An intracellular antigen that reacts with MO2, a monoclonal antibody to CD14, is expressed by human lymphocytes

Boris Tartakovsky, Mordechai Fried, Margalit Bleiberg, Dan Turner, Michael Hoffman, Israel Yust

Research Unit, Clinical Immunology and AIDS Center, Tel Aviv Sourasky Medical Center, 6 Weizmann Str., Tel Aviv 64239, Israel

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Abstract

CD14, a lipopolysaccharide (LPS) receptor, is present on the surface membrane of phagocytic leukocytes; it is also present in a soluble form in serum. Recently published results confer to this molecule novel functions that are linked to T-cell activation and to apoptosis. We report here that we have defined and characterized a novel lymphocyte population in human peripheral blood, a population that expresses an intracellular antigen detectable with MO2, a monoclonal antibody directed against the human CD14 molecule. This population is composed primarily of CD8-positive T-cells. We found surprisingly that this novel MO2-positive population of lymphocytes was greatly enhanced in asymptomatic, untreated HIV-positive individuals.

1. Introduction

CD14 is a 55 kDa glycosylphosphatidylinositol (GPI)-linked protein present on the surface membrane of phagocytic leukocytes; it is also present in a soluble form in serum. CD14 is one major molecule responsible for the innate host inflammatory response to microbial infection. As a receptor for lipopolysaccharide (LPS) on the surface of monocytes and macrophages, the CD14 molecule was thought, until recently, to be involved primarily in non-specific host defense mechanisms against gram-negative bacteria [1–5].

A number of recently published results confer to this interesting molecule novel functions that are linked to T-cell activation and also to apoptosis. Thus, CD14 may function as an “apoptotic cell receptor” on the surface of phagocytes since it seems to detect and bind to phosphatidylserine which is externally exposed by apoptotic cells [6,7] and it may also be linked to susceptibility of monocytes or other cells to apoptosis [8,9,17]. Particularly interesting, CD14 either as a recombinant protein or as a native molecule, secreted by monocytes in vitro, has been recently shown to bind to the surface of in vitro activated human T-cells [10,11]. Most importantly, the interaction of CD14 with T-cells was shown to convey a negative signal onto these T-cells [12], in the form of IL2, IL4 and IFN gamma inhibition, probably due to the inactivation of NFkB [11]. In this study, some evidence was produced as to the internalization of recombinant CD14 by T-cells, in vitro.

However, these results have been reported only with cells cultured in vitro and artificially activated. We have been particularly interested by the implication of these findings and sought to assess the possible contribution of CD14 to T-cell activities and functions, in vivo, in human.

As a first step, we looked for any evidence for the in vivo expression of CD14 by human lymphocytes and
particularly by T-cells. The results obtained form the matter of this present paper.

2. Materials and methods

Peripheral blood mononuclear cells (PBMCs), freshly obtained on a ficoll-hypaque gradient (UNI-CEP tubes Novamed Ltd., Jerusalem, Israel), were washed in PBS and then fixed in 4% paraformaldehyde (Merck, Germany) in PBS, for 30 min at 4 °C. Cells were washed in PBS and then permeabilized in PBS supplemented with 0.1% saponin (from Quillaja bark, Sigma) and 1% bovine serum albumin (BSA, Sigma) for 30 min at room temperature. Thereafter, cells were divided into staining tubes and processed in the saponin buffer for dual color fluorescence staining.

Cells (in 50 μl saponin buffer) were incubated with 2 μg/ml of anti-CD14 monoclonal antibodies (RD1-conjugated, detailed in Table 1) or isotype monoclonal antibody controls and same amount of anti-CD3 or CD4 or CD8 or CD19 monoclonal antibodies or appropriate isotype controls, for 30 min at room temperature, followed by one washing in saponin buffer and two washings in PBS. Cells were resuspended in PBS and immediately analyzed with the FACScalibur (Becton Dickinson).

3. Results

We have tested six different anti-human CD14 monoclonal antibodies for their capacity to specifically stain human lymphocytes. In order to maximize the chances to detect CD14, we tested cells that were fixed and permeabilized before staining. Thus, PBMCs were fixed in 4% paraformaldehyde and then permeabilized in saponin buffer. Cells were incubated with labeled anti-CD14 antibodies and appropriate isotype control and subsequently analyzed by flow cytometry. Results of such an analysis are shown in Table 1. As demonstrated, all monoclonals stained monocytes but only two monoclonals gave positive staining of lymphocytes (UCHM1 and MO2). Only one of them, however, MO2 [13], demonstrated a significant and specific binding to these cells. We therefore concentrated on this antibody and further found that the staining of lymphocytes was evident only if the cells were fixed and permeabilized before hand. PBMCs either live or only fixed in paraformaldehyde failed to show any significant staining with this particular monoclonal antibody (Fig. 1), thus suggesting that the cellular antigen was not accessible to the MO2 antibody unless the membrane was perforated.

Additional controls were run; first, we looked at whole blood cells (stained following erythrocyte lysis, fixation and permeabilization) and not at PBMCs. Results demonstrated that the ficoll-hypaque gradient separation of the mononuclear cells had no effect on the MO2 staining and similar results were obtained whether the lymphocytes were separated or not (17% vs. 25% of CD3 cells stained positive for MO2).

Next, we compared two different permeabilization reagents, saponin and Tween 20. In order to control for any artifactual effect of the saponin, we performed parallel experiments in which the saponin was substituted with Tween 20. Although the saponin was found to be superior in its visualization of the binding (15% vs. 8% CD3+/MO2+ cells), the permeabilization with Tween 20 resulted in a positive and specific binding of the MO2 antibody to lymphocytes.

We then compared two different fluorochromes. In order to control for the fluorescent probe, we used two different preparations of the MO2 antibodies, one

<table>
<thead>
<tr>
<th>Anti-CD14 mAb</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
<th>Isotype control</th>
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<tbody>
<tr>
<td>MY4 (Coulter, USA)</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>UCHM1 (IQP, Holland)</td>
<td>±</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>MO2 (Coulter, USA)</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>M5E2 (Pharmingen, USA)</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>TUK4 (DAKO, Denmark)</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>RMO52 (Coulter, USA)</td>
<td>–</td>
<td>+</td>
<td>–</td>
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PBMCs were fixed, permeabilized and stained with various monoclonal antibodies directed against human CD14 or appropriate isotype controls. Lymphocytes and monocytes were differentiated by scatter. –, no staining; +, positive, significant staining; ±, marginal staining.
conjugated to RD1 and one conjugated to FITC. No significant differences could be demonstrated (9% vs. 7.5%, respectively).

Finally, we looked at the stained cells by fluorescence microscopy in order to try and localize the cellular antigen. Thus, PBMCs were fixed and permeabilized and then double-stained for CD3 (red fluorescence) and for MO2 (green fluorescence), and also with the appropriate isotype controls. Cells were mounted on a glass slide and immediately analyzed under a fluorescence microscope. As shown in Fig. 2, various combinations of staining were evident. CD3-positive as well as CD3-negative cells were either positive or negative for the MO2 antibody. Moreover, the MO2 antigen, in the CD3-positive cells, was found to be at the periphery of the cells, close to the outer membrane. Some cells were heavily stained and others were stained in a distinct, granulated manner also at the submembrane domain. No nuclear staining was observed. Isotype control staining was negative (data not shown).

Next, we looked at the cellular distribution of the MO2-positive cells in terms of the various subpopulations of peripheral blood lymphocytes. Thus, PBMCs were fixed and permeabilized and then double-stained for the MO2 antigen and for several other markers such as CD3, CD8, CD4 and CD19. These analyses are illustrated in Figs. 3 and 4. It is evident from the results shown in Fig. 3 that there are two main populations of lymphocytes that express the MO2 antigen: a proportion of CD3-positive cells and a proportion of CD3-negative cells. An extensive analysis demonstrated that there were no CD19- (Fig. 3), CD20- or CD22-positive (data not shown) B-cells which expressed this antigen. The main population of the CD3-negative cells was found to be composed of CD16- or CD56-positive lymphocytes (not shown) probably belonging to the NK cell lineage. The CD3-positive population was demonstrated to be composed mainly of CD8 (brightly positive for CD8)-positive T-cells (Fig. 4). Only a minority of the CD4-positive T-cells was found to be MO2-positive (Fig. 4).

The possibility that the intracellular MO2 antigen detected in lymphocytes may be CD14 implies that soluble CD14, from plasma, may be passively or actively uptaken by these cells. If so, one would assume that the higher the concentration of soluble CD14 in plasma, the higher its chances to be uptaken by lymphocytes.

In order to test this hypothesis in a clinical setting, we set out to investigate the rate of expression of MO2 by lymphocytes obtained from various patients suffering from inflammatory processes, bacterial sepsis, autoimmune diseases and also HIV-positive patients, as it is known that there is a substantial elevation of soluble CD14 in these patients [18,19]. For that purpose, the expression of MO2 in CD3-, CD4- and CD8-positive lymphocytes in a group of asymptomatic and untreated HIV-positive individuals, as compared with a matched group of healthy controls, was assessed. The results are shown in Table 2. As evident from the table, the percent of lymphocytes expressing MO2 was considerably higher in HIV patients. This was evident in particular in the CD8 population, although even the rate of expression by CD4 cells was enhanced in these patients.

4. Discussion

We describe in this paper a novel lymphocyte subpopulation in human peripheral blood. This subpopulation is characterized by the presence of an antigen, detected with the MO2 mAb [13] and to a lesser extent with the UCHM1 mAb, which can be visualized following fixation and permeabilization of the cells. Upon fluorescence microscopy analysis, the binding was observed to occur at the periphery of the permeabilized cells, close to the membrane region. Further attempts to accurately localize the antigen are currently in process. The fluorescence obtained upon binding of the MO2 mAb is not dependent on the
fluorescent probe (FITC or RD1) or on the detergent used for permeabilization (saponin or Tween20), although saponin seems to preserve better the integrity of this antigen, as reported recently for other lymphocyte antigens as well [14,15]. Neither is it depending on the methodology used for cell separation, as lymphocytes separated by density gradient or directly stained in whole blood, gave the same results. In a separate group of experiments (not shown), we observed that within 2 h of blood drawing, when whole blood in heparin was left at room temperature, the MO2 signal dropped significantly, thus suggesting a degradation process.

The healthy individual-derived lymphocytes positive for the MO2 antigen, segregated into two groups: CD3-positive cells and CD3-negative cells. The CD3-negative ones are primarily CD56- and CD16-positive (not shown), thus probably belonging to the NK cell lineage. The CD3-positive ones segregate further into subgroups: the main population in terms of absolute number is CD8 cells. Very few CD4 cells were stained with this antibody. As shown, no B-cells (CD19, CD20 or CD22) could be detected as positive for this MO2 antigen.

Interestingly, a vast proportion of the gamma-delta T-cell receptor-positive cells, found in peripheral blood, were also found to be MO2-positive (data not shown). The MO2 antibody is a commercially available anti-CD14 antibody for in vitro diagnostic use. We performed a PCR analysis on highly purified CD8 cells for the CD14 transcript and a band of the appropriate size was obtained. But, since a small contamination with other cells, probably monocytes, could not be completely prevented, we cannot draw unequivocal conclusions from this experiment (not shown). Other known CD14 monoclonal antibodies, apart from UCHM1, which gave marginal results, were incapable of demonstrating any binding. We do not believe that CD14 is synthesized by lymphocytes and we have no reason to suppose that our results point to this direction. In addition, we only show, in this present communication, that a certain epitope of CD14 (the MO2 epitope) and not the entire molecule is internally expressed. Thus, our hypothesis is that if this MO2 antigen is indeed CD14, it must probably source from the plasma, bind to the lymphocytes possibly via a receptor differentially expressed by

![Fig. 3. MO2 is expressed by T-cells and by non-T-/non-B-cells: FACS analysis of gated lymphocytes. PBMCs were fixed, permeabilized and stained for MO2-RD1 (FL2, left panels) or IgM-RD1 (FL2, right panels) and CD3-PercP (FL3, upper panels) or CD19-PercP (FL3, lower panels). Note two populations of MO2-positive cells, a CD3-positive one and a CD3-negative one. Note no CD19-positive, MO2-positive cells, only CD19-negative, MO2-positive cells.](image)
various lymphocytes [16], and be rapidly internalized by the cells, as shown in vitro ([10,11]). As a partial and indirect support to this hypothesis, we show that HIV patients express a higher percentage of lymphocytes expressing the MO2 antigen. It has been established by others that monocyte and plasma CD14 are significantly elevated in HIV patients [18,19]. Thus, this elevation may be correlated to the substantial increase in MO2 expression observed in HIV patients by us, in this study. This as well as other directions are being evaluated in our laboratory.

Regardless, the final identification of the antigen in question will have to be elucidated in a different manner, probably by purification and sequencing.

To conclude, this communication describes lymphocytes in human peripheral blood that express internally MO2, a monoclonal epitope cross-reacting with human CD14. The T-cell subpopulation is composed mainly of CD8 cells and is greatly expanded in HIV patients. The final identification of this antigen and the possible function of the cells in which it is expressed are currently under investigation.

Table 2
MO2 antigen is overexpressed in HIV-positive individuals

<table>
<thead>
<tr>
<th>CD3+/MO2+</th>
<th>CD4+/MO2+</th>
<th>CD8+/MO2+</th>
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<tr>
<td>Healthy</td>
<td>10.1% (7.2)</td>
<td>0.4% (0.9)</td>
</tr>
<tr>
<td>HIV-positive</td>
<td>26.6% (14.4)</td>
<td>6.7% (6.1)</td>
</tr>
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</table>

PBMCs were obtained from 17 untreated and asymptomatic HIV-positive individuals (mean age 32 years ± 6) and 20 healthy individuals (mean age 27 ± 7), fixed, permeabilized and double-stained for MO2-positive CD3, CD4 and CD8 cells. Numbers represent the mean percent of positive cells in the various subpopulations. S.D. are shown in parenthesis. P < 0.02 (Student’s t-test) in all groups.

Fig. 4. MO2 is expressed mainly by CD8 cells and not by CD4 cells: FACS analysis of gated lymphocytes. PBMCs from one individual were fixed, permeabilized and stained for MO2-RD1 (FL2) and CD4-FITC (FL1) or MO2-RD1 (FL2) and CD8-FITC (FL1). Note that the majority of the MO2-positive cells belong to the CD8 lineage and not to the CD4 one. Lower panels are controls stained with IgM-RD1.

References


