50% higher expression of GCR in these cells. The expression of GCR is only modestly higher in the $CD14^{+}CD16^{+}$ monocytes, and this may contribute to the higher sensitivity of these cells to GC-induced cell death. Still, it is clear that as-yet unrecognized differences in receptor properties or other factors that regulate receptor function will have to come into play to explain the profound differences in GC sensitivity. Also, there may exist differences in pro- or antiapoptotic mechanisms. For eosinophils, which undergo high spontaneous and induced apoptosis, it was shown that the antiapoptotic molecule Bcl-2 is absent and that the increased survival of these cells after IL-5 treatment is associated with induction of Bcl-2 mRNA and protein [28, 29]. In the $CD14^{+}CD16^{+}$ monocytes, the Bcl-2 protein was, however,

readily detectable at levels similar to those found in the classical $CD14^{++}$ monocytes (data not shown). It is still unclear whether there is differential GC-induced expression of other pro- or antiapoptotic molecules in the $CD14^{+}CD16^{+}$ monocytes.

GCs are not the only drugs that induce a selective depletion of the $CD14^{+}CD16^{+}$ monocytes. Studies by Hotta et al. [30] have shown that deoxyspergualin will deplete the $CD14^{+}CD16^{+}$ monocytes. Although this drug decreases the classical monocytes as well, the effect on the proinflammatory monocytes is much more pronounced. It will be important to analyze whether GCs and deoxyspergualin will act on the $CD14^{+}CD16^{+}$ monocytes by the same caspase-dependent mechanism.

As the $CD14^{+}CD16^{+}$ monocytes are potent, proinflammatory cells, their drug-induced depletion as shown herein may be an important element of the immunosuppressive action of GCs. The depletion of the $CD14^{+}CD16^{+}$ cells may be a useful tool to monitor immunosuppression in patients treated with GCs or deoxyspergualin.

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