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Tracking the extramedullary PML-RARα-positive cell reservoirs in a preclinical model: Biomarker of long-term drug efficacy

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ABSTRACT

Using an acute promyelocytic leukemia (APL) preclinical model, we show that oncogene–specific PCR (Polymerase Chain Reaction)-based assays allow to evaluate the efficacy of immunotherapy combining all-trans retinoic acid (ATRA) and a DNA-based vaccine targeting the promyelocytic leukemia-retinoic acid receptor alpha (PML-RARα) oncogene. Kaplan–Meier survival analysis according to the peripheral blood PML-RARα normalized copy number (NCN) clearly shows that ATRA + DNA-treated mice with an NCN lower than 10 (43%) formed the group with a highly significant ($p < 0.0001$) survival advantage. Furthermore, a PCR assay was used to assess various tissues and organs for the presence of PML-RARα-positive cells in long-term survivors ($n = 15$). As expected, the majority of mice ($n = 10$) had no measurable tissue level of PML-RARα. However, five mice showed a weak positive signal in both the brain and spleen ($n = 2$), in the brain only ($n = 2$) and in the spleen only ($n = 1$). Thus tracking the oncogene-positive cells in long-term survivors reveals for the first time that extramedullary PML-RARα-positive cell reservoirs such as the brain may persist and be involved in relapses.

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1. Introduction

Preclinical mouse models of cancers are often required to validate novel therapeutic approaches and sensitive monitoring procedures are necessary to assess and quantify tumor regression or clearance. The transplantable transgenic mouse model of acute promyelocytic leukemia (APL) is a well characterized preclinical model which mimics human APL, both in its biological characteristics and its response to conventional therapeutic drugs such as all-trans retinoic acid (ATRA) and arsenic trioxide [1–3]. We have previously reported that a DNA-based vaccine targeted to the promyelocytic leukemia-retinoic acid receptor α (PML-RARα) fusion gene combined with ATRA has a pronounced effect on survival [4–6]. The aim of the study was to better characterize the efficacy of this treatment regimen and to determine molecular markers of clinical outcome. We established a minimal residual disease (MRD) monitoring based on the high sensitivity of detection of PML-RARα transcripts by Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) technology in APL mice based on the technique routinely used for the diagnosis, follow up and prediction of disease evolution in APL patients [7,8]. We also aimed at tracking the presence of PML-RARα-positive cells in various tissues in long-term survivor mice.

2. Materials and methods

2.1. Animal model

All procedures complied with national regulations on the use of animals for experimentation. Transgenic mice bearing the human PML-RARα cDNA (bcr1) were previously constructed in FVB/N inbred strain of mice and a transplantable model was established in which 100% of the mice die of the disease [1]. To allow reproducible APL development, we injected intravenously 10^4 APL spleen blast cells into 8- to 10-week old syngeneic recipients (FVB/N mice bred and maintained under pathogen free conditions in the

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Département d'Expérimentation Animale, Institut Universitaire d'Hématologie, Université Paris-Diderot). Establishment and follow up of leukemia was assessed by the appearance of high leucocyte and low platelet counts (CELL-DYN 3700, Abbott Laboratories, Chicago, IL, USA). We administered retinoic acid by subcutaneous implantation of 21-day release pellets containing 5 mg of ATRA (Innovative Research of America, Saratosa, FL, USA) as previously described [1,4]. For vaccination, we injected 50 μg of DNA (prepared as described in [4]) intramuscularly into the quadriceps of each mouse. As indicated in Fig. 2, APL blast cells were injected on day 0, a week later ATRA pellets were introduced subcutaneously and the following day, DNA was injected intramuscularly at 20-day intervals for three cycles. For second transfer experiments, we injected 10^4 bone marrow (BM) or spleen (SPL) blast cells from untreated APL mouse or 10^7 BM or SPL cells from the ATRA + DNA-treated long-term survivor into 2 (BM) or 3 (SPL) syngeneic (FVB/N) recipients.

2.2. PML-RARα-specific PCR assays

Total RNA was isolated from peripheral blood samples using TRIZOL LS® (Invitrogen, Carlsbad, CA, USA) or bone marrow samples using TRIZOL® (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed on total RNA using GeneAmp RNA PCR Core Kit (Applied Biosystems, Branchburg, NJ, USA). 5 μl of cDNA or plasmid dilutions were used for the subsequent qPCR reaction as described previously [9]. Briefly, we used 1.25 U AmpliTaq Gold DNA polymerase, 0.5 U AmpErase uracil N-glycosylase (UNG), 200 μM dATP, dCTP, dGTP, 400 μM dUTP, forward and reverse primers (5′-GTCTTCCTGCCCAACAGCAACC-3′, 5′-CTCACAGGCGCTGACCCCATAGT-3′) at 200 nM, specific TaqMan probe (5′-6FAM-CAGCCCTCCCTCGCCACCCCCTCTA-TAMRA-3′) at 100 nM, in TaqMan Buffer A with 6 mM $MgCl_2$, in a final volume of 50 μl. All the reagents were from Applied Biosystems, qPCR reactions were performed in an ABI7500 fast thermocycler (Applied Biosystems). After 2 min at 50 °C to allow the destruction by UNG of potential contaminant PCR products, and 10 min at 95 °C to denaturate UNG and activate AmpliTaq Gold, the amplification was carried out by 50 cycles at 95 °C for 15 s and 65 °C for 1 min. In order to correct variations linked to differences in the amount and quality of RNA the 18S rRNA gene, a ubiquitously expressed housekeeping gene, was used as reference gene (18S rRNA Endogenous Control primer and probe set, Applied Biosystems). The number of PML-RARα transcripts was normalized to 10^6 copies of 18S rRNA transcripts. Standard curves were performed using 10-fold serial dilutions of a plasmid containing the complete PML-RARα bcr1 cDNA. Each sample was analyzed in triplicate. The standard curve allowed us to interpolate the unknown copy number of specific cDNA in each sample. The sensitivity of our method allowed us to detect up to 1 PML-RARα-positive cell among 10^4 negative cells (Fig. 1). In some experiments DNA was used instead of RNA because the transplanted leukemia cells contained the cDNA coding for PML-RARα (bcr1). Then, single cell suspension (bone marrow, spleen) or tissue samples (skin, salivary glands, thymus, kidney, muscle, heart, spleen, liver and brain) were lysed using proteinase K (Invitrogen) and DNA was precipitated with ethanol after phenol-chlorophorm-isoamyl alcohol (Invitrogen) extraction. One microgram of DNA was used for qPCR analysis using the same PML-RARα (bcr1) specific primer pair and TaqMan probe as for cDNA. The number of PML-RARα copies was normalized to 10^2 copies of β-actin gene. For β-actin, following forward primer 5′-ACGGCCAGGTCATCAC TATTG-3′, reverse primer 5′- CAAGAAGGAAGGCTGGAAAAGA-3′ and TaqMan probe 5′-6FAM-CAACGAGCGGTTCCGATGCCCT-TAMRA-3′ were used. All assays were run in triplicate. Amplification of at least two of three replicates with PML-RARα Ct values ≤40 (threshold 0.1) was required to define result as PCR positive. There were no significant differences in the Ct values of housekeeping genes 18S rRNA or β-actin between various organs or tissues and between PML-RARα negative and positive samples.

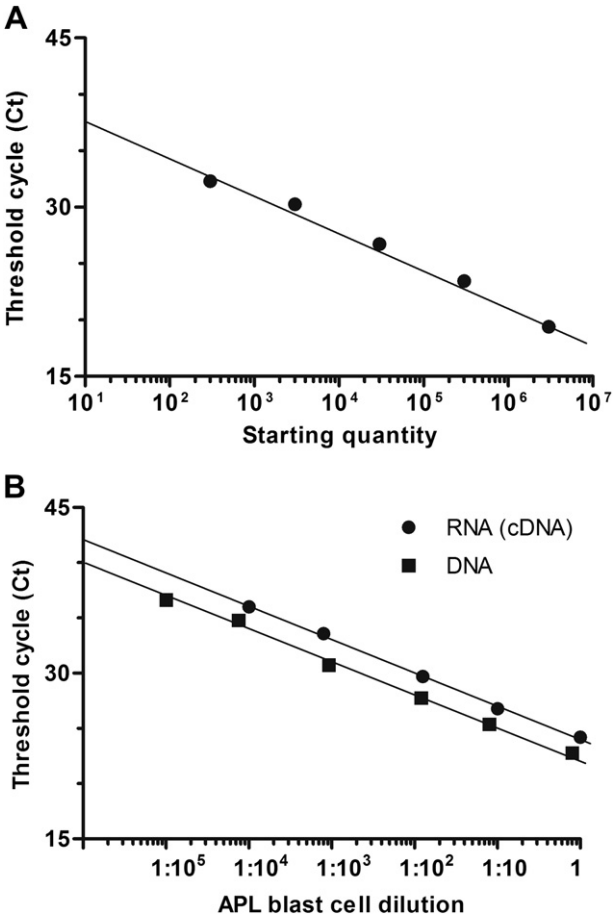

Fig. 1. *Definition of sensitivity*. Standard curve of PML-RARα (bcr1 cDNA) plasmids (A). Plasmids were diluted 10-fold to obtain 3,000,000 to 300 copies. The standard curve shows linear correlation between the Ct values and the initial amounts of PML-RARα plasmids. Serial dilutions of APL blast cells (B). Results of the amplification of cDNA (RNA) or DNA obtained from a dilution series of PML-RARα-positive blast cells in FVB/N normal cells (total number of cells 10^7 in each sample). The curve shows the Ct values for PML-RARα in correlation to the dilution of APL blast cells. There are linear correlations between the log number of APL blast cells and the Ct value from 10^7 to 10^2 for DNA and 10^7 to 10^3 for cDNA (RNA).

3. Results and discussion

3.1. Assessment of leukemic clearance by RT-qPCR assay to monitor disease development and to predict clinical outcome

Syngeneic mice transplanted with the spleen cells from APL transgenic mice expressing a 2,853 bp of the human PML-RARα cDNA (bcr1) develop an APL [1]. These APL mice were treated by ATRA alone ($n = 55$), combined with PML-RARαFrC DNA-based vaccine ($n = 94$) or left untreated ($n = 65$). To monitor the PML-RARα-positive cell clearance, RNA was isolated from peripheral blood (PB) samples and standardized MRD monitoring protocol was applied. Thus we show that in this model, disease status is well reflected by PB platelet counts and PML-RARα transcript levels (Fig. 2A–C).

Treatment with ATRA alone controls disease up to 50 days when relapses are seen with the recurrence of thrombopenia and

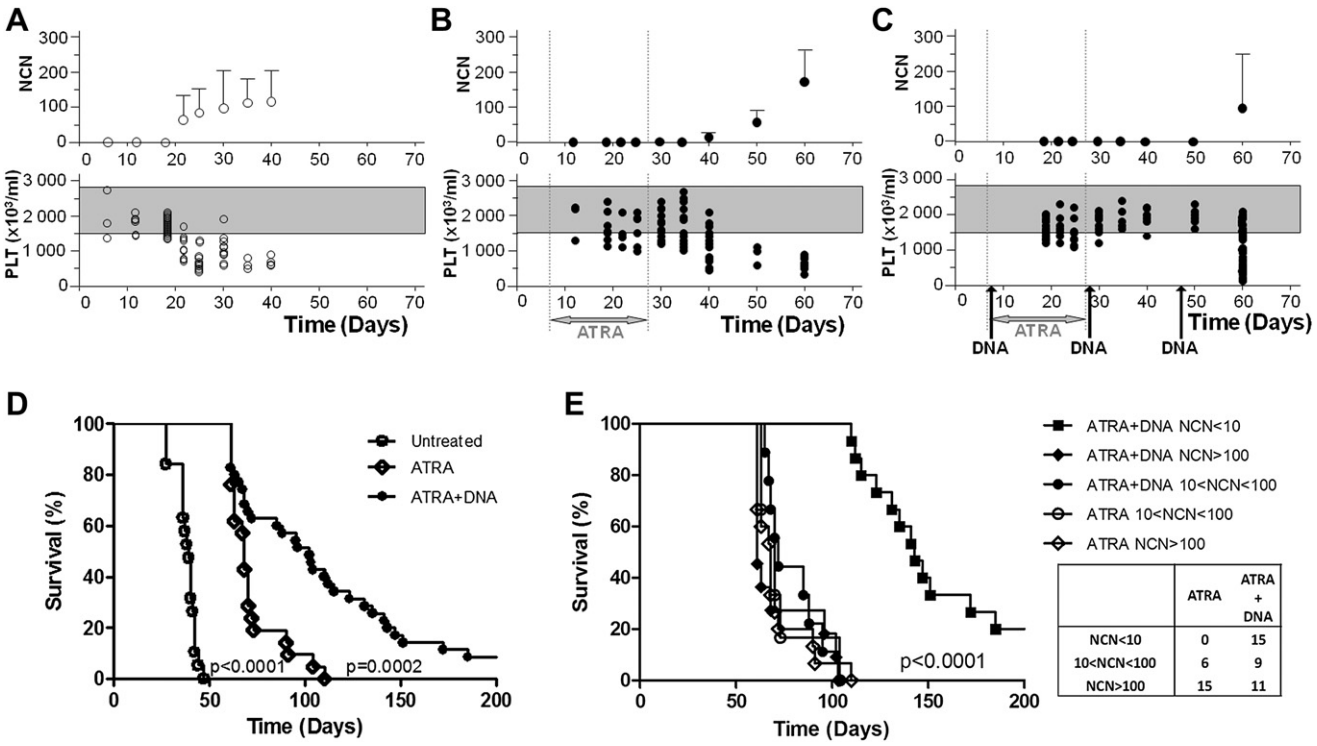

Fig. 2. *Follow up of PML-RARα transcripts in APL mice is consistent with platelet counts and is of prognostic value.* FVB/N mice were injected intravenously with 10^4 APL cells on day 0 and were left untreated ($n = 65$) (A), treated with ATRA alone ($n = 55$) (B) or in combination with PML-RARα-FrC DNA vaccine ($n = 94$) (C). ATRA (5 mg) was administered on day 7 by subcutaneous implantation of 21-day release pellet. Effect of ATRA is depicted by dotted lines in panels (B) and (C). DNA vaccination was injected intramuscularly (50 μg/leg) on days 8, 28 and 48 as indicated by black arrows in panel (C). Peripheral blood samples were collected during the life span of untreated mice or until day 60 in ATRA- and ATRA + DNA-treated group. PML-RARα transcripts and platelet (PLT) counts were examined in each sample. Results are expressed as normalized copy number (NCN) of PML-RARα transcripts per 10^6 18S rRNA copies. Each point represents mean value of at least 3 individuals, error bars represent SD. PLT counts per microliter of peripheral blood were determined by automated counting, results are shown as individual values. Gray rectangular box in panels (A–C) represents values obtained from non-leukemic FVB/N mice. (D) Kaplan–Meier survival analysis of ATRA- ($n = 21$), ATRA + DNA-treated ($n = 35$) or untreated ($n = 18$) mice. (E) Mice treated with ATRA alone or ATRA + DNA were grouped according to the level of NCN at day 60 after leukemia engraftment. Survival was analyzed by the Kaplan–Meier method; comparison between groups was analyzed by log-rank tests. Number of individuals within each group is given in table (inset).

detectable levels of PB PML-RARα transcripts (Fig. 2B). Survival of APL mice treated by the combination ATRA + DNA is significantly ($p = 0.0002$) superior to that of mice treated by ATRA alone [4,6] (Fig. 2D). Treatment with ATRA + DNA prolongs PML-RARα negative life span up to 60 days (Fig. 2C) correlating with the achieved survival advantage (Fig. 2D).

Interestingly, of the ATRA-treated mice still alive on day 60 ($n = 21$), 15 mice (71%) had PML-RARα normalized copy number (NCN) > 100, 6 mice (29%) an NCN between 10 and 100 and none an NCN < 10 (Fig. 2E). In contrast, in the ATRA + DNA-treated mice still alive on day 60 ($n = 35$), 11 mice (31%) had NCN > 100, 9 (26%) an NCN between 10 and 100 and 15 mice (43%) an NCN < 10. Kaplan–Meier analysis of APL mouse survival according to the stratification based on PML-RARα NCN clearly indicates that in ATRA + DNA-treated mice those with RT-qPCR results lower than 10 NCN (43%) constituted the group with a significant survival advantage ($p < 0.0001$), confirming thus the therapeutic benefit of the combined therapy. Therefore, monitoring PB cells by RT-qPCR analysis of PML-RARα offers a measurable evaluation of disease control during treatment pointing up the power of minimal disease quantitative assessment to differentiate therapeutic strategies in preclinical mouse models as well as in patients [7,10].

Furthermore, it is well known that in this preclinical model, suboptimal ATRA treatment leads to differentiation of APL cells and complete remission but relapses always occur and no cure is achieved with any mice alive after 120 days [3,4,6]. On the contrary in the ATRA + DNA-treated groups, 30% of the mice are still alive with blood cell counts and spleen sizes within the normal range (data not shown) and referred to as long-term survivors (LTS) [4,6] (Fig. 2D, E). In order to further assess tumor burden, LTS mice were sacrificed at different time intervals (Table 1). RNA was isolated from PB and BM samples and the standardized MRD monitoring protocol was applied (Table 1). Our data show the absence of cells expressing PML-RARα transcripts in PB and BM in all LTS tested suggesting a complete molecular remission. When we inoculated 10^7 of BM or spleen cells originating from ATRA + DNA-treated LTS ($n = 6$) into secondary recipients ($n = 30$), none of the injected mice developed APL (followed up > 200 days). Injection of 10^4 BM or spleen cells from untreated APL mice was however sufficient to establish APL and all recipients died between 25 and 37 days after inoculation (Table 1).

3.2. Assessment of tissue clearance of APL cells

The combined results of MRD monitoring and absence of leukemia in secondary transplanted mice strongly suggest that the leukemia-initiating cells (LIC) of this mouse model are targeted by ATRA + DNA combined therapy. We have investigated the involved immune responses [6] and we attributed the anti-leukemic effect to CD4-mediated response as depletion of CD4+ T-cells in LTS leads to relapse and death of all mice within 3 months. Thus the immune responses elicited by ATRA + DNA vaccine may control either residual PML-RARα leukemic cells or PML-RARα LIC as none are detectable in PB and BM of LTS. These previously published results

Table 1
Investigation of long-term survivors.

Treatment	Mouse no.	Sac. (day)	PML-RARα RNA		PML-RARα DNA				2nd transfer		
			PB	BM	SPL	Brain	Liver	Lung	Transferred cells	Diseased /total	Survival (days)
No	9457	12	Neg	0.07	0.80	Neg	0.05	0.02	n.d.		
	9460	22	167.29	5.92	36.12	1.29	37.02	7.07	n.d.		
	9485	33	203.05	9.38	152.03	3.44	198.44	23.66	BM	12/12	29–37
									SPL	12/12	25–32
ATRA + DNA	5530	131	Neg	Neg	Neg	Neg	Neg	Neg	BM	0/2	>200
									SPL	0/3	>200
	5584	151	Neg	Neg	Neg	Neg	Neg	Neg	BM	0/2	>200
									SPL	0/3	>200
	5585	172	Neg	Neg	Neg	Neg	Neg	Neg	BM	0/2	>200
									SPL	0/3	>200
	4084	326	Neg	Neg	Neg	n.d.	n.d.	n.d.	BM	0/2	>200
									SPL	0/3	>200
	5516	411	Neg	Neg	Neg	Neg	Neg	Neg	BM	0/2	>200
									SPL	0/3	>200
	5581	427	Neg	Neg	Neg	Neg	Neg	Neg	BM	0/2	>200
									SPL	0/3	>200
	9540	148	Neg	Neg	Neg	Neg	Neg	Neg	n.d.		
	4944	235	Neg	Neg	Neg	Neg	Neg	Neg	n.d.		
	6828	243	Neg	Neg	**0.02**	**0.13**	Neg	Neg	n.d.		
	7089	243	Neg	Neg	**0.01**	**0.01**	Neg	Neg	n.d.		
	4086	336	Neg	Neg	Neg	n.d.	n.d.	n.d.	n.d.		
	4081	344	Neg	Neg	Neg	n.d.	n.d.	n.d.	n.d.		
	5293	473	Neg	Neg	Neg	**0.12**	Neg	Neg	n.d.		
	5565	498	Neg	Neg	Neg	**0.02**	Neg	Neg	n.d.		
	5537	516	Neg	Neg	**0.02**	Neg	Neg	Neg	n.d.		

Mice were sacrificed (sac) at indicated day and samples obtained from peripheral blood (PB) and bone marrow (BM) were examined for the presence of PML-RARα transcript (RNA) and spleen (SPL) and other organs (BRAIN, LIVER and LUNG) for the presence of PML-RARα DNA (DNA). Positive results are expressed as numbers and indicate PML-RARα copies normalized to housekeeping gene copies (NCN) as decribed in Materials and Methods. Three representative individual APL mice from the untreated experimental group are shown as positive controls. Not done (n.d.). Bold values represent positive values.

[6] have prompted us to track PML-RARα-positive cells in the other organs and tissues. We took advantage of the presence of PML-RARα cDNA transgene in the transplanted leukemia cells and used genomic DNA as template for a qPCR assay. Using DNA as an alternative to RNA enabled us to use 10 times more template in order to increase the detection rates of leukemic cells potentially including also the quiescent ones. Various tissues were examined such as skin, salivary glands, thymus, kidney, muscle, heart (data not shown), spleen, liver, lung and brain (Table 1) in LTS ($n = 15$) and APL controls ($n = 3$). Only 5 mice were positive: 4 mice with a weak positive signal in the brain (2 of these mice had also a weak signal in spleen) and one mouse with only a weak signal in the spleen (Table 1). Thus the efficacy of ATRA + DNA vaccine may lie in the control by a CD4-mediated immune response of leukemic cells which are not detectable in PB and BM but may reside in the brain or spleen.

Interestingly, although the most common site of relapse in APL patients is the BM, cases of extramedullary (EM) relapses are observed [11]. The occurrence of EM disease has long been considered a rare event in APL patients treated with chemotherapy alone, whereas this phenomenon has been increasingly reported in the ATRA era [12]. Central nervous system (CNS) is the preferential site of EM involvement in APL. EM relapse, including the CNS, can occur either in isolation or associated with BM involvement [13], though ATRA has been documented to cross the blood–brain barrier and to be efficient in glioblastomas and neuroblastomas [14,15].

4. Conclusion

Our study underlines the strong potential of the APL preclinical model to validate and optimize therapeutic treatments. We have applied PCR-based techniques allowing us to track the leukemic cells in long-term survivors obtained by immunotherapy combining ATRA and DNA-based vaccine targeting the PML-RARα fusion gene. Our results show that in a complete molecular response assessed on peripheral blood and bone marrow, extramedullary PML-RARα-positive cell reservoirs such as the brain may persist and be involved in relapses. Targeting these reservoirs of residual or leukemia-initiating cells should be the goal of the future therapeutic regimens in APL.

Conflict of interest disclosure

RAP and CC have patents pending through Université Paris-Diderot related to technology employed in this present study. RAP and CC are founding members of and have financial interest in a company (Vivavacs). The remaining authors declare no conflict of interest.

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