

Letter to the Editor

Divergent autologous T cell responses to leukaemic dendritic cells during remission in acute promyelocytic leukaemia

To the Editor:

Most acute myeloid leukaemia (AML) blasts fail to present antigens to T cells due to, e.g., downregulation of MHC molecules and lack of expression of co-stimulatory molecules [7]. Lack of these molecules prevents cross priming, which may result in tolerance or energy. Acute promyelocytic leukaemia (APL), a subset of AML, is typified by a unique chromosomal translocation t(15;17)(q22;q21) generating the fusion of the promyelocytic leukaemia gene PML and the retinoic acid receptor RAR α , a potential tumour target [10]. Although all trans-retinoic acid (ATRA) and anthracyclin containing cyto-reduction regimens cure the majority of APL patients, relapses occur. Immunotherapy using dendritic cell (DC) based vaccines could provide an effective tool to control or eradicate minimal residual disease in AML. DCs have strong expression of MHC class I, MHC class II and co-stimulatory molecules. Moreover, leukaemia-derived DCs are thought to conserve the characteristic cytogenetic and phenotypic aberrations of the malignant cells relevant for antigen presentation. In a phase-I pilot study on DC vaccination in advanced chronic myeloid leukaemia (CML) strong DTH-responses could be detected representing autologous CML-specific T cell responses *in vivo* [9].

The aim of this study was to examine the immunogenicity of APL-derived antigen presenting cells (APCs) in an autologous setting. For this purpose, we obtained bone marrow of one particular patient, a 22-year-old female, diagnosed with AML classified as t(15;17)-positive APL. All samples were drawn after informed consent. Mononuclear cells, consisting of 90% blast cells, were isolated and cultured in serum-free StemSpanTM H2000 medium (Stemcell Technologies, Meylan, France) supplemented with calcium ionophore A23187 (375 ng/ml, Sigma, St. Louis, MO) and rhIL-4 (250 U/ml, Serva GmbH, Heidelberg, Germany) for two days as described previously [5]. Directly after achieving complete remission (CR) fol-

lowing two courses of chemotherapy and after prolonged remission duration (20 mo. CR), autologous peripheral blood mononuclear cells (PBMCs) were isolated. Plastic adherent monocytes were cultured into remission MoDCs (remMoDCs) by 7 days of culture in StemSpanTM H2000 medium supplemented with 800 U/ml rhGM-CSF (250 U/ml, Peprotech, Rocky Hill, NJ) and 500 U/ml rhIL-4. Non-adherent peripheral blood lymphocytes (PBLs) were used as source of remission T cells.

Blast cells showed typical APL morphology: hypergranularity and bundles of Auer rods (Fig. 1A). Flow-cytometric analysis revealed that none of the blasts expressed HLA-DR, co-stimulatory molecules CD40, CD80 and CD86, CD54, CD1a and CD83. Previous data showed that APL cells can be induced to significantly upregulate CD40, CD80, CD86 and CD54 ($n = 7$, $p < 0.05$). HLA-DR and CD83 were also upregulated although significance was not reached ($n = 7$, $p = 0.09$ and $p = 0.07$, respectively (data not shown) [5,14]. In the blasts of the particular patient in this study, culture with A23187 and IL-4 induced expression of CD40 (21%), CD80 (17%), CD86 (38%), CD54 (22%), HLA-DR (20%) and the maturation marker CD83 (16%); CD1a remained low. Persistent bundles of Auer rods in the cytoplasm of the APL-APCs indicated their leukaemic origin (Fig. 1B). This was confirmed by detection of the PML/RAR α -fusion protein in sorted APCs (FACS-VANTAGE, Becton Dickinson) [14]. In 98 out of 100 sorted APCs the microgranular staining pattern of PML/RAR α -fusion protein was detected by anti-PML monoclonal antibody PG-M3 (Santa Cruz Biotechnology, CA) thereby proving the presence of t(15;17) in these APCs (data not shown).

The availability of APL cells as well as sampling of remission T cells from this patient immediately after achieving CR enabled the study of functional properties of APL-APCs. Non-leukemic remMoDCs were used as a control. Irradiated APL-APCs and remMoDCs were allowed to stimulate autologous PBLs

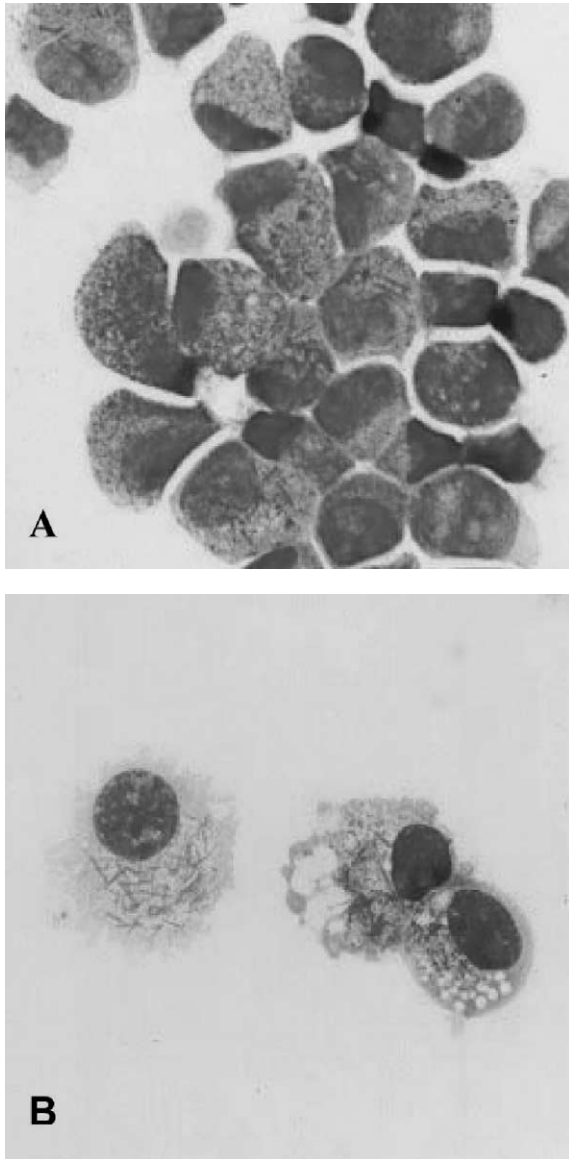


Fig. 1. Morphology of leukaemic cells and leukaemia-derived APCs in APL. APL blast cells are shown in panel A, the CI-cultured APL-APCs in panel B. Cells were spun onto slides and stained with May-Grünwald-Giemsa and evaluated by light microscopy. APCs are enlarged, veiled cells with a decreased nucleus to cytoplasm ratio as compared to blast cells. Auer rods are visible in both cell types.

obtained at CR in separate cultures at a 1:5 ratio in the presence of rhIL-7 (5 ng/ml) and rhIL-2 (10 U/ml, both from Strathmann Biotech GmbH, Hannover, Germany). Autologous T cells, re-stimulated at day 7, were harvested after 14 days. Co-culture of autologous remission T cells with APL-APCs or autologous remMoDCs resulted both in a 3.2-fold expansion of total cell number. Distribution of T cell subpopulations

before and after co-culture was characterised by flow-cytometry. Results are depicted in Table 1. Initially, the majority of the CD4⁺ and CD8⁺ T cells had a naïve phenotype (CD45RA⁺CD27⁺). After co-culture with APL-APCs and remMoDCs, the CD4⁺ subpopulation shifted towards central-memory (CD45RA⁻CD27⁺) and effector-memory cell (CD45RA⁻CD27⁻) phenotypes. The predominant CD8⁺ subpopulation found after co-culture was one with central-memory phenotype (CD45RA⁻CD27⁺) [4,11]. The CD8⁺-effector-(CD45RA⁺CD27⁻) and effector-memory population (CD45RA⁻CD27⁻) expanded approximately 29-fold and 24-fold after stimulation with APL-APCs and remMoDCs, respectively. The percentage T- $\gamma\delta$ cells within the CD3⁺-population increased 2.4-fold and 2.6-fold for APL-APC and remMoDC, respectively. The fluorescence intensity of HLA-DR, a late activation marker of T- $\gamma\delta$ cells inclined approximately 5-fold after co-culture with APL-APCs and remMoDCs as compared to the unstimulated T- $\gamma\delta$ cells (data not shown) [15]. The percentage NK cells increased but remained low. Intracellular flow-cytometric detection of cytokine production showed that the fraction of IFN- γ -producing T cells inclined from 8% before stimulation to 33% and 35% after stimulation with APL-APCs and remMoDCs, respectively (Fig. 2A). The percentage of IL-4-producing T cells remained low; IL-4 production was only detected in the CD4⁺ fraction (Fig. 2B). Cell free culture supernatants harvested 24 h after re-stimulation of T cells were analysed by ELISA (Sanquin, Amsterdam, The Netherlands) to detect the amount of cytokines produced. APL-APCs induced highest production of IFN- γ as compared to remMoDCs (Fig. 2C) indicating a preferential Th1 response.

Control cultures with cytokines alone showed 13-fold expansion of total cell number. These cultures barely showed differentiation towards memory cells in the CD4⁺ as well as in the CD8⁺ subpopulation. A slight increase in the percentage of CD8⁺ effector-memory and effector T cells was observed, however less as compared to APL-APC and remMoDC stimulation. The same accounted for the population of effector-memory CD4⁺ cells. The percentage of the T- $\gamma\delta$ cells, their activation state and the percentage of NK cells remained low (data not shown).

Co-culture of APL-APCs and remMoDCs with remission autologous PBLs obtained at a later time point in remission (20 mo. CR) resulted in a 5.1 and 3.1-fold expansion of total cell number, respectively. A higher percentage of memory T cells was observed before co-

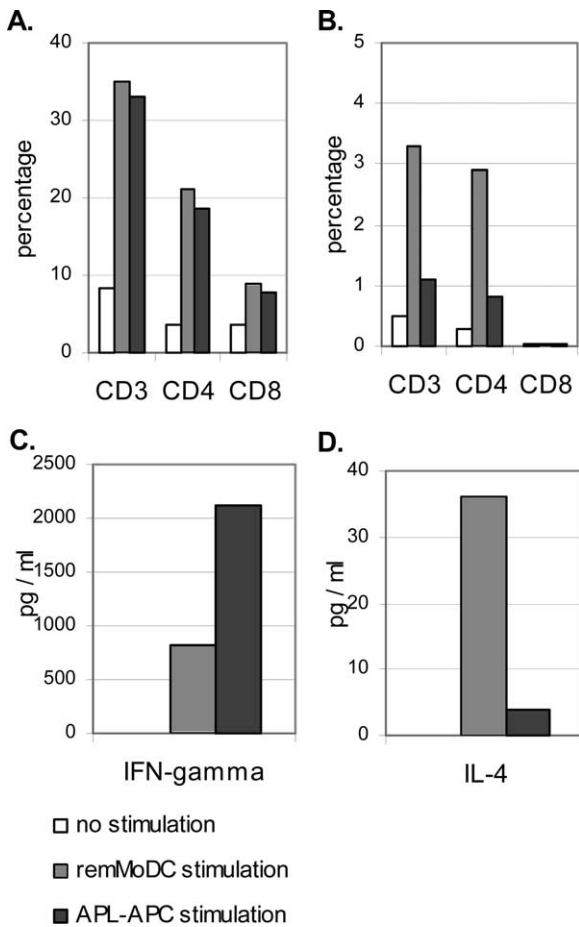


Fig. 2. Cytokine analysis of autologous remission T cells. In panel A, percentages of IFN- γ -producing T cells are shown; in panel B, percentages IL-4-producing T cells. No IL-4 production could be found in CD8-positive cells. Panel C and D depict cytokine production of remMoDC- and APL-APC-stimulated T cells and unstimulated T cells 24 h after re-stimulation as assessed by ELISA. The number of lymphocytes was equal in all cultures.

culture as compared to the PBL-sample obtained directly after achieving CR (Table 1). Co-culture with APL-APCs resulted in a higher percentage of CD8⁺ cells and hence a lower CD4/CD8-ratio (from 2.5 to 0.4 at 0 mo. and 20 mo. CR, respectively). In the total cell population, the percentage of CD8⁺ effector T cells (CD45RA⁺CD27⁻) increased from 0.2% in the first to 1.7% in the second PBL sample after co-culture with APL-APCs. The percentage of cells with the ability to lyse in a non-MHC-restricted way, NK cells (CD3⁻CD56⁺), NK-T cells (CD3⁺CD56⁺) and T- $\gamma\delta$ cells was remarkably higher at 20 mo. of CR as compared to the first remission sample, before as well as after co-culture. The T- $\gamma\delta$ cells were even more activated; the mean fluorescence intensity of HLA-DR was

12.1-fold (APL-APCs) and 14.7-fold (remMoDCs) higher as compared to unstimulated T- $\gamma\delta$ cells. Furthermore, analysis of supernatants of co-cultures of remission PBLs harvested at 20 mo. after CR with either APL-APCs or remMoDCs showed almost similar IFN- γ levels (85 and 75 ng/ml, respectively). IL-4 production was low for both APL-APCs and remMoDCs stimulation (below 7 pg/ml). As in the first PBL sample, stimulation by IL-2 and IL-7 alone induced a slight increase in percentages CD8⁺ effector-memory and effector cells and effector-memory CD4⁺ cells. In contrast to the first sample, IL-2 and IL-7 induced expansion of NK, NK-T and T- $\gamma\delta$ cells. The increase in total cell number was 2.4 fold.

In this particular patient, cells of the innate system were more frequent at a later point during remission (20 mo.) than directly after achieving CR, even before stimulation. NK cells are reported to reappear during immune reconstitution after autologous bone marrow transplantation in AML and their cytotoxic activity may restore immunity and hence contribute to maintenance of CR [7]. This might indicate enhanced immune reconstitution at this time point.

APL-APC- and remMoDC-stimulated autologous T cells and unstimulated autologous T cells were tested for their cytotoxic activity in a flow-cytometric assay [13]. The autologous remission PBLs used for this assay were isolated directly after achieving CR. Target leukaemia cells, cryopreserved at diagnosis, and effector cells were co-cultured for 6 h, stained by a myeloid marker (CD13) and a specific T cell marker (CD3), respectively, followed by a SYTO-16/7-AAD viability staining [13]. Blast cell apoptosis/secondary necrosis increased from 4% spontaneous apoptosis towards 29% and 55% apoptosis/secondary necrosis after co-culture with unstimulated autologous T cells and remMoDC-stimulated T cells, respectively (E:T 20:1, Fig. 3). The cytotoxic activity of APL-APC-stimulated T cells was highest in all E:T ratios up to 71%. Remarkably, already unstimulated T cells exerted a moderate level of cytotoxicity towards APL blasts while only a minority had a memory phenotype. One might speculate that during chemotherapy destruction of leukaemic cells resulted in uptake of leukaemia-associated antigens by endogenous DC and presentation to T cells. These in vivo stimulated T cells might be accounted for the observed cytolytic activity. As shown previously, AML cells are susceptible to non-MHC-restricted cytotoxic activity of T- $\gamma\delta$ cells [2]. We observed that T- $\gamma\delta$ cells were activated during co-culture with APL-APC as well as remMoDC. This sup-

Table 1

Immunophenotype of autologous remission PBL isolated in complete remission (CR) before and after stimulation by remMoDC or APL-APC, and cytokines alone

Sub population	PBL directly after achieving CR				PBL at 20 mo. of CR			
	Stimulator				Stimulator			
	None	IL-2 and IL-7	MoDC	APL-APC	None	IL-2 and IL-7	MoDC	APL-APC
CD3	72.4%	98.6%	95.4%	94.7%	66.9%	76.8%	72.2%	68.2%
CD4 ⁺ of CD3 ⁺ population	57.2%	44.4%	57.4%	57.5%	63.1%	45.7%	39.5%	23.9%
CD8 ⁺ of CD3 ⁺ population	33.0%	52.9%	21.2%	23.2%	32.9%	42.2%	42.7%	56.8%
T $\gamma\delta$ ⁺ of CD3 ⁺ population	8.6%	2.7%	24.4%	23.3%	3.9%	12.0%	17.8%	19.2%
CD56 ⁺ of CD3 ⁺ population	0.2%	1.2%	4.2%	5.2%	1.9%	9.6%	13.3%	19.8%
CD3 ⁻ CD16 ⁺ CD56 ⁺ (NK cells)	0.5%	1.0%	3.5%	4.0%	13.7%	18.9%	21.0%	24.7%
CD3 ⁺ CD4 ⁺ subpopulations:								
CD45RA ⁺ CD27 ⁺ naïve	72.0%	50.4%	8.1%	4.6%	54.9%	74.3%	61.4%	63.3%
CD45RA ⁻ CD27 ⁺ memory	27.4%	36.1%	30.8%	22.9%	40.9%	12.2%	10.8%	17.7%
CD45RA ⁻ CD27 ⁻ effector-memory	0.3%	10.4%	59.7%	71.3%	4.1%	13.4%	27.8%	19.0%
CD3 ⁺ CD8 ⁺ subpopulations:								
CD45RA ⁺ CD27 ⁺ naïve	87.6%	92.9%	21.8%	18.6%	62.3%	58.9%	44.5%	22.4%
CD45RA ⁻ CD27 ⁺ central memory	11.9%	2.4%	63.8%	64.2%	35.0%	23.9%	29.3%	45.4%
CD45RA ⁻ CD27 ⁻ effector-memory	0.1%	4.4%	13.7%	16.4%	1.6%	14.8%	24.5%	27.6%
CD45RA ⁺ CD27 ⁻ effector	0.5%	0.4%	0.8%	0.8%	1.1%	2.4%	1.7%	4.5%
T $\gamma\delta$ ⁺ , NK-T and NK cells	6.8%	4.9%	30.8%	30.9%	17.6%	35.5%	43.4%	51.2%

ports the observation that DCs participate in activation and expansion of T- $\gamma\delta$ cells *in vitro* [16]. Furthermore, activated T- $\gamma\delta$ cells might have contributed to the cytolytic capacity of remMoDC- as well as APL-APC-stimulated T cells.

To investigate the role of MHC-restriction during apoptosis induction in APL-cells the cytotoxicity assay was repeated in the presence of MHC class I-blocking antibody W6.32 (2.5 $\mu\text{g/ml}$, a kind gift of Dr. S.M. van Ham, Department of Immunopathology, Sanquin Research at CLB, Amsterdam, The Netherlands) or the appropriate isotype control (mouse IgG2a, 2.5 $\mu\text{g/ml}$, Sanquin). MHC class II inhibition of cytotoxic activity was not studied since APL blast cells do not express MHC class II molecules. Due to limited availability of autologous remission PBLs directly after achieving CR, only PBLs isolated at 20 mo. of CR could be used in this assay. Similar co-cultures with autologous APL-APCs and remMoDCs were performed to stimulate the PBLs. In contrast to the first cytotoxicity assay no significant additive effect was observed in the cytolytic capacity of APL-APC-stimulated as compared to remMoDC-stimulated PBL: 58% and 62% apoptosis, respectively. Blocking of MHC class I did not significantly inhibit this cytotoxic activity indicating a predominant non-MHC restricted killing.

It is known that immune cells are suppressed at time of leukaemia presentation and subsequently depleted due to intensive chemotherapy. Newly produced cells are exported from the thymus bearing a naïve phenotype; memory T cells emerge due to homeostasis-driven T cell proliferation and differentiation [3,8]. In our study, the majority of T cells isolated early in lymphocyte recovery were naïve. In contrast, predominantly memory and effector cells were found in the second remission sample (20 mo. CR) which might indicate immune reconstitution. During reconstitution of lymphopenia naïve T cells are more sensitive to activation by weak self-antigens creating a window during which immunotolerance may be broken [6]. In addition, Asavaroengchai et al. showed that immunisation of mice with tumour-lysate pulsed DC during early periods of lymphoid reconstitution induces an effective immune response against breast tumours [1]. Tumour-specific T cells were shown to be more frequent in vaccinated lymphopenic than in vaccinated normal mice. Moreover, delayed vaccination resulted in the induction of T cells with less therapeutic activity [6]. On the other hand, the innate immune system, supplying cells that are able to produce stimuli that activate APCs, and the adaptive immune system, supplying T cells, can

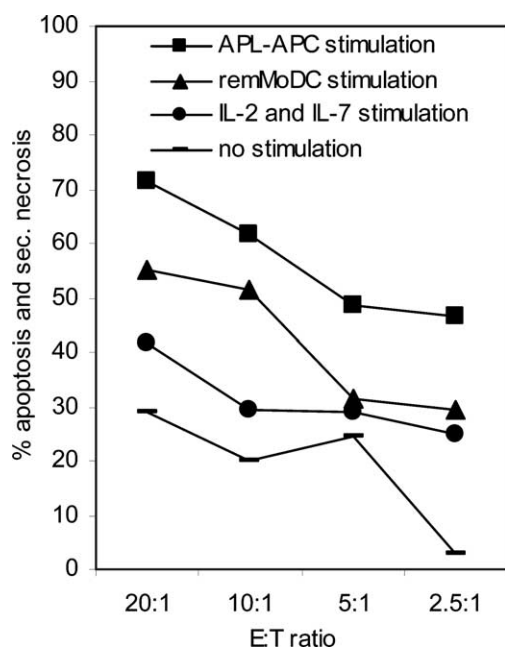


Fig. 3. Cytolytic capacity of autologous remission T cells and rem-MoDC- and APL-APC stimulated T cells towards corresponding autologous blast cells. The assay was performed with remission PBL isolated directly after achieving complete remission. Effector to target ratio is depicted on the X-axis; the Y-axis depicts spontaneous apoptosis and secondary necrosis in the leukaemic cell population as assessed by a flow-cytometric cytotoxicity assay [13]. Spontaneous apoptosis and secondary necrosis of the leukaemic blast cells in the APL sample was 4%.

collaborate in the induction of effector T cells [12]. This might explain the higher amount of CD8⁺ effector cells that was found after APL-APC stimulation of PBLs isolated after 20 mo. as compared to stimulation directly after achieving CR.

To summarise, we showed that APL-APCs induced an autologous immune response against corresponding APL cells *in vitro*. APL-APC-stimulation of remission T cells, obtained directly after achieving CR, elicited a preferential Th1 response, as indicated by an increased IFN- γ production. Moreover, these APL-APC-stimulated T cells were superior to remMoDC-stimulated cells with regard to IFN- γ production and cytotoxic activity suggesting leukaemia antigen-reactive responsiveness. On the other hand, our data illustrated different effects of leukaemia-derived APCs on autologous remission T cells isolated after prolonged remission. Restoration of the innate immune system at this time point might have contributed to this effect. Thus, timing of active specific immunisation with leukemic APCs might be of critical importance to optimise the induction of leukaemia specific T cell responses.

In conclusion, the potential of developing APC-vaccines for treatment of minimal residual disease in APL may contribute to effective immune surveillance.

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