Dendritic cell counts and their subsets during treatment of multiple myeloma

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Human dendritic cells have distinct roles in the regulation of immunity. In this study we analysed the kinetics and the proportion of myeloid and plasmacytoid subsets of dendritic cells (DC) in peripheral blood of 15 patients with multiple myeloma (MM) before and during treatment that included autologous transplantation. Control group of 15 healthy volunteers was evaluated by using the same approaches. Flowcytometric determination of relative and absolute cell counts in unmanipulated peripheral blood was based on the expression of surface antigens CD83 and HLA-DR. Depending on the expresion of CD11c or CD123, we divided these cells into $CD11c^+$ dendritic cells type 1 (DC1) and $CD123^+$ DC type 2 (DC2).

Significant differences were found in initial relative counts of $CD83^+$ cells and of the DC2 subtype between the group of controls and the group of patients before treatment. In absolute counts, there was a difference only in the DC2 subtype. After induction treatment (vincristine, doxorubicin, and dexamethasone), the mean percentage of $CD83^+$ DC and the DC1 percentage were significantly higher than initially, but there was no significant difference in absolute counts. Administration of G-CSF again increased the total DC numbers. Intermediate DC counts were found in the apheresis products. After engraftment, we found the highest relative DC numbers, but absolute counts were not very high because of leukopenia. Within six months after transplantation, normal relative and absolute DC counts were found in patients.

Untreated patients with MM have significantly lower relative numbers of peripheral blood DC in comparison with healthy volunteers. The highest number of total DC was found after engraftment. The DC1/DC2 ratio showed relative predominance of DC1 subtype and the lowest DC1/DC2 ratio was found in the apheresis products. DC counts comparable with those of healthy volunteers were found in patients six months after transplantation.

Key words: dendritic cells, multiple myeloma, flow cytometry

Dendritic cells (DC) are the most potent antigen-presenting cells. They have an ability to take up and process antigens (Ag) and to initiate primary immune responses by stimulating naive resting T cells [1]. After loading with relevant Ag, anti-tumor immunity can be induced in patients with multiple myeloma (MM) [2, 3]. Thus, DC are of interest for potential immunotherapeutic application [4].

Properties, phenotype and function of DC depend on the type of bone marrow precursors and on the stage of DC maturation. Monitoring of DC is complicated by the lack of a defined method for counting DC which are classified as lineage negative cells (lin^-) with strong expression of HLA-DR molecules or as $CMRF44^+CD19^-CD14^-$ cells [5]. DC precursors reach peripheral tissues through blood stream and at this time express only a restricted subset of antigens. Activation or differentiation of dendritic cells are commonly associated with surface expression of CD83.

At least three different subtypes of DC have been characterized in humans, $CD14^+CD11c^+$ monocytes, $CD14^-CD11c^-IL3Ra^+$ plasmacytoid (lymphoid) cells and $CD14^-CD11c^+$ myeloid (circulating) DC [6]. We have studied the DC1 subset which is $CD11c^{high}$ $CD123^{low}$ cells and the $CD11c^-CD123^+$ DC2 subset. The $CD14^-CD11c^+CD13^+CD33^+$ DC1 subset requires the presence of GM-CSF for survival and after stimulation it produces high levels of IL-12. These DC activate naive T lymphocytes, drive T-cell differentiation into the Th1 subtype, and thereby generate antitumor immunity. The $CD14^-CD11c^-CD4^+CD123^+$ DC2 subset requires IL-3 for

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Tab. 1. Relative and absolute DC counts – comparison of healthy volunteers (controls) and patients (initial values as start and the sixth month after transplantation as after 6M).

Controls vs. Patients	%			x10^9/l		
	control	start	after 6M	control	start	after 6M
CD 83	0.26	0.15	0.24	0.41	0.29	0.42
CD83/HLA-DR/CD11c	0.06	0.04	0.05	0.09	0.06	0.09
CD83/HLA-DR/CD123	0.05	0.02	0.04	0.06	0.02	0.08
DC1/DC2	1.54	4.25	1.57	1.54	4.25	1.57

maturation and appears to be an important source of type I interferons in response to viruses [7]. DC2 can induce differentiation of T-cells into the Th2 subtype and modulate the development of acute „graft versus host disease“ (aGVHD) after allogeneic hemopoetic cell transplantation or induce tolerance. It has been suggested that the development of acute and chronic GVHD might be associated with selective loss of DC2 from peripheral blood [7, 8, 9, 10].

There is evidence that the number of circulating precursors of DC in patients with hematological malignities is lower than in healthy persons [11, 12] and also that in the elderly the numbers of DC2 are decreased [13]. We examined and compared relative and absolute counts of dendritic cells and their subtypes in healthy volunteers and in newly diagnosed untreated patients with multiple myeloma. We also monitored DC numbers during the treatment of patients undergoing autologous transplantation of peripheral blood stem cells (PBSC) to find the optimal time for separation of DC for immunotherapy.

Patients, materials and methods

Healthy volunteers. Peripheral blood of healthy volunteers was analyzed as a control for initial values; n = 15; 12 women and 3 men; the mean age was 57 ± 9,7 years.

Patients. Peripheral blood (PB) and/or peripheral blood stem cells (PBSC) of 15 patients (10 women and 5 men) who were transplanted were obtained after informed consent; the mean age was 56 ± 8 years. PB samples and also concentrates of PBSC were collected in EDTA at following timepoints: 1. at the time of the diagnose; 2. after induction chemotherapy (vincristin, doxorubicin, and dexamethasone); 3. after stimulation by cyclophosphamide (5 g/m^2) and subsequent G-CSF (5 – 10 μg/kg), when the white blood cell count (WBC) became higher than 5x10^9/l; 4. in the peripheral blood stem cell (PBSC) apheresis product; 5. after autologous transplantation (T) when WBC was > 5x10^9/l; 6. in the sixth month after transplantation.

Methods. Samples of 70 μl peripheral blood (or 10 μl PBSC) were incubated with appropriate amounts of monoclonal antibodies. The following combinations of antibodies were used: HLA-DR-FITC/CD11c-PE/CD83-PC5 (Immunotech, Marseille, France), HLA-DR-FITC/CD123-PE/CD83-PC5 (Immunotech and Becton-Dickinson, Franklin Lakes, NJ, USA). As an isotype control, mouse IgG1-FITC/IgG1-PE/IgG1-PC5 (Immunotech) was used to calibrate the flowcytometer. After 20 min at room temperature, erythrocytes were lysed and samples were fixed with 1% paraformaldehyde in PBS. Flow cytometric analysis was performed on FC500 Cytomics (Beckman Coulter, Hialeah, FL, USA).

Identification of DC and their subtypes. Measurements of DC subpopulations were done on leucocytes of peripheral blood. Mononuclear cells were identified according to forward scatter (FS) and side scatter (SS) properties. Analysis was performed on 100.000 cells after adjustment of instrumental fluorescent voltages with an isotype control. Analysed subpopulations was distinguished according to their expression of CD83 and HLA-DR and also subtypes CD11c$^+$ and CD123$^+$ cells were counted after appropriate gating (Fig. 1). Absolute counts of DC were determined from the total white blood cell numbers acquired on hematological analyser Abbot CD3500. Triple measurements of the same sample were performed.

Statistics. The data were plotted in Box-Whisker plots, categorised columnal and correlational diagrams. In coherential parameters, basic statistic value counts were determined (mean, median, standard deviation and appropriate kvantils). The Spearman's correlation coefficient R_S was used in correlation relations analysis, Mann-Whitney U test was applied for comparison of the healthy control group with initial values of the untreated group. For comparison of expressed markers during treatment, Wilcoxon's pair test was used. Statistical tests were evaluated on the level of significance α=0.05.

Results

Comparison of initial DC counts between healthy volunteers and patients with MM before treatment and in the sixth month after transplantation. Flowcytometric analysis of CD83 positive cell population was complicated because of the weak expression of the antigen. Mean relative expression of CD83 marker was significantly higher in healthy volunteers than in patients (0.26% of mononuclear cells vs. 0.15%; p<0.01), no difference was observed in absolute counts CD83$^+$ cells (0.41x10^9/l of blood vs. 0.29x10^9/l; p=0.44). We found higher relative (p=0.01) and absolute (p<0.01) counts of DC2 cells in controls than in patients (Tab. 1). No differences in DC1/

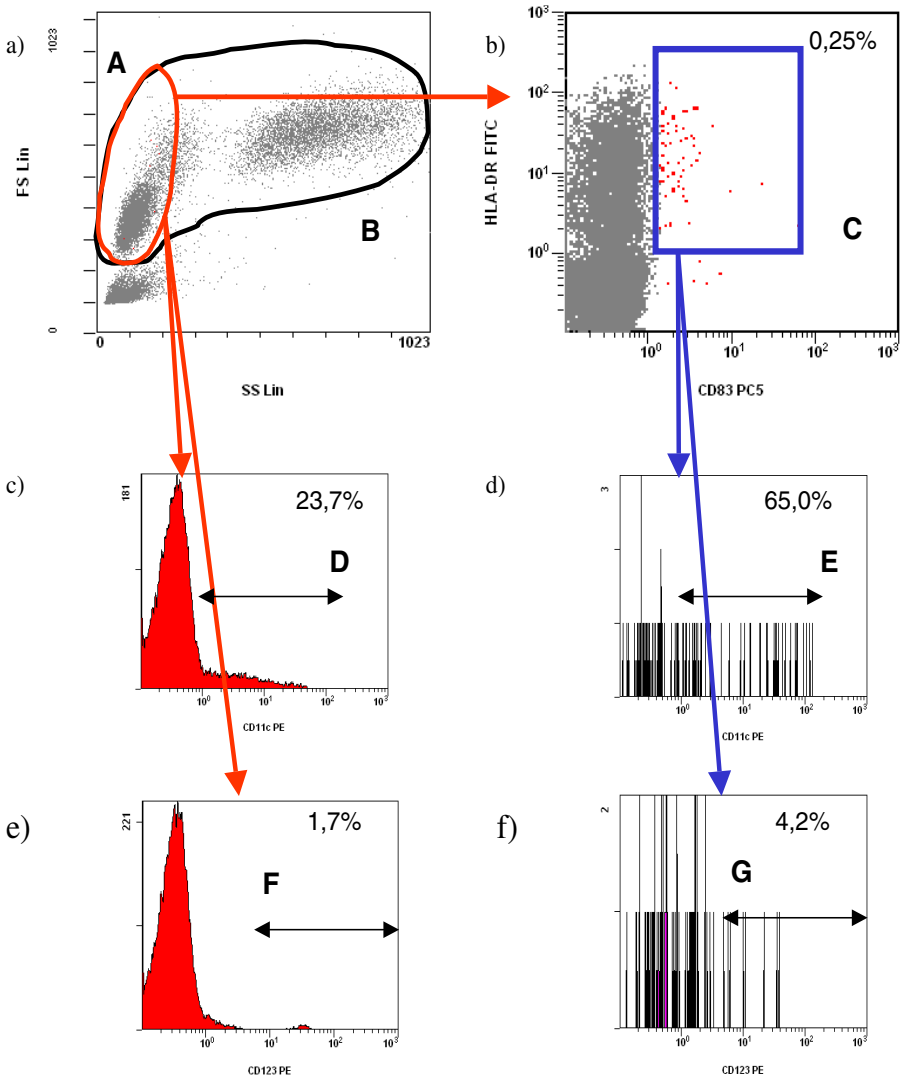

Fig. 1. Gating strategy: a) Selection of viable leukocytes and mononuclear cells according to FS and SS; b) Expression of HLA-DR and CD83 on mononuclear gate A, population HLA-DR$^+$CD83$^+$ in gate C form 0,25% of mononuclear gate A; c) Expression of CD11c on mononuclear gate A; d) Expression of CD11c on HLA-DR$^+$CD83$^+$ population; e) Expression of CD123^{hi} on mononuclear gate; f) Expression of CD123^{hi} on HLA-DR$^+$CD83$^+$cells.

DC2 ratio was found. Relative and absolute DC counts of patients acquired in 6th month after transplantation (0.24%; 0.42x10^9/l) were compared with initial values in healthy volunteers and there was found no significant difference.

DC counts during treatment. We observed significant differences in relative and absolute DC counts and also in DC1/DC2 ratios at these timepoints: after induction treatment, after the administration of G-CSF, in the apheresis products, and during engraftment (Tab. 2, 3, 4; Fig. 2). We observed higher relative and absolute DC counts after induction treatment when compared with initials values (0.18%; 0.36x10^9/l of CD83$^+$ DC). Also, after application of G-CSF, relative DC counts increased (0.34%; 0.29x10^9/l of CD83$^+$ DC), but absolute DC counts and DC1/DC2 ratios decreased. Apheresis samples of PBSC contained similar relative DC counts (0.22%; 3,74x10^9/l of CD83$^+$ DC) as peripheral bloods after induction chemotherapy, but absolute counts were higher then after G-CSF. The lowest DC1/DC2 ratio was in the graft. After engraftment we observed the highest relative DC counts (0.5%; 0.2x10^9/l of CD83$^+$ DC), but absolute counts were not high due to leukopenia.

Discussion

Measurement of DC counts in peripheral blood is complicated by the absence of their specific markers. Usually DC precursors are identified as HLA-DR$^+$ lin$^-$ cells (1). However, the populations of lineage$^-$ (lin$^-$) HLA-DR$^+$ cells were found

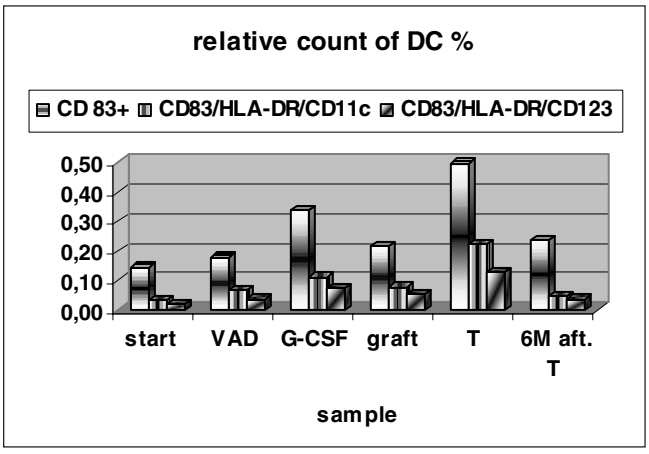

Fig. 2. Relative DC counts during the treatment

to be non-homogenous and give rise to a number of different cell populations and also to non-DC fibroblasts [14] The population of non-DC, lin$^-$HLA-DR$^+$ may vary considerably in patients, creating a potential for error when this method is used to monitor blood DC (5). The amount of lin$^-$HLA-DR$^+$ cells may increase in some diseases, especially when hematopoetic progenitors are mobilized to peripheral blood, further illustrating the inadequacy of this method for determination of DC counts [15].

The objective of this work was to quantify unmanipulated early dendritic cells in peripheral blood and also to test their ability to mature. We focused on the expression of CD83, a marker of activation, normally found on differentiated/activated DC and sometimes also on freshly isolated DC. This marker together with subset of main histocompatibility complex is used for identification of DC (5). CD83 can be found preferentially on activated or differentiated dendritic cells (1). Expression of CD83 can be induced by multistep purification procedure, including overnight culture of peripheral blood mononuclear cells (MNC) [16, 17]. In uninduced peripheral blood MNC, however, errors can occurs in evaluation of CD83. A small population of DC will express this marker due to sporadic encounter with specific Ag followed by CD83 up-regulation. Such CD83$^+$ DC might be circulating through peripheral blood during their migration into secondary lymphatic tissues following activation. There is also strong correlation between CD83$^+$ and CMRF-44$^+$ cells ($r^2 = 0.89$), even thought CD83 expression is about 15% lower (1). A clinically applicable procedure for blood DC isolation using monoclonal antibodies CMRF-44 and CMRF-56 to obtain DC in sufficient quantity and quality for cancer immunotherapy has been recently reported [18]. This procedure should facilitate the evaluation of CD83$^+$ DC in peripheral blood.

We used unmanipulated peripheral blood because of potential cell losses during separation. The expression of CD83 marker was weak and our CD83$^+$ cells were mostly HLA-DR$^+$ and this population likely corresponds to early activated DC. DC counts exhibited intraindividual variability (data not shown) that may reflect fluctuations within a relatively broad range or cyclic variations in blood DC counts (1). Also, interindividual differences were found (data not shown).

There is not much knowledge about kinetics of peripheral blood DCs. It has been reported that counts of the DC2 subtype are age-dependent and they decrease with age (13). Our control group were healthy volunteers with similar mean age as patients. We found that newly diagnosed MM patients have significantly decreased initial relative DC numbers both CD83$^+$ cells and DC2 subtype in peripheral blood when compared with healthy volunteers. When the absolute counts were evaluated, only DC2 numbers were significantly lower in patients compared to controls. In the 6th month after transplantation, there were no significant differences in DC numbers. Decreased absolute counts of DC (HLA-DR$^+$Lin$^-$) have been reported previously in chronic hepatitis C infection. These DC1 and DC2 had impaired ability to produce IL-12 and IFNα [19]. Evaluating both DC1 and DC2 subtypes in children with newly diagnosed solid tumors showed significant reduction in absolute counts of DC1 in addition to lower relative counts for both subtypes (12). Children with allergy had lower absolute DC2 counts [20]. On the other hand, surgery (laparoscopic cholecystectomy) and psychological stress (exercise using the Bruce protocol) increased circulating blood dendritic cells independently of monocyte counts without phenotypic or functional chages [21]. Brown et al. studied DC subtypes in MM patients according to expression of CMRF-44 and found only trend of lower DC count in patients with progressive disease when compared to patients with

Tab. 2. Relative and absolute DC counts during the treatment

Patients		start	VAD	G-CSF	graft	T	6M aft. T
%	CD 83+	0.15	0.18	0.34	0.22	0.50	0.24
	CD83/HLA-DR/CD11c	0.04	0.07	0.11	0.08	0.22	0.05
	CD83/HLA-DR/CD123	0.02	0.04	0.07	0.05	0.13	0.04
x10^9/l	CD 83+	0.29	0.36	0.29	3.74	0.20	0.42
	CD83/HLA-DR/CD11c	0.06	0.15	0.10	1.34	0.10	0.09
	CD83/HLA-DR/CD123	0.02	0.07	0.07	1.11	0.05	0.08
	DC1/DC2	4.25	4.77	1.99	0.88	1.63	1.57

Tab. 3. Comparison of differences of relative (A) and absolute (B) counts of CD83$^+$ cells. DC1 (C. D). DC2 (E. F) during the treatment. Bold numbers are used when differences are statisctically significant (p≤0.05).

A.

relative CD83$^+$ counts					
	start	VAD	G-CSF	T	6M aft. T
start					
VAD	**0.009**				
G-cSF	**0.001**	**0.001**			
graft	**0.004**	0.112	**0.001**		
T	**0.001**	**0.001**	**0.036**	**0.001**	
6M aft. T	**0.001**	**0.035**	**0.006**	0.394	**0.001**

B.

absolute CD83$^+$ counts					
	start	VAD	G-CSF	graft	6M aft. T
start					
VAD	0.496				
G-CSF	0.650	0.173			
graft	**0.001**	**0.001**	**0.001**		
T	0.191	**0.027**	0.173	**0.001**	
6M aft. T	**0.020**	0.112	0.088	**0.001**	**0.017**

C.

relative DC1 counts (CD83/HLA-DR/CD11c)					
	start	VAD	G-CSF	graft	T
start					
VAD	**0.008**				
G-CSF	**0.015**	0.394			
graft	**0.015**	0.510	0.191		
T	**0.001**	**0.003**	0.173	**0.005**	
6M aft. T	0.256	0.148	**0.036**	**0.041**	**0.001**

D.

absolute DC1 counts (CD83/HLA-DR/CD11c)					
	start	VAD	G-CSF	graft	T
start					
VAD	0.069				
G-CSF	0.955	0.256			
graft	**0.001**	**0.001**	**0.001**		
T	0.733	0.088	0.363	**0.001**	
6M aft. T	0.233	0.570	0.733	**0.001**	0.427

E.

relative DC2 counts (CD83/HLA-DR/CD123)					
	start	VAD	G-CSF	graft	T
start					
VAD	0.112				
G-CSF	**0.019**	0.140			
graft	0.064	0.514	0.272		
T	**0.005**	**0.001**	0.112	**0.019**	
6M aft. T	0.061	0.865	0.088	0.460	**0.003**

F.

absolute DC2 counts (CD83/HLA-DR/CD123)					
	start	VAD	G-CSF	graft	T
start					
VAD	**0.031**				
G-CSF	0.074	0.650			
graft	**0.004**	**0.006**	**0.003**		
T	0.256	0.069	0.427	**0.004**	
6M aft. T	**0.017**	0.427	0.460	**0.005**	0.125

stable disease [22]. Do found normal counts of preDC in MM patients, but myeloid DC counts were about 50% lower than in healthy control [23]. Our data fit well with these results.

Our results showed an increase in DC count (DC1 and DC2) after induction treatment with VAD compared to initial values. Savary et al. found lower Lin$^-$HLA-DR$^+$ cells in patients after chemotherapy [24]. We hypothesize that the increase in DC counts is due to the reduction of bone marrow infiltration by myeloma cells which can produce inhibition factors and block the mobilisation of preDC to the blood stream. After application of G-CSF we observed increased relative counts of DC83$^+$ cells as well as DC1 and DC2 subtypes. At the same time, their absolute counts decreased. Vuckovic et al. found no change in the CD11c$^+$ DC population after stimulation of healthy volunteers using G-CSF and also an increase in the DC2 population which correlated with a decrease of CD62L-selectin expression

Tab. 4. DC1/DC2 ratio during the treatment. Bold numbers are used when differences are statistically significant (p≤0.05).

DB1/DB2					
	start	VAD	G-CSF	graft	T
start					
VAD	0.510				
G-CSF	0.150	0.249			
graft	**0.030**	**0.041**	0.155		
T	**0.040**	0.657	0.721	0.590	
6M aft. T	**0.050**	**0.031**	0.701	0.920	0.328

[25]. Bolwell et al. observed also an increase in DC2 counts in G-CSF stimulated donors in comparison to G-CSF + etoposide stimulation [26]. It is known that administration

of G-CSF stimulates the production of DC2 subtype but the administration of GM-CSF increases DC1 counts [11, 25, 27]. However, these studies were done on $Lin^{-}HLA\text{-}DR^{+}$ population and not on $CD83^{+}$ cells. The administration of G-CSF and GM-CSF decreased relative and absolute DC2 counts. Flt3 is an agent that increases both DC1 and DC2 counts [28].

The relatively "normal" percentage of $CD83^{+}$ DC was found in PBSC apheresis product. The increase was not detected probably due to the rise in $CD34^{+}$ cell counts (1). However, we did find increased absolute DC counts in the apheresis product. PBSC and also monocytes are a source of cells for *in vitro* expansion of DC when characteristic DC phenotype is expressed after the addition of various cytokines. In a study with donor DC, Tzolas *et al* found that a higher donor DC1 content in the stem cell graft was associated with increased incidence and severity of acute GVHD [29]. Reddy et al. evaluated DC count at engraftment and found that higher percentages of circulating DC (DC1 and DC2) in peripheral blood at the time of engraftment correlate with relapse and survival after allogeneic peripheral blood stem cell transplantation [27, 30]. For these reasons, it is possible that monitoring and comparison of peripheral blood DC count before and after transplantation can predict and identify patients at risk for relapse after allogenic transplantation.

The reconstitution of the blood pool after transplantation is demonstrated by increased relative $CD83^{+}$ DC counts and also DC1, DC2 subtype counts in patients in comparison to values of healthy control. The absolute counts were affected by lymphopenia. Reddy et al. evaluated the percentage of DC in allogenic transplantations and found that higher percentage of circulating DC correlate with later relapses and prolonged survival. On the other hand, lower DC counts were associated with higher incidence of aGVHD and earlier death [30].

No statistical difference was found in sequential evaluation of $CD83^{+}$ DC numbers at the 6th month after transplantation suggesting that the DC1 and DC2 counts were reconstituted to normal levels and are similar to those of healthy controls. Similar results have been reported by Arpinati et al. who monitored $CD11^{+}$ and $CD123^{+}$ DC reconstitution after PBSC transplantation [31].

In conclusion, newly diagnosed multiple myeloma patients have decreased percentage of $CD83^{+}$ DC cells in peripheral blood, especially of the DC2 subtype, but after treatment the counts normalize. Peripheral blood stem cell apheresis product contains the highest absolute counts of $CD83^{+}$ cells and is therefore the most suitable for immunotherapeutic applications.

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