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Monocyte Differentiation and Accessory Function: Different Effects on the Proliferative Responses of an Autoreactive T Cell Clone as Compared to Alloreactive or Antigen-Specific T Cell Lines and Primary Mixed Lymphocyte Cultures

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Abstract

An autoreactive T cell clone derived from a patient with reactive arthritis, two alloreactive T cell lines, two antigen-specific T cell lines and allogeneic resting T cells were analyzed for their responses to monocytes and macrophages derived from monocytes by *in vitro* differentiation.The autoreactive T cell clone strongly proliferated in response to fresh monocytes and to macrophages derived from a 7 day culture, but only poorly to monocytes cultured for 2 days. In contrast, alloreactive and antigen-specific T cell lines proliferated to all stimulator cells equally well. Finally, primary mixed lymphocyte reactions could be stimulated by both fresh and 2-day cultured monocytes, but not by *in vitro* derived macrophages. The impaired response of the autoreactive T cell clone to 2-day cultured monocytes could not be attributed to reduced expression of several well-defined surface molecules nor to induction of nonresponsiveness. Neither allogeneic monocytes nor cytokines (IL-1, IL-2, IL-4, IL-6) could correct the defective response of the autoreactive T cell clone. However, preculture of monocytes in the presence of interferon-gamma, IL-1, IL-4 or IL-6 retained their stimulatory capacity. Our interpretation of the selectively impaired response of the autoreactive T cell clone is that it most likely recognizes a differentiation-dependent monocyte/macrophage-specific peptide.

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Introduction

T cell activation requires a complex interplay of antigen-specific T cell-accessory cell interactions via TCR/CD3-MHC/peptide complex and antigen-independent interactions via adhesion molecules, costimulatory ligands and cytokines (1). Monocytes and dendritic cells are considered to be professional accessory cells capable of stimulating both primary and secondary T cell responses (2). On the other hand, the dominant function of tissue macrophages is phagocytosis and cytotoxicity, while accessory functions are strongly reduced (3–5). Monocyte differentiation to macrophage- or dendritic-like phenotypes can be induced by *in vitro* culture in the presence of serum (6–9). Similar to tissue macrophages, *in vitro* matured macrophages show impaired accessory functions in mitogen-induced T cell responses (9, 10) and mixed lymphocyte reactions (6). The present study was performed to test the ability of *in vitro* differentiated monocytes to stimulate different secondary T cell responses. It turned out that antigen-specific and alloreactive T cell lines could be equally restimulated by different monocyte differentiation stages, while an autoreactive T cell clone was selectively unable to recognize monocytes cultures for 2 days.

Materials and Methods

Blood donors

Mononuclear cells (MNC) and T cell lines were derived from donor UA (HLA A2,32; B27, 51; DR11.1, 11.3) (11) and donor MS (HLA A1; B8, w60; DR3, 11.1). Additional HLA-taped MNC and EBV-transformed B cell lines were kindly provided by Dr. ARLETTE URLACHER (Blood Bank, Strasbourg, France).

Establishment of T cell lines

The origin and specificity of T cell lines used in this study is summarized in Table 1. The autorective T cell clone UA-S2 was derived from IL-2-supplemented limiting dilution cultures of synovial fluid lymphocytes of patient UA suffering from reactive arthritis (11). UA-S2 recognizes a so far unknown peptide in association with HLA-DR 11.3 (11, 12). The expressed TCR α and β chains of UA-S2 have been sequenced (13). The alloreactive T cell clone MS-UA-B12 was derived by stimulation of MNC from donor MS with irradiated MNC from donor UA under limiting dilution conditions. It recognizes HLA-DR 11.3-typed and some DR 11.4-typed MNC or B cell lines (not shown). The alloreactive cell line PLT–>UA was produced by a primary stimulation of lymphocytes of donor NN (not HLA-typed) with irradiated MNC from donor UA in bulk culture and shows a proliferative response to all DR 11 positive MNC or B cell lines (not shown). Similarly, cell line PLT–>MS was established using stimulator cells from donor MS. Antigen-specific T cell clones MS-PPD-B5 and MS-PEP-N1 were derived from primary cultures of MNC from donor MS stimulated with PPD (25 μg/ml, Behring, Marburg, Germany) or pork pepsin (1 μg/ml, Sigma, München, Germany) and subsequent limiting dilution cloning and subcloning (14). The phenotype of all T cell lines was $TcR\alpha\beta^+CD3^+CD4^+CD8^-CD29^{high}$.

T cell lines were maintained in medium RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), glutamine (2 mM), 10 % FCS (all reagents from Biochrom, Berlin, Germany), 2.5 % human AB serum and highly purified human natural IL-2 (20 U/ml, Biotest, Dreieich, Germany). For primary cultures 10 % autologous serum was used instead of FCS. Every two weeks, the T cell lines were restimulated with irradiated (30 Gy) allogeneic MNC and monoclonal anti-CD3 antibody BMA030 (10 ng/ml, Behring). Mycoplasma contaminations were excluded by periodical assays for mycoplasmal adenosine phosphorylase (MycoTect, Gibco, Grand Island, NY, USA) using mouse 3T3 fibroblasts as indicator cells for toxic substrate formation.

Separation of mononuclear cell fractions

MNC were isolated from cytapheresis concentrates of donors UA or MS by Ficoll density gradient centrifugation. Lymphocyte- and monocyte-enriched fractions were prepared by countercurrent centrifugal elutriation in a Beckman J2/21 as described in detail by ANDREESEN et al. (15) and cryopreserved in small aliquots in liquid nitrogen in the presence of 50 % FCS and 10 % DMSO. The monocyte-enriched fractions from donors UA and MS contained 70 % and 83 % $CD14^+$ cells, respectively. The lymphocyte-enriched fractions contained less than 0.3 % $CD14^+$ cells.

Monocyte culture

Monocyte-enriched cell fractions were carefully thawed at appropriate time points by stepwise addition of RPMI 1640 at 4 °C and washed twice with medium (300 × g, 10 min, 4 °C). The recovery of vital cells (trypan blue exclusion) was always greater than 80 %. Cells were resuspended in RPMI 1640 supplemented with antibiotics, glutamine and 2 % human AB serum and seeded at different cell concentrations (1×10^3 to 4×10^4) into flat bottom microtiter ELISA plates (Greiner, Nürtingen, Germany). After 2 h non-adherent cells were removed by washing the plates with medium twice. Cultures were continued in RPMI 1640 + 2 % AB serum for 2 or 7 days. Where indicated, recombinant human cytokines were added from the start of cultures (interferon-gamma, rhIFN-gamma, 200 U/ml, Bioferon, Laupheim, Germany; interleukin-1 β, rhIL-1, 1 ng/ml, kindly provided by Dr. W. CONCA, Freiburg; interleukin-4, rhIL-4, 100 U/ml, kindly provided by Dr. S. GILLIS, Immunex, Seattle, WA, USA; interleukin-6, rhIL-6, 100 U/ml, Boehringer, Mannheim, Germany).

Proliferative response

After 2 h, 2 days or 7 days of monocyte culture the medium was replaced by test medium (RPMI 1640 + 10 % FCS + 2.5 % human pooled serum). At these time points, viability of the cultures was checked microscopically and in some experiments by MTT reduction tests (not shown) of parallel cultures. T cell lines were added to the adherent cell layer at 2×10^4 per well. In the case of antigen-specific T cell lines, assays were performed in parallel with and without antigens (25 µg/ml PPD or 1 µg/ml pepsin). Some assays were supplemented with cytokines (concentrations indicated above), anti-CD3 antibody (BMA030, 10 ng/ml), anti-CD 28 antibody (9.3, 1:8000, kindly provided by Dr. J. A. LEDBETTER, Seattle, WA, USA), or allogeneic monocyte enriched cell fraction (2×10^4/well). In some experiments, anti-CD3 antibody or fresh autologous monocytes were added 24 h later. The cultures were pulsed (18 h) with ^{3}H-thymidine after 48 h. For MLC cultures allogeneic lymphocytes, depleted from monocytes by counterflow centrifugation, were added at 1×10^5 per well and pulsed with ^{3}H-thymidine after 5 days. Proliferative responses are given in counts per min (cpm) and represent the mean of triplicates; standard deviations were sometimes elevated due to inhomogenous distribu-

tion of monocytes in the wells but always below 25 %. Background ^{3}H-thymidine uptake of monocytes was usually below 200 cpm and was subtracted.

Surface antigen expression

T cell lines were phenotyped by flow cytometry (FACStar Plus, Becton-Dickinson) using phycoerythrin- or fluorescein-conjugated monoclonal antibodies: anti-TcR$\alpha\beta$ (BMA031, Behring), anti-CD3 (UCHT1) and anti-CD8 (DK25, Dako), anti-CD4 (IOT4, Dianova-Immunotech), anti-CD29 (4B4, Coulter). Monocyte surface antigen expression was evaluated either by cell ELISA or by flow cytometry. The cell ELISA system has been described previously (15). Briefly, monocytes were cultured in microtiter plates as described above, fixed at 4 °C with 0.05 % glutaraldehyde for 10 min and treated with monoclonal antibodies against β_2-microglublin (β2M, Becton Dickinson), CD14 (My4, Coulter), HLA-DR/DP (Tü39, Biotest) and the differentiation-dependent antigen gp68-MAX.3 (16). Specifically bound antibodies were detected by peroxidase-conjugated second antibody, developed with phenyl-diaminedichloride and optical density was read at 486 nm. Results are given as percentage of β2M expression (antigen expression index). For flow cytometric analysis, monocytes were cultured in teflon bags (7). Cells were stained by direct (CD14, My4, Coulter; HLA-DR, Becton-Dickinson; ICAM-1, CD54, Dianova-Immunotech; all antibodies fluorescein-conjugated) or indirect immunofluorescence (LFA-3, TS2.9, kindly provided by Dr. S. C. MEUER, Heidelberg; second antibody: fluorescein-conjugated goat-F(ab)$_2$-anti-mouse-IgG, Dianova, Hamburg, Germany). Staining was performed in the presence of 1 % human IgG to reduce unspecific binding. Isotype matched mouse IgG (Becton-Dickinson) was used for control staining.

Results

We used a monocyte culture system in microtiter plates to investigate the effect of differentiation on auto-, allo- and soluble antigen induced T cell proliferation. Monocyte differentiation into mature macrophages was monitored microscopically and by expression of the macrophage marker MAX.3 (Tab. 2). Analysis of monocyte surface markers known to be involved in T cell activation (HLA-DR, CD14, ICAM-1, LFA-3) did not reveal any major changes of expression during monocyte cultures (Tab. 2).

Various T cell lines with different specificities (Tab. 1) and resting monocyte-depleted T cells were cocultured with monocytes or accessory

Table 1. Characterization of T cell lines.

	UA-S2[a]	MS-UA-B12	PLT–>UA	MS-PPD-B5	MS-PEP-N1[b]
Donor	UA	MS	NN	MS	MS
Primary stimulation	IL-2	UA	UA	PPD	pepsin
Specificity	autoreactive	alloreactive	autoreactive	PPD	pepsin
Restriction	DR 11.3	DR 11.3	DR 11	DR 3	DR 3

[a] SCHLESIER et al. (11); [b] LACOUR et al. (14, 34)

Table 2. Phenotypical characterization of cultured monocytes.

Monocyte culture	Phenotype								
	Cell ELISA[a]				Flow cytometry[b]				
	β2M	CD14	MAX. 3	HLA-DR	Control	CD14	HLA-DR	ICAM-1	LFA-3
Day 0	1.8	85	12	96	190	600 (90)	510 (93)	390 (97)	455 (99)
Day 2	1.8	86	27	85	210	610 (82)	590 (87)	620 (87)	520 (87)
Day 7	2.2	112	58	110	300	670 (96)	590 (77)	690 (90)	610 (95)

[a] Antigen expression index calculated on the basis of β2-microgobulin (β2M) expression (OD_{486}) (15).
[b] Mean channel fluorescence (% positive cells) measured with a FACStar Plus (Becton-Dickinson). The table shows representative data from 1 out of 2 experiments that are in accordance with previously published results (15).

cells derived from monocytes by culture for 2 or 7 days. Experiments with monocytes derived from 2 donors (Fig. 1) revealed that the alloreactive T cell clone MS-UA-B12 and 2 alloreactive T cell lines (PLT – > UA and PLT – > MS) were stimulated equally by all monocyte differentiation stages (Fig. 1A). The same was true for the antigen-specific T cell clones MS-PPD-B5 and MS-PEP-N1 (Fig. 1B). On the contrary, primary alloreactive T cell responses (MLC) could be equally stimulated by fresh and 2-day cultured monocytes but not by macrophages derived from 7-day cultures (Fig. 1C).

In clear contrast to the alloreactive and antigen-specific responses, the autoreactive T cell clone UA-S2 of synovial fluid origin did not respond to 2-day cultured monocytes in several independent experiments, although strongly proliferating in response to fresh monocytes and *in vitro* derived macrophages (Fig. 1D). The impaired stimulatory capacity of monocytes

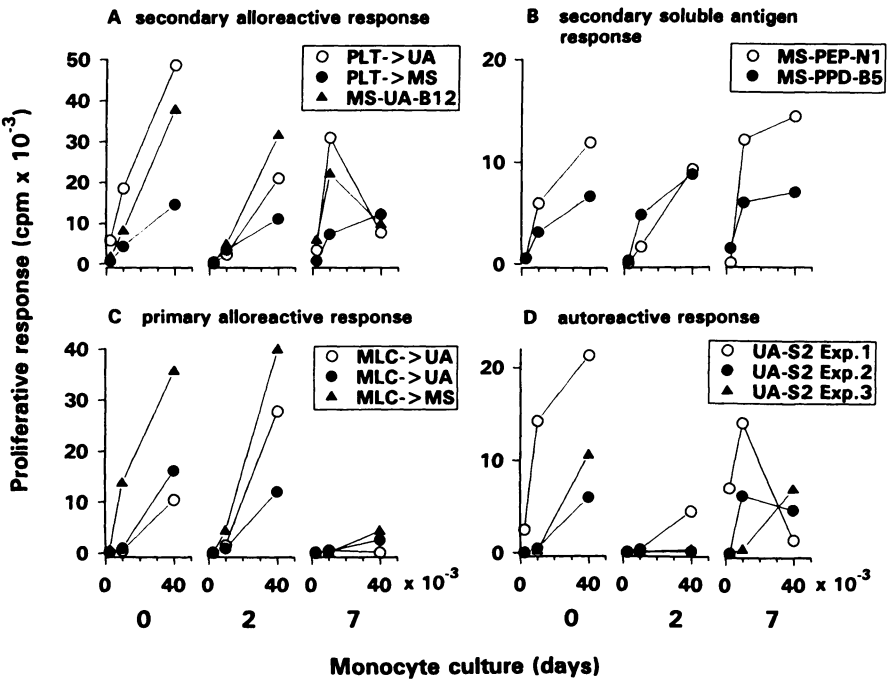

Figure 1. Response of alloreactive, antigen-specific and autoreactive T cells to different maturation stages of monocytes. Appropriate elutriation-enriched monocytes of donor UA or MS were seeded in microtiter plates at 2.5×10^3, 1×10^4 and 4×10^4 cells/well. (A, B, D) Various T cell lines (2×10^4) or (C) monocyte depleted lymphocytes (MLC, 1×10^5) were added either immediately (day 0) or after 2 days or 7 days of monocyte preculture. Antigens (pepsin or PPD) were included in assays with antigen-specific T cell clones (B); proliferative responses in the absence of antigens were always below 200 cpm. Proliferative responses were quantified by a 18 h pulse of ^{3}H-thymidine after 48 h in the case of secondary responses or after 5 days in the case of MLC. Individual experiments are shown for all cell types.

Table 3. Impaired stimulation of UA-S2 by 2 day cultured monocytes is not due to suppression.

Stimulus	Proliferative response (cpm)
Medium	10
Anti-CD3	10
Fresh monocytes	5910
Fresh monocytes + anti-CD3	16560
2d monocytes	140
2d monocytes + anti-CD3	10440
2d monocytes + fresh monocytes	7710
7 d monocytes	6210
7d monocytes + anti-CD3	9360
7d monocytes + fresh monocytes	5600

UA monocytes were seeded at 4×10^4 per well and precultured for 2 or 7 days in RPMI 1640 medium supplemented with 2 % AB serum. Fresh UA monocytes were added at 4×10^4 per well and anti-CD3 antibody (BMA030) at 10 ng/ml. UA-S2 (2×10^4 per well) was cocultured for 48 h and then pulsed with ^{3}H-thymidine for additional 18 h.

already became obvious after a one day culture and lasted for 4 days (not shown) and was observed with a wide range of monocyte concentrations tested (up to 1×10^5 cells/well). In contrast, primary and secondary alloreactive responses tested in parallel during the same experiment were never decreased at day 2 of monocyte culture, thus excluding experimental variations in monocyte cultures as the cause of impairement. Addition of anti-CD3 antibody or fresh autologous monocytes completely restored the response of UA-S2 to 2-day cultured monocytes (Tab. 3) excluding suppressive effects of the «medium-aged» macrophages. More importantly, even the delayed addition of anti-CD3 or fresh monocytes restored the proliferative response of UA-S2 also excluding induction of nonresponsiveness as the underlying mechanism (Tab. 4). In contrast, the impaired response to 2-day cultured monocytes could neither be restored by addition of cytokines (IL-1, IL-4, IL-6) or anti-CD28 antibody, nor by addition of allogeneic monocytes. However, monocytes precultured during 2 days in the presence of IFN-gamma were still able to induce UA-S2 proliferation (3310 cpm vs. 740 cpm with IFN-gamma vs. medium cultured monocytes). A similar but less pronounced effect was observed after monocyte precultures in the presence of IL-1, IL-6, and IL-4 (not shown).

Discussion

In this study we evaluated the effect of *in vitro* maturation on accessory function of monocytes/macrophages. In contrast to monocytes, *in vitro* differentiated macrophages were only weak stimulators of primary alloreac-

Table 4. Impaired stimulation of UA-S2 by 2-day cultured monocytes is not due to induction of nonresponsiveness and cannot be restored by cytokines or allogeneic monocytes.

Presence of 2-day cultured UA monocytes	Supplementation	Proliferative response	
		UA-S2	B12
–	Medium	12	11
–	IL-2 (20 U/ml)	22	180
–	Fresh UA monocytes	4680	3910
+	Medium	740	4510
+	anti-CD3 (10 ng/ml) after 24 h	6690	6150
+	fresh UA monocytes after 24 h	3510	7610
+	IL-2 (20 U/ml)	2170	5110
+	rhIL-4 (100 U/ml)	880	5680
+	rhIL-1 (1 ng/ml)	1320	6320
+	rhIL-6 (100 U/ml)	1170	5950
+	anti-CD28 (9.3; 1:8000)	960	4020
+	allogeneic monocytes	860	7090

UA monocytes were seeded at 4×10^4 per well and precultured for 2 days in RPMI 1640 medium supplemented with 2 % AB serum. At the day of assay the medium was replaced by RPMI 1640 medium with 10 % FCS and 2 % AB serum; UA-S2 or MS-UA-B12 were added at 2×10^4 per well. Cytokines, monoclonal antibodies, allogeneic monocytes from donor MS (2×10^4 per well) or fresh UA monocytes (2×10^4 per well) were added either simultaneously or after 24 h. 48 h after onset the assay was pulsed with ^{3}H-thymidine for additional 18 h. The data represent a typical experiment out of 3 similar experiments.

tive T cell responses which is consistent with other reports (5, 10). Similarly, primary T cell responses cannot be induced by fixed APC (17), B cell lines (18) or interferon-gamma-treated endothelial cells, fibroblasts or chondrocytes (19, 20), a failure supposed to be due to insufficient or altered production of necessary costimulatory signals, cytokines or processed antigen peptides (21).

On the other hand, it is widely accepted that induction of secondary T cell responses is not strictly dependent on professional APC. Human T cell lines have been shown to respond to alloantigens and soluble antigens presented by B cell lines (22) or IFN-gamma-treated fibroblasts, endothelial cells (19) or chondrocytes (20). In this study we extend this listing by demonstrating that *in vitro* derived macrophages, in contrast to their inability to stimulate primary responses, are clearly capable of stimulating secondary alloreactive and antigen-specific responses as efficiently as monocytes. Similarly, in the murine system, antigen-specific T cell lines could be restimulated by antigens presented on peritoneal macrophages (23, 24). On the basis of the above-mentioned facts the response pattern of the autoreactive T cell clone UA-S2 is most striking. The inability of mono-

cytes cultured for 2 days to stimulate UA-S2 cannot be attributed to a deficient costimulatory signal because of the following reasons: i) various secondary and even primary T cell responses were stimulated equally well by cultured monocytes, ii) molecules necessary in T cell-monocyte interactions like HLA-DR, CD14, LFA-3, and ICAM-1 were not deficient on cultured monocytes, iii) allogeneic monocytes, cytokines (25, 26), and anti-CD28 antibody (27) could not restore the impaired response, and most importantly, iv) nonresponsiveness that is induced by APC in the absence of adequate costimulation (27) was not observed with UA-S2, since the clone was fully responsive to fresh monocytes or anti-CD3 antibody after preincubation with cultured monocytes for 24 h. Therefore we propose that the only plausible explanation for the failure of cultured monocytes to stimulate UA-S2 is the lacking presentation of the peptide recognized by the T cell receptor of UA-S2. This peptide may be either derived from a differentiation-dependent monocyte-specific protein or produced by a differentiation-dependent protease. This suggestion is in agreement with the observed major changes in phenotype and enzyme content after 2 days of culture during *in vitro* differentiation of monocytes to macrophages (8, 15, 28). It also explains the failure of an autologous B cell line to stimulate UA-S2 (11). Although cell type-restricted autoreactive T cells have been described, the nature of the peptides recognized has not been uncovered (29). This is, to our knowledge, the first report on a differentiation-dependent autoreactive response. Culture-dependent loss of antigen-presenting ability for exogenous soluble antigens has been described for murine macrophages and explained by the inability of cultured APC to produce the correct peptides due to changes in the proteolytic system (24).

The cytokines IFN-gamma, IL-1, IL-6, and IL-4 to some extent retained the stimulatory potential of cultured monocytes in the UA-S2 system. This may be due to the documented effects of these cytokines on differentiation and accessory function. Especially IFN-gamma is a strong enhancer of accessory activity due to upregulation of several surface molecules like MHC class II antigens, ICAM-1 and B7/BB1 (19, 30–32). IL-1, IL-6, and IL-4 have been shown to augment the accessory potency of cultured monocytes in mitogen-driven T cell responses (28, 33). Thus, culture of monocytes in the presence of these cytokines may induce and/or maintain the presentation of the antigenic peptide to UA-S2.

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