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An Fcγ receptor I (CD64)-negative subpopulation of human peripheral blood monocytes is resistant to killing by antigen-activated CD4-positive cytotoxic T cells

It has been demonstrated that in monocyte/T cell co-cultures activated with recall antigens, cytotoxic T cells were generated which are able to reduce the number of antigen-presenting monocytes. In previous studies we could show that a minor subset of monocytes, the Fcγ receptor I-negative (CD64⁻) monocytes, exhibits significantly higher antigen-presenting capacity than the main population of monocytes (> 90 %) which are Fcγ receptor I-positive (CD64⁺). Therefore, we addressed the question whether they are also differentially susceptible to T cell-mediated killing. In the present study we demonstrate that the CD64⁻ monocyte subset is more resistant to killing by antigen-activated T cells than CD64⁺ monocytes, as indicated by a higher viability and recovery of CD64⁻ monocytes. This mechanism involves CD95 (Fas) antigen, since monocyte death in co-cultures with antigen-activated T cells could be partially reduced by blocking anti-Fas monoclonal antibodies (mAb). In agreement with this finding, although CD95 antigen was expressed on CD64⁺ and CD64⁻ monocytes at comparable levels, killing of CD64⁻ monocytes by activating anti-Fas mAb was lower than of CD64⁺ monocytes.

1 Introduction

The effective activation of T cells by triggering the T cell receptor and interaction with co-stimulatory molecules on antigen-presenting cells (APC) requires several hours [1]. Therefore, the generation of CD4⁺ cytotoxic T cells which are able to kill APC [2–5] is considered to represent an immunoregulatory mechanism. Moreover, APC resistant to killing by cytotoxic T cells may be responsible for an enhanced immune response [6, 7]. The mechanism of killing involves interaction of CD95 (Fas/APO-1) antigen and Fas ligand which leads to apoptosis [8, 9]. Recently, we found CD4⁺CD45RO⁺ cytotoxic T cells able to induce apoptosis of APC in cultures of peripheral blood lymphocytes activated by the recall antigens tetanus toxoid and the purified protein derivative of tuberculin (PPD) [10]. Since peripheral blood monocytes are heterogeneous in phenotype and in their capacity to function as APC [11], the question arises whether monocyte subpopulations are equally susceptible to T cell-mediated killing. In particular, monocytes lacking the Fcγ receptor I (CD64⁻), comprising less than 10 % of all monocytes, were found to be more effective as APC than the predominant population of CD64⁺ monocytes [12]. Thus, we investigated whether

CD64⁺ and CD64⁻ monocytes differ with respect to their survival in antigen-activated co-cultures with autologous T cells, expression of Fas antigen, and their susceptibility to Fas-mediated killing. The data presented show that CD64⁻ monocytes are more resistant to killing by PPD-activated T cells and to apoptosis induced by anti-Fas APO-1 mAb.

2 Materials and methods

2.1 Culture medium

All cell cultures were performed in RPMI 1640 medium supplemented with 100 U/ml penicillin G, 100 µg/ml streptomycin, 2 mmol/l L-glutamine and 10 % heat-inactivated FCS, all obtained from Biochrom (Berlin, Germany).

2.2 Cell preparations

Purified monocytes, lymphocytes, and subsets of monocytes were isolated from peripheral blood of normal healthy donors. For stimulation experiments with PPD, only blood from tuberculin skin test-positive donors was taken. Peripheral blood mononuclear cells (PBMC) were first isolated from heparinized whole blood by Ficoll/Isopaque (1.077 g/ml, Pharmacia, Freiburg, Germany) density gradient centrifugation (465 × g, 45 min, 22 °C). PBMC, harvested from the interface, were washed once in HBSS (Biochrom) (465 × g, 15 min, 4 °C) and once in HBSS through an FCS cushion (298 × g, 15 min, 4 °C) to remove cell fragments and residual platelets. Monocytes and lymphocytes were further separated by counterflow centrifugation using the JE-6B-elutriator system (Beckman Instruments Inc., Palo Alto, CA) as described [12]. The purity of the resulting fraction of pooled monocytes was approximately 90 ± 3 % and that of the pooled lymphocytes 99 ± 2 % as examined by α-naphthyl acetate esterase or immunofluorescence staining with anti-CD14-specific

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Abbreviations: CFSE: 5-(6)-Carboxyfluorescein succinimidylester 7-AAD: 7-Aminoactinomycin D MO: Monocytes

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mAb (MO-P9, IgG2b, Becton Dickinson, Heidelberg, Germany). For the isolation of CD64⁺ and CD64⁻ subsets from monocytes, the elutriation-derived, purified monocytes were directly labeled with FITC-conjugated anti-CD64 mAb (clone 22, IgG1, Medarex Inc., Annandale, NJ) which recognizes an epitope of the Fc γ receptor I on human monocytes outside the Fc binding region [13] and, therefore, does not block the Fc receptor function. Briefly, monocytes were incubated with FITC-conjugated anti-CD64 mAb for 15–30 min on ice in PBS (Merck, Darmstadt, Germany), washed once in ice-cold PBS through an FCS cushion, and finally resuspended in ice-cold PBS. Then CD64⁺ and CD64⁻ monocytes were separated by cell sorting using cytofluorograf 50H cell sorter system (Ortho Diagnostics Systems, Westwood, MA). Monocytes stained with FITC-conjugated control IgG1 (Dako, Hamburg, Germany) were used as negative control to define sorting criteria. Cell fragments could be gated out according to their reduced forward and side scatter signals compared to intact cells. The subsequent flow cytometric reanalysis revealed a purity of $98 \pm 1\%$ for the CD64⁺ sorted monocytes and of $76 \pm 5\%$ for the CD64⁻ sorted subset. In each experiment unstained elutriated monocytes were further depleted of residual lymphocytes by cell sorting using the light scatter signals as sort criteria, since both the forward scatter and side scatter signals are stronger in monocytes than in lymphocytes. These sorted monocytes termed “unseparated monocytes” were $95 \pm 2\%$ pure, as judged by α -naphthyl acetate esterase staining. In most of the experiments elutriated lymphocytes were further depleted of B cells, HLA-DR⁺ cells, and NK cells by magnetic cell sorting (MACS) as described by Abts et al. [14] and Miltenyi et al. [15]. Briefly, lymphocytes (10^7 cells/ml) kept on ice were stained in three consecutive incubation steps of 15 min each: first with anti-CD20 (B-Ly1, IgG1, Dako, Hamburg, Germany), anti-HLA-DR (B8.12.2., IgG2b, Dianova, Hamburg, Germany), and anti-CD16 (3G8, IgG1, Dianova) fluorochrome-unconjugated mAb (50 – $100\ \mu\text{l}$ mAb/ 10×10^7 cells), then with biotinylated goat anti-mouse IgG (Dianova) diluted 1:100, and finally with streptavidin-conjugated magnetic microbeads (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). Each incubation step was followed by washing the cells through an FCS cushion. The cells were then adjusted to 20×10^6 cells/ml with medium containing 1% BSA followed by magnetic cell separation through an appropriate column (Miltenyi Biotech equilibrated as described by the manufacturer. The efficiency of depletion was proven by fluorescence analysis of the “non-magnetic” fraction of cells stained with PE-conjugated anti-CD3, anti-CD19, anti-CD16, and anti-HLA-DR mAb. Subsequent investigation by flow cytometry revealed an enrichment from $69 \pm 6\%$ to $92 \pm 3\%$ CD3⁺ T cells and contamination with CD19⁺, CD16⁺ or HLA-DR⁺ cells did not exceed $2 \pm 2\%$.

2.3 Cell culture and stimulation with PPD

Unseparated, CD64⁺, or CD64⁻ monocytes (2.5×10^4) were co-cultured with purified T cells (2×10^5) in Falcon 2057 polystyrene tubes in $250\ \mu\text{l}$ of culture medium in the presence or absence of PPD (Statens Serum Institut, Copenhagen, Denmark) at a final concentration of $10\ \mu\text{g/ml}$. For determination of IFN- γ , culture supernatants were harvested after 3 days of culture.

2.4 Labeling of cells with 5-(6)-carboxyfluorescein succinimidylester (CFSE)

To differentiate between monocytes and lymphocytes during flow cytometric analysis, two main criteria were used. First, lymphocytes were discriminated from monocytes due to their smaller forward and side scatter signals. Second, in the majority of experiments monocytes or lymphocytes were labeled before co-culture with CFSE [16, 17]. This fluorochrome passes the cell membrane and binds to cytoplasmatic proteins. Freshly isolated monocytes or lymphocytes ($1 \times 10^7/\text{ml}$) were incubated with CFSE at a final concentration of $5\ \mu\text{M}$ for 15 min in PBS at 37°C and 5% CO₂, washed twice in ice-cold PBS through an FCS-cushion, and resuspended in culture medium. These cells were then co-cultured with unstained monocytes or lymphocytes for 72 h in the presence or absence of stimuli. Cells stained in this way had higher fluorescence intensity than anti-CD64-FITC-stained CD64⁺ cells. Moreover, the intensity of CFSE-stained cells decreased only slightly during the culture period of 72 h.

2.5 Evaluation of cell viability

To detect dead or apoptotic cells in cell cultures, 7-aminoactinomycin D (7-AAD) (Sigma, Deisenhofen, Germany) was used as described elsewhere [10]. Briefly, after harvesting of culture supernatants and prior to flow cytometry cells were incubated on ice with $20\ \mu\text{g/ml}$ of 7-AAD and the cells were analyzed 15 min after labeling. We have shown previously that, when applying this technique to monocytes co-cultured with T cells, staining is predominantly found in apoptotic cells as verified by the terminal deoxynucleotidyl transferase assay [10]. The use of this dye in combination with CFSE-staining enabled us to discriminate apoptotic cells (red fluorescence) from non-apoptotic cells (no red fluorescence) within CFSE-stained (green fluorescence) or CFSE-unstained lymphocytes or monocytes. In addition, due to different light emission spectra of 7-AAD and PE [18] 7-AAD was used in combination with PE-conjugated mAb. In these cases the cells were first labeled with PE-conjugated mAb and then incubated with 7-AAD for 15 min before flow cytometric analysis.

2.6 Determination of the effect of blocking and activating anti-CD95 mAb on monocyte killing

For Fas blocking experiments, unseparated PBMC ($2 \times 10^5/200\ \mu\text{l}$) were cultured in the presence or absence of PPD for 72 h. Fifteen minutes prior to stimulation with PPD $1\ \mu\text{g/ml}$ of blocking IgG1 anti-Fas mAb ZB4 [19] was added to the cultures. As a negative control $1\ \mu\text{g/ml}$ of an IgG1 isotype control mAb was added to parallel cultures. For the induction of Fas (CD95)-induced apoptosis, unseparated monocytes ($1 \times 10^6/500\ \mu\text{l}$) and isolated CD64⁺ and CD64⁻ monocyte subsets were cultured alone for 12 h in the presence or absence of 2.5 – $10\ \mu\text{g/ml}$ of activating IgG3 anti-CD95 mAb APO-1 [20] purified on protein G-agarose from the supernatant of SB6-2B1 cells. As a negative control, cells in parallel cultures were incubated with anti-Ki-67 (kindly provided by Prof. J. Gerdes, Forschungszentrum Borstel, Germany), an IgG3 mAb specific for an intracellular proliferation-associated nuclear anti-

gen. In both kinds of experiments monocyte viability was determined by staining with 7-AAD at the end of the culture period and subsequent flow cytometry, as described in Sect. 2.5.

2.7 Anti-CD95 staining of cells by immunofluorescence labeling

For the determination of CD95 expression cells were labeled with three different anti-Fas mAb: PE-conjugated anti-CD95 mAb (DX2, IgG1, PharMingen, Hamburg, Germany) at a dilution of 1:10, or the unconjugated mAb SB6-2B (IgG3), or ZB4 (IgG1, Immunotech, Hamburg, Germany) at a final concentration of 10 µg/ml in PBS for 15 min on ice. As negative controls, cells were incubated with PE-conjugated and unconjugated IgG1 control mAb (Dako, Hamburg, Germany) or unconjugated IgG3 anti-Ki-67 mAb. Then the cells were washed once in ice-cold PBS through an FCS-cushion (298 × g, 10 min, 4°C) and resuspended in PBS. Cells stained with unconjugated mAb were incubated with PE-conjugated goat F(ab')₂ anti-mouse IgG_{Fc} in a second step and washed as described before. Finally, cells were fixed with 1.5% paraformaldehyde-PBS solution and kept in the dark at 4°C until flow cytometric analysis.

2.8 Flow cytometric analysis

The analysis of immunofluorescence-stained cells was always performed with the FACStar Plus (Becton Dickinson, Heidelberg, Germany) equipped with an argon laser.

2.9 Measurement of IFN-γ in culture supernatants

The concentrations of IFN-γ in supernatants were determined using a quantitative ELISA, kindly provided by Dr.

H. Gallati (Hoffmann-LaRoche, Basel, Switzerland). The assay was carried out as recommended by the manufacturer (Hoffmann-LaRoche) and as described by Gallati [21].

3 Results

3.1 CD64⁺ monocytes survive better in PPD-stimulated cultures than unseparated or CD64⁺ monocytes

To compare the survival of CD64⁺ and CD64[−] monocytes in PPD-stimulated cultures, cell viability was evaluated by exposure to 7-AAD as described in Sect. 2.5. To distinguish between monocytes and T cells, one population was labeled with CFSE before the co-cultures were set up. Such treatment had no apparent influence on monocyte APC function, nor on T cell response. As shown in Table 1, the PPD-induced IFN-γ release in cultures of CFSE-stained monocytes or T cells was comparable to that of parallel cultures of CFSE-unstained cells. Flow cytometric analysis of these monocyte/T cell co-cultures revealed a clear distinction of four cell populations which differ in green (CFSE) versus red (DNA staining by 7-AAD) fluorescence (Fig. 1). Such analysis enabled the calculation of apoptotic (7-AAD⁺) cells within monocytes. As shown in Table 2, in cultures to which unseparated or CD64⁺ monocytes had been added the proportion of 7-AAD⁺ monocytes increased after PPD stimulation [ratio PPD/none (unseparated) = 1.68 ± 0.33 ; PPD/none (CD64⁺) = 2.98 ± 1.83]. In contrast, in cultures with CD64[−] monocytes the ratio (PPD/none) did not change (1.05 ± 0.19). Thus the increase of monocyte apoptosis in activated cultures was higher in unseparated monocytes and their CD64⁺ subset than in CD64[−] monocytes. In addition, the monocyte recovery presented as percentage of CFSE-stained monocytes (Fig. 1, number in parenthesis) within total cells collected was usually lower in PPD-activated

Figure 1. Flow cytometric analysis of monocyte viability and recovery in monocyte/T cell co-cultures. T cells (2×10^5) were co-cultured with monocyte subsets (2.5×10^4) in 250 µl of culture medium for 72 h. Dot plots of unstimulated control cultures (upper) and PPD-activated cultures (bottom) are shown. Monocytes were labeled before culture with CFSE (x-axis, green fluorescence), and prior to flow cytometric analysis cultures were preincubated with 7-AAD (red fluorescence, y-axis) which enabled calculation of the monocyte viability (% red cells within population of green cells, upper numbers in each dot plot). The calculated proportion of monocytes in culture at time 0 h was 11.1%, the percentage of monocytes within the monocyte/T cell co-cultures is given in parenthesis (calculated as % of green cells in the whole population).

Table 1. The influence of CFSE labeling of monocytes or T cells on the PPD-induced IFN- γ release by T cells co-cultured with different monocyte subsets^{a)}

Experiment	Co-cultured cells ^{b)} (treatment) ^{c)}	IFN- γ (pg/ml) PPD
1	Unseparated MO + T	6043 $\pm$ 213
	Unseparated MO + T (CFSE)	5119 $\pm$ 221
2	Unseparated MO + T	5280 $\pm$ 11
	Unseparated MO (CFSE) + T	6574 $\pm$ 428
	CD64 ⁺ MO + T	2178 $\pm$ 224
	CD64 ⁺ MO (CFSE) + T	1745 $\pm$ 92
	CD64 ⁻ MO + T	11783 $\pm$ 317
	CD64 ⁻ MO (CFSE) + T	12830 $\pm$ 511

a) Data shown are the mean values $\pm$ SD of independent measurements of at least two dilutions performed from the same culture supernatant. The IFN- γ release by unstimulated cells was always less than the detection limit (< 78 pg/ml).

b) Unseparated monocytes (MO) as well as their CD64⁺ and CD64⁻ subsets (2.5×10^4) were co-cultured with a constant number of T cells (2×10^5) in 250 μ l medium in the presence of 10 μ g/ml PPD. IFN- γ was measured in supernatants of 3 days cultures by ELISA.

c) Monocytes or T cells were preincubated with the fluorochrome CFSE (20 μ g/ml) before co-culture.

Table 2. Comparison of unseparated monocytes and CD64⁺ and CD64⁻ subsets in their susceptibility to cell death in PPD-activated co-cultures with T cells^{a)}

Experiment	Monocyte subset ^{c)}	Cell death (% 7-AAD ⁺ monocytes) ^{b)}		
		Medium	PPD	Ratio (PPD/none)
1	Unseparated	8,2	13,3	1,6
	CD64 ⁺	31,9	56,5	1,8
	CD64 ⁻	32,9	30,6	0,9
2	Unseparated	12,9	26,6	2,1
	CD64 ⁺	7,7	43,6	5,7
	CD64 ⁻	31,2	35,4	1,1
3	Unseparated	25,0	42,5	1,7
	CD64 ⁺	8,8	20,9	2,4
	CD64 ⁻	19,0	25,2	1,3
4	Unseparated	22,7	30,2	1,3
	CD64 ⁺	19,9	39,3	2,0
	CD64 ⁻	45,0	41,9	0,9

a) Unseparated monocytes or their CD64⁺ and CD64⁻ subsets (2.5×10^4 cells) were co-cultured with T cells (2×10^5) in 250 μ l medium in the presence or absence of 10 μ g/ml PPD for 3 days.

b) 7-AAD staining and flow cytometric analysis were performed as described in Sects. 2.5 and 2.8.

c) In experiments 1–3, monocytes and in Experiment 4, T cells were labeled with CFSE prior to co-culture with unstained monocytes or T cells.

compared to unstimulated samples. As depicted in Fig. 1, the reduction of the proportion of monocytes was more apparent in cultures to which CD64⁺ monocytes (reduction from 9.4% to 4.6%) or unseparated (not shown) were added than in cultures containing CD64⁻ monocytes (reduction from 9.7% to 8.5%). Similar results were obtained in PPD-activated co-cultures of elutriated monocytes and T cells labeled after 72 h of culture with a cocktail of mAb FITC-conjugated CD64, PE-conjugated anti-CD3 and -CD2) instead of CFSE to differentiate between monocyte subsets and T cells. When analyzed after staining with 7-AAD, the percentage of apoptotic cells was higher in the population of CD64⁺/CD3⁺/CD2⁻ than in CD64⁻/CD3⁺/CD2⁻ cells. Thus, the viability of CD64⁻ monocytes was in this case also found to be higher as compared to CD64⁺ monocytes (not shown). Thereby we could exclude

that the separation procedure *per se* caused the observed differences in monocyte apoptosis. Since anti-CD64 labeling was performed after culture, we could also rule out that antibody binding on CD64⁺ monocytes itself was responsible for their higher susceptibility to T cell-mediated apoptosis compared to CD64⁻ monocytes.

3.2 Blocking of Fas antigen partially reduces killing of monocytes in co-cultures with PPD-activated T cells

Since it is known that the interaction of CD95 (Fas) antigen and Fas ligand are involved in the mechanism of killing leading to apoptosis [8, 9], we tested the effect of blocking anti-Fas mAb in unseparated unstimulated or PPD-activated PBMC. Therefore, cells were cultured in the presence or absence of blocking anti-Fas mAb ZB4 [19] for 72 h and killing was again determined by 7-AAD labeling. In order to identify all monocytes (including CD64⁻) within the whole population, cells were stained with a cocktail of mAb (FITC-conjugated anti-HLA-DR, PE-conjugated anti-CD3, -CD19 and -CD56) and only the HLA-DR⁺/CD3⁺/CD19⁻/CD56⁻ population was analyzed. Similar to reconstituted monocyte/T cell co-cultures (Sect. 3.1), the number of 7-AAD⁺ monocytes was enhanced in PPD-activated compared to unstimulated cultures (Table 3). In both experiments shown, the addition of anti-Fas mAb ZB4 reduced the number of 7-AAD⁺ monocytes in the PPD-stimulated cultures when compared to parallel cultures containing no mAb or an isotype control mAb in the same concentration. In experiment 1 the spontaneous killing was also reduced. In some donors who were found to be high responders to PPD (> 10 ng/ml IFN- γ) monocyte death was unaffected by the addition of mAb ZB4 in the same concentration (data not shown).

3.3 Expression of Fas antigen on CD64⁺ and CD64⁻ monocytes

Having found that monocyte killing by PPD-activated T cells can be reduced by blocking anti-Fas mAb, the differential susceptibility of monocyte subsets could be related to differential expression of CD95 (Fas) on CD64⁺ and CD64⁻ monocytes. Therefore we measured the expression

Table 3. The inhibition of monocyte death by blocking anti-CD95 mAb ZB4 in PPD-activated cultures of PBMC^{a)}

Experiment	mAb	Cell death (% 7-AAD ⁺ monocytes) ^{b)}	
		Medium	PPD
1	–	63	77
	IgG1	57	72
	ZB4	45	57
2	–	51	75
	IgG1	50	75
	ZB4	53	67

a) PBMC (2×10^5 cells) were cultured in 200 μ l medium in the presence or absence of 10 μ g/ml PPD and 1 μ g/ml mAb for 3 days.

b) 7-AAD staining and flow cytometric analysis were performed as described in Sects. 2.5 and 2.8.

of Fas antigen on freshly isolated CD64[–] and CD64⁺ monocytes and after their co-culture with T cells in the presence of PPD. As shown in Table 4 for staining with anti-Fas mAb clone DX2, freshly isolated (0 h) as well as cultured (16 h and 72 h) unseparated and CD64[–] and CD64⁺ monocytes contained comparable proportions of CD95⁺ monocytes. While in PPD-activated monocyte/T cell co-cultures the proportion of CD95-expression remained unchanged during a 72-h stimulation period, the antigen density per cell (given by mean fluorescence intensity; number in parenthesis) increased and was enhanced compared to unstimulated cultures in all monocyte populations to a similar degree. In addition T cells of these cultures responded by increased expression of CD95 during the incubation times of 16 h and 72 h and due to stimulation with PPD. In T cells, both the proportion of CD95⁺ cells and antigen density per cell increased, irrespective of the monocyte subset (CD64⁺ and CD64[–]) which was present in the culture (Table 4). In addition, immunofluorescence labeling with anti-CD95 mAb clone SB6-2B used for the induction of apoptosis (Sect. 3.4) revealed a comparable staining pattern of T cells as well as on freshly isolated

monocytes and their subsets (data not shown). Labeling with anti-Fas blocking mAb ZB4 also stained similar numbers of freshly isolated monocytes and T cells (not shown).

3.4 Anti-Fas antibodies are not cytotoxic for CD64[–] monocytes

Since CD64⁺ and CD64[–] monocytes show similar CD95 (Fas antigen) expression (Sect. 3.3), we investigated whether CD64⁺ and CD64[–] monocytes differentially respond to anti-Fas antibodies which are able to trigger apoptosis [8, 20, 22–24]. Therefore, first freshly isolated elutriated monocytes were incubated for 12 h with IgG3 anti-CD95 mAb clone SB6-2B. After incubation cells were counted and labeled with FITC-conjugated anti-CD64 mAb and a cocktail of mAb (CD3, CD2, CD19, CD56, all PE-conjugated) to exclude any cells which could contaminate the monocyte preparation during flow cytometric analysis. After exposure to 7-AAD, the multiple staining allowed gating and analysis of the viability (percentage of 7-AAD⁺) of CD64⁺ (FITC-green) and CD64[–] (not green, not PE-red) cells. As shown in Table 5, the CD64⁺ monocytes were more sensitive to anti-Fas mAb-mediated apoptosis which was indicated by the threefold higher percentage of 7-AAD⁺ cells in CD64⁺ (47%) than in CD64[–] monocytes (14.1%). In a separate experiment the anti-Fas mAb was added to sorted unseparated monocytes and to isolated CD64⁺ and CD64[–] subsets (Fig. 2). In this case CD64[–] monocytes were also resistant to anti-Fas mAb-mediated killing (12.8% 7-AAD⁺ compared to 7.9% in control cultures), whereas in unseparated and CD64⁺ monocytes the proportion of 7-AAD⁺ cells was markedly enhanced (40.3% in unseparated and 44.8% in CD64⁺). Since both kinds of experiments revealed comparable results and, moreover, Fas-induced killing of CD64⁺ sorted and unseparated unstained monocytes (containing > 90% CD64⁺ cells) was identical, an influence of the isolation procedure or antibody binding on CD64⁺ monocytes *per se* can be excluded.

Table 4. Kinetics of CD95 (FAS antigen) expression on monocytes and T cells in PPD-stimulated co-cultures^{a)}

Time period of co-culture ^{b)}	PPD	0 h ^{d)}		CD95 ⁺ cells (%) ^{b)}		72 h	
		MO	T	MO	T	MO	T
Monocyte subset							
Unseparated	–	95 (56)	–	88 (53) ^{e)}	21 (25)	67 (89)	49 (36)
CD64 ⁺	–	96 (47)	–	92 (45)	23 (28)	60 (113)	54 (34)
CD64 [–]	–	82 (47)	–	84 (30)	23 (22)	67 (136)	92 (48)
Unseparated	+	–	–	87 (69)	40 (22)	84 (309)	97 (90)
CD64 ⁺	+	–	–	88 (74)	49 (24)	85 (296)	97 (80)
CD64 [–]	+	–	–	78 (57)	48 (27)	82 (371)	97 (79)
T cells alone	–	–	30 (29)	–	19 (28)	–	37 (34)
	+	–	–	–	23 (20)	–	33 (31)

a) Data shown are from one out of three experiments performed (–: not done).

b) Cells were immunofluorescence labeled with anti-CD95 mAb clone DX2.

c) Unseparated monocytes or their CD64⁺ and CD64[–] subsets (2.5×10^4) were co-cultured with 2×10^5 T cells in 250 μ l medium in the presence or absence of 10 μ g/ml PPD.

d) Freshly isolated cells were labeled without further cultivation.

e) Mean fluorescence intensity (channel number of logarithmic scale).

Table 5. Comparison of the cytotoxic activity of anti-CD95 (APO-1) mAb on CD64⁺ and CD64⁺ subsets determined with the whole monocyte population by differential staining with mAb

Monocyte subset ^{a)}	Cell death (% 7-AAD ⁺ cells)	
	– Anti-Fas mAb	+ anti-Fas mAb
Total monocytes	6,6 ± 0,6	44,3 ± 9,2
CD64 ⁺	6,1 ± 2,2	47,0 ± 8,8
CD64 [–]	13,8 ± 1,2	14,1 ± 2,7

a) Elutriation-derived monocytes ($1 \times 10^6/500 \mu\text{l}$ medium) were cultured for 12 h in the absence or presence of $10 \mu\text{g/ml}$ of APO-1 mAb (clone SB6-2B). Thereafter, cells were labeled with PE-conjugated (red) anti-CD3, -CD2, -CD19, -CD56 and FITC-conjugated (green) anti-CD64 mAb to distinguish between different monocyte subsets within the whole cell population. Subsequently, after preincubation with 7-AAD cell death was measured by flow cytometric analysis. During analysis unstained cells were considered as total monocytes, cells stained only green as CD64⁺, and cells stained neither red nor green as CD64[–] monocytes. Data are the mean values $\pm$ SD from three independent experiments.

4 Discussion

In cultures activated with recall antigens monocytes are eliminated by CD4⁺CD45RO⁺ T lymphocytes which induce apoptosis of APC [10]. Since CD64⁺ and CD64[–] monocyte subpopulations differ in their capacity to present PPD to autologous T cells, as shown previously [12], we investigated in this study whether CD64⁺ and CD64[–] monocyte subpopulations are equally susceptible to T cell-mediated killing. It has been demonstrated that CD64[–] monocytes are less efficiently killed by PPD-activated T cells than the CD64⁺ subset. This is based on data revealing that the viability and recovery of CD64[–] monocytes were comparable in control and PPD-activated cultures. In contrast, in case of unseparated or CD64⁺ monocytes, the percentage of apoptotic cells was higher in PPD-stimulated than in control cultures. It is generally accepted that CD4⁺ cytotoxic T cells eliminate APC by inducing

their apoptosis, probably due to interaction between CD95 (Fas) antigen and its ligand [7–9]. This also seems to be involved in our studies, since mAb blocking CD95 (Fas) partially reduced the killing of monocytes within PPD-activated PBMC of some skin test-positive donors. The question why killing was not completely inhibited and why CD95-blocking mAb was ineffective in PPD high responders is still unresolved. It might indicate that other killing principles are involved. Apoptosis induced by TNF- α appears to be very unlikely in the case of human monocytes, since it has been shown recently that TNF- α prevents spontaneously occurring apoptosis in human monocyte cultures [25]. The perforin/granzyme pathway is the major killing mechanism of cytotoxic CD8⁺ T cells. This pathway, however, seems to be of minor significance in cytotoxic CD4⁺ T cells [7] which are the major mediators of monocyte killing in PPD-activated co-cultures [10]. In our study we did not investigate the possible contribution of the perforin/granzyme pathway, but we focussed our interest on the Fas-mediated killing by CD4⁺ T cells. The observed differences in the survival of monocyte subpopulations could be due to variable inducibility of cytotoxic T cells in the presence of monocyte subsets, different expression of CD95 antigen on CD64⁺ and CD64[–] monocytes, or functional differences in pathways leading to cell apoptosis in monocyte subsets. Our data cannot be explained by a differential induction of cytotoxic T cells by monocyte subsets, since CD64[–] monocytes were also less efficiently eliminated in PPD-stimulated cultures of T cells and unseparated monocytes, where CD64⁺ monocytes are in excess (more than 90 %) and could induce T cell cytotoxicity. In agreement with data reported by others [22, 23], in our hands almost all freshly isolated monocytes expressed CD95 (Fas antigen). This expression increased dramatically after culture (as judged from increase of fluorescence intensity), and CD64⁺ and CD64[–] behaved comparably. Similarly, the expression of Fas antigen increased on co-cultured T lymphocytes, irrespective of the monocyte subset present. Although we observed no substantial difference in the expression of CD95 antigen on CD64⁺ and CD64[–] monocyte subsets, the CD64[–] monocytes were

Figure 2. Comparison of anti-CD95 (APO-1) mAb-induced killing of monocyte subsets. Unseparated monocytes (left), CD64[–] (middle) or CD64⁺ (right) monocytes were incubated with $10 \mu\text{g/ml}$ of anti-CD95 (APO-1) mAb clone SB6-2B for 12 h, and after preincubation with 7-AAD flow cytometric analysis was performed. Histograms of viability measurements (7-AAD-positive cells) in control cultures treated with $10 \mu\text{g/ml}$ of an isotype-matched IgG3 Ki-67 mAb (upper row) and in the presence of APO-1 mAb (bottom) are shown.

resistant to killing by IgG3 anti-CD95 mAb clone SB6-2B, suggesting that intracellular pathways leading to cell apoptosis may be different in CD64⁺ and CD64⁻ cell populations. The question of possible differences in intracellular pathways of CD64⁺ and CD64⁻ monocytes was not addressed directly in this study. Since cysteine proteases are supposed to be involved in apoptosis [26, 27], we tested IL-1 β converting enzyme (ICE) as one of them. It is of interest that lysates derived from CD64⁻ monocytes are inactive when tested for the ability to cleave the IL-1 β precursor, indicating that this monocyte subset may lack or contain less active ICE/CED-3 proteases (Loppnow et al., unpublished). Although the presence of ICE *per se* is not essential for cell apoptosis to occur [28–30], the finding suggests that the composition of cysteine proteases may differ in CD64⁺ and CD64⁻ monocyte subsets. We have shown that CD64⁻ monocytes may serve as potent APC, a finding which corresponds to high expression of MHC class II antigens [12] and of CD86 co-stimulatory molecule on these cells (E. Grage-Griebenow, in preparation). The data presented above indicate that the more efficient activation of T cells in the presence of CD64⁻ compared to CD64⁺ monocytes may also be associated with their higher resistance to cytotoxic T cells, as reported for antigen-presenting cell lines resistant to killing by activated T cells [6]. CD64⁻ monocytes share many features of peripheral blood dendritic cells (DC) [31–34], as they are poorly phagocytic but very potent APC, have low expression of CD14 antigen, high expression of HLA-DR antigen [12], a phenotype similar to that of the CD14⁺/CD16⁺ monocyte subset, as described by Ziegler-Heitbrock [35], and CD64⁻ monocytes express high levels of CD86 co-stimulatory molecule (E. Grage-Griebenow, in preparation). In addition, CD64⁻ monocytes have high antiviral activity [12, 36], as described also for DC [32, 34]. Interestingly, reticular DC which are considered as very efficient APC are also not lysed by CD4⁺ cytotoxic cells (cited after Hahn et al. [7]). In this line our data point to the possibility that the resistance to killing by activated T cells may be a common feature of efficient APC. This could have important biological significance, such as providing efficient (high expression of class II MHC and of co-stimulatory molecules) and longer lasting (resistance to Fas-mediated killing) presentation of peptides by non-phagocytic APC (DC, CD64⁻ monocytes). Similar to the expression of MHC class II antigens and co-stimulatory molecules, resistance to killing by antigen-triggered CD4⁺ T cells is probably of utmost importance during induction of primary immune responses. In contrast, apoptosis of monocytes induced by phagocytosis of bacteria [37] or triggered by the contact with activated T cells ([10], CD64⁺ monocytes, this report) may be advantageous for the host to avoid an unnecessary immune response and to limit an infection by intracellular parasites [7]. Taken together, these data may also indicate different immunoregulatory tasks for CD64⁻ and CD64⁺ monocytes, in the sense that the CD64⁻ subset is more privileged for efficient and long lasting presentation of antigen to T cells, whereas the CD64⁺ subset is important for the removal of parasites by ingestion and cooperation with inflammatory (Th1) T cells.

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