Brief report

DNA vaccination with all-*trans* retinoic acid treatment induces long-term survival and elicits specific immune responses requiring $CD4^+$ and $CD8^+$ T-cell activation in an acute promyelocytic leukemia mouse model

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DNA vaccination and all-*trans* retinoic acid (ATRA) result in a survival advantage in a mouse model of acute promyelocytic leukemia (APL). Depletion of $CD4^+$ or $CD8^+$ cells abolished this effect. $CD4^+$ depletions of long-term survivors resulted in relapse and death within 3 months, thus demonstrating the need of both $CD4^+$ and $CD8^+$ subsets for the generation of DNA-driven antileukemic immune responses and underscoring a crucial role of $CD4^+$ cells in the maintenance of durable remissions. Degranulation and cytotoxic carboxyfluorescein diacetate succinimidyl ester–based assays showed major histocompatibility complex–restricted APL-specific T cell–mediated immune responses. Sorted APL-specific $CD8^+CD107a^+$ T cells showed an increase of antileukemic activity. Effectors from ATRA + DNA–treated mice were shown to secrete interferon-γ when stimulated with either APL cells or peptides from the promyelocytic leukemia-RARα vaccine-derived sequences as detected by ELISpot assays. Our results demonstrate that DNA vaccination with ATRA confers the effective boosting of interferon-γ–producing and cytotoxic T cells in the leukemic mice. (Blood. 2010; 115:653-656)

Introduction

Despite improved molecular characterization of malignancies and development of targeted therapies,[1] acute leukemia is not curable and few patients survive more than 10 years after diagnosis.[2,3] To further improve outcome, we studied the potential efficacy of boosting the patient's immune response. Therapeutic vaccination aimed at promoting T-cell immunity requires: activation of the innate system, optimal presentation of major histocompatibility complex (MHC) class I–binding peptides, and provision of $CD4^+$ T-cell help. DNA vaccines have the potential to supply all of these. In an animal model of acute promyelocytic leukemia (APL),[4] we developed a promyelocytic leukemia-RARα (PML/RARα)–targeted DNA-based vaccine[5] and show that DNA combined with all-trans retinoic acid (ATRA) has a pronounced survival advantage, concomitant with time-dependent antibody production,[5,6] and an increase in interferon-γ (IFN-γ).[5] A similar approach confirmed these findings.[7] The role of ATRA as an immunomodulator is well documented.[8-11] Furthermore, Westervelt et al[12] show that ATRA responses are influenced by the presence of an intact adaptive immune response.

The present study is aimed at investigating the immune responses involved in the antileukemic effect of the combined ATRA + DNA therapy, particularly those mediated by $CD4^+$ and $CD8^+$ T cells.

Methods

We extended our previous study using the same protocol illustrated in supplemental Figure 1A (available on the *Blood* website; see the Supplemental Materials link at the top of the online article) where we combined ATRA (5 mg) and a PML-RARαFrC DNA construct in an APL mouse model.[5] Peripheral blood (PB) was collected approximately every 20 days from day 19 after APL engraftment to follow the mice clinically. At specific days, mice were killed to evaluate responses. Methods are detailed in the figure legends. Animal studies were undertaken according to the guidelines of the institutional animal care committee of Hôpital Saint-Louis.

Results and discussion

DNA vaccination combined with ATRA induces long-term survival

Our finding of ATRA + DNA treatment significantly extending survival compared with ATRA alone was confirmed in additional protocols (Figure 1A-B; supplemental Figure 1B-C). We have previously reported that DNA alone elicited a modest survival advantage[5] and that FrC alone failed to give long-term survival[13] (supplemental Figure 1D).

ATRA + DNA–treated mice had increased white blood cell counts on day 21 after leukemia engraftment, possibly resulting from the

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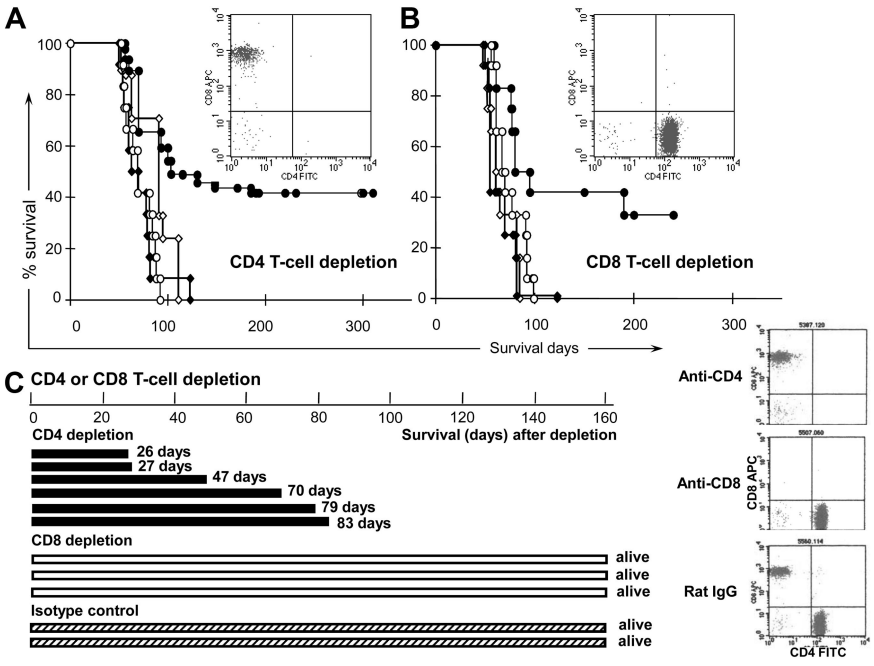

Figure 1. Requirement of CD4$^+$ and CD8$^+$ T cells for prolonged survival induced by combined therapy. Survival was analyzed by the Kaplan-Meier method; comparison between groups was analyzed by log-rank tests. Survival curves of (A) APL mice treated with ATRA + PML-RARαFrC DNA (ATRA + DNA; ●, isotype control undepleted, n = 12; and ○, CD4-depleted, n = 12; $P < .001$) and APL mice treated with ATRA alone (◇, isotype control undepleted, n = 8; and ◆, CD4-depleted, n = 12). (B) APL mice treated with ATRA + DNA (●, isotype control undepleted, n = 12; and ○, CD8-depleted, n = 12; $P < .001$) and APL mice treated with ATRA alone (◇, isotype control undepleted, n = 8; and ◆, CD8-depleted, n = 12). APL mice were depleted of either CD4$^+$ (A) or CD8$^+$ (B) T cells by repeated (5-day interval) intraperitoneal injections of 0.2 mg of subset-specific monoclonal antibodies starting from day 10 after leukemia engraftment (1 day after the DNA vaccination). Isotype control group mice received 0.2 mg of rat IgG per injection. The efficiency of depletion was monitored by flow cytometry (representative dot plots of PB gated on CD3$^+$ populations). These early depletions were conducted at least twice. (C) LTSs from ATRA + DNA–treated groups were CD4- or CD8-depleted as described. The injection of subset-specific (CD4 [■]; and CD8 [□]; depletion, respectively) or rat IgG (isotype control, ▨) started on day 120 after engraftment of leukemic cells. Days of death after the beginning of depletion are indicated. The efficiency of depletion was monitored by flow cytometry (representative dot plots of PB gated on CD3$^+$ populations). ATRA (5 mg) was administered by subcutaneous implantation of 21-day release pellets.

increases in CD4$^+$ and CD8$^+$ cells (supplemental Figure 2A). ATRA- and nearly half of the ATRA + DNA–treated mice had reduced platelets by day 60 (supplemental Figure 2A). Long-term survivors (LTSs) from the ATRA + DNA–treated group (> 120 days) had significantly reduced bone marrow blast counts (2%-12%; supplemental Figure 2B). ATRA-alone–treated mice died by day 90.

PB absolute counts of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ subsets, as well as natural killer (NK), Tγδ, and B lymphocytes, were measured by flow cytometry. Mice treated with ATRA alone had lower CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ absolute counts compared with ATRA + DNA–treated mice at day 21 (supplemental Figure 3). The increased CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ absolute counts in ATRA + DNA–treated animals were associated with improved survival (supplemental Figure 3A-B), probably reflecting mobilization of immune responses.

Increasing the number of T cells of ATRA-treated mice by infusion of CD3$^+$CD4$^+$ cells originated from ATRA- or ATRA + DNA–treated mice did not rescue these mice from relapse and death (supplemental Figure 4). This indicates that the protective effect of the combined therapy is not quantitative but qualitative.

No differences of NK, Tγδ, and B cells in the PB were detected among untreated, ATRA-, or ATRA + DNA–treated APL mice (data not shown).

CD4$^+$ and CD8$^+$ T cells are required for prolonged survival induced by ATRA + DNA therapy

To examine the implication of CD4$^+$ or CD8$^+$ T cells, leukemic mice were depleted of either CD4 or CD8$^+$ T cells.

Injections of CD4$^+$ or CD8$^+$ subset-specific monoclonal antibody (mAb) starting after the first DNA vaccination did not affect the survival of untreated (data not shown), ATRA-treated, or rat IgG–treated vaccinated mice (isotype control; Figure 1A-B). The depletion of CD4$^+$ or CD8$^+$ subsets completely abolished the antileukemic effect of the combined therapy. None of the CD4- or CD8-depleted ATRA + DNA–treated mice survived after day 100 (Figure 1A, $P < .001$; and Figure 1B, $P = .045$, respectively). These data reflect the need of these T cells for the generation of DNA vaccine driven anti–APL-protective immune responses.

Depletions of CD4$^+$ T cells in LTSs from the ATRA + DNA–treated group led to relapse and death of all mice (n = 6) between 26 and 83 days after the initiation of depletion (Figure 1C). All CD8-depleted LTSs as well as those treated with rat IgG were alive on day 160 after depletion. These results show the crucial role of CD4$^+$ T cells in the maintenance of the antileukemic effect of DNA vaccination and suggest that, although these mice are in complete remission, they still need CD4$^+$ T-cell activity for maintenance.

APL-specific T-cell responses in ATRA- and ATRA + DNA–treated leukemic mice

To evaluate the cytotoxic activity, we used the carboxyfluorescein diacetate succinimidyl ester (CFSE)–based assay.[14] Effectors from ATRA-treated (Figure 2A) or ATRA + DNA–treated (Figure 2B) APL mice had specific cytotoxic activity against APL assayed on days 34 to 57. Effectors from LTSs from ATRA + DNA–treated mice showed less than 60% target survival at a ratio of 50:1 (Figure 2C). To demonstrate that the killing of APL cells was MHC-restricted, we performed cytotoxic assays in the presence of blocking mAb. The specific cytotoxicity observed with the isotype control Ab was significantly reduced when APL target cells were preincubated with

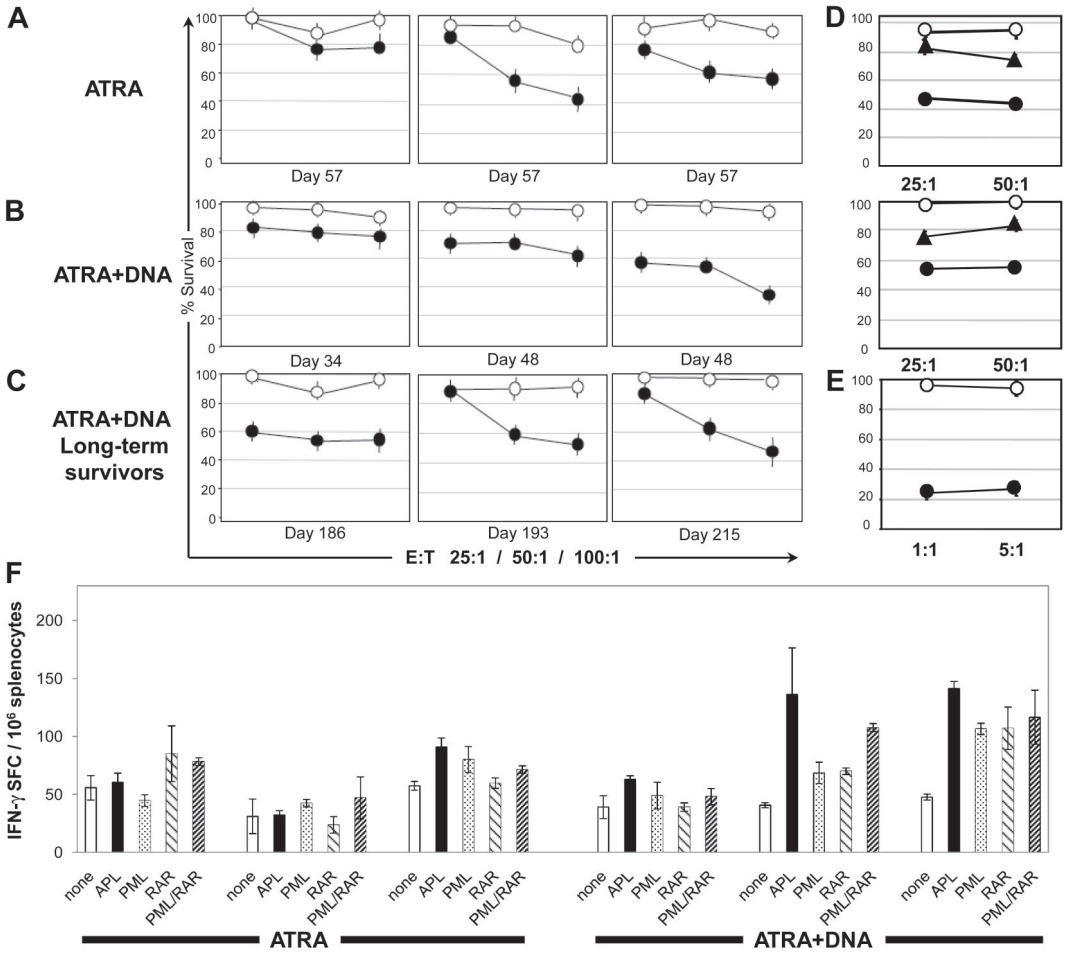

Figure 2. ATRA- and ATRA + DNA–treated mice develop specific immune responses against APL cells and PML-RARα sequences. Cytotoxic CFSE-based assays were performed as previously described.[14] APL cells and control FVB/N Con A blasts were resuspended at 2×10^7/mL in Dulbecco phosphate saline buffer (Eurobio) and labeled with 10μM CFSE (Molecular Probes Europe) for 20 minutes at 37°C. The reaction was stopped by adding an equal volume of FCS, followed by incubation for 2 minutes at room temperature. After 2 washes, 10^4 labeled targets were incubated at the following E:T ratio: 0:1, 25:1, 50:1, and 100:1, for 6 hours at 37°C in 200 μL of culture medium (RPMI); 10 000 Trucount beads were then added with propidium iodide (PI; 1 μg/mL) to quantify the number of viable cells. The samples were then immediately analyzed by flow cytometry. For each sample, 2000 microbeads were acquired. Only $CFSE^+$ and PI^- cells were considered as good survivors. The percentage survival was calculated as follows: % survival (y-axis) = [absolute count of viable CFSE + PI^- targets with effector (t = 6 hours)]/[absolute count of viable CFSE + PI^- targets only (t = 6 hours)] × 100. Effector cells from ATRA- and ATRA + DNA–treated mice have a specific cytotoxicity against APL cells (●) compared with 48-hour induced syngeneic (FVB/N) Con A blasts (○) in a CFSE-based assay. (A) Effectors from 3 ATRA-treated mice assayed at day 57. (B) Effectors from 3 ATRA + DNA–treated mice assayed at days 34 and 48. (C) Effectors from 3 LTSs (ATRA + DNA–treated group), assayed at days 186, 193, and 215; E:T ratio, 25:1, 50:1, and 100:1. Assays were done in triplicate, and values are mean ± SD. (D) The cytotoxicity of effector cells from ATRA + DNA–treated mice is MHC restricted. CFSE-based cytotoxic assays were performed with a blocking anti–H-$2D^q$/H-$2L^q$ monoclonal antibody (KH117 clone). After staining with CFSE, APL and CBF (○) targets were preincubated with saturating amounts (10 μg/mL) of either the anti–H-2^q mAb (▲) or the isotype control IgG2aκ antibody (●); E:T ratio, 25:1 and 50:1. Assays were done in triplicate, and values are mean ± SD. (E) $CD107a^+$ activated T cells from ATRA + DNA–treated LTSs are endowed with an APL-specific cytotoxic activity. APL-activated T cells from LTSs (day 298) were separated by sorting $CD3^+CD8^+CD107a^+$ cells, using the FACSVantage cell sorter and assessed in a CFSE assay using as targets FVB/N cells (○) and APL cells (●); E:T ratio, 1:1 and 5:1. Assays were done in triplicate, and values are mean ± SD. (F) Ex vivo detection of T-cell responses in APL mice after treatment. Splenocytes were harvested from individual ATRA- and ATRA + DNA–treated mice at day 22 after APL engraftment. A total of 10^6 splenocytes were incubated with 0.25×10^6 APL cells or with 1μM each of 3 different peptides (PML, RARAα, and PML/RARα) for 18 hours at 37°C in 5% CO_2 to assess T-cell responses to the PML/RARα sequences of the vaccine. The peptide sequences are: PML, EVFLPNSNHVASGAGEAA (18-mer); RARα, AIETQSSSSEEIVPSPPS (18-mer); PML/RARα, ASGAGEAAIETQSSS (15-mer). The numbers of spot-forming cells (SFC) secreting IFN-γ were assessed ex vivo by ELISpot assay. Assays were done in triplicate; baseline responses without stimulation (none) are indicated. The mean values with SD of SFC per million splenocytes are shown on the y-axis. Data from individual mice are shown; representative data from 1 of 2 identical experiments are shown. ATRA (5 mg) was administered by subcutaneous implantation of 21-day release pellets.

the anti–H-$2D^q$/L^q Ab (increased survival from 52% with isotype control Ab to 80% with H-2^q–specific mAb at a 25:1 effector/target [E:T] ratio), thus demonstrating that the cytotoxic activity was MHC-restricted (Figure 2D). The remaining cytotoxic activity observed in the presence of the blocking mAb could reflect the residual H-$2K^q$ MHC-restricted activity or nonrestricted cytotoxicity mediated by NK or Tγδ cells.

Because degranulation of cytotoxic T cells and killing of targets are correlated,[15,16] we performed the CD107a mobilization assay using LTS splenocytes. APL-specific activation of $CD3^+CD8^+$ T cells (1.5%-17%) was observed (supplemental Figure 5E).

$CD3^+CD8^+CD107a^+$ sorted cells from an ATRA + DNA–treated LTSs induced cytotoxicity, with less than 25% of viable APL targets rescued at a 1:1 E/T ratio (Figure 2E) demonstrating APL-specific cytotoxic activity.

DNA vaccines usually stimulate T_H1 cytokines. The cytokine release profile of ATRA + DNA–treated LTSs was analyzed earlier than 300 days assayed previously.[5] Analysis showed APL-specific

increases in T_H1 cytokines (23-fold for IFN-γ and 5-fold for TNF-α; supplemental Figure 6).

To assess priming of APL-specific T cells, effectors from ATRA- and ATRA + DNA–treated APL mice were assayed in an ELISpot IFN-γ assay. None of the ATRA-treated APL mice showed any substantial secretion of IFN-γ secretion compared with the baseline secretion. In contrast, DNA vaccine induced IFN-γ secretion (Figure 2F). Interestingly, when the LTSs from the ATRA + DNA group were assayed, increases of IFN-γ–producing cells were observed (supplemental Figure 7).

Overall, using complementary methods, we show that APL-specific T cells remained active in vaccinated animals long after the last boost of DNA. The aim of DNA vaccination is to target tumor cells not eradicated by current protocol, preferably in the setting of minimal disease load. The power of the immune system is clear from the effectiveness of passive immunity. DNA vaccination as an approach for immunotherapy is increasing.[17,18] Although ATRA may be a good adjuvant for boosting immune responses,[10,11,19,20] the mounted immune responses were insufficient to contain the disease. Combining DNA with ATRA resulted in increased APL-specific immune responses, with MHC-restricted $CD8^+$ T-cell responses and increased IFN-γ production, which rescued the disease. Together with the finding of a major role for $CD4^+$ T cells in maintaining the durable remissions, these data suggest that, in human clinical trials, the combination of ATRA + DNA vaccine should control minimal residual disease. This study provides insights into the immunology of DNA vaccines, and this may be relevant for targeting other malignancies.

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Authorship

Contribution: K.F. and K.P. performed research and analyzed data; C.L.P., M.R., V.B., and P.K. performed research; M.A. performed research and contributed to statistical design and analysis; A.J. reviewed the histology slides; M.-E.N. reviewed the bone marrow slides; R.W. contributed to statistical design and analysis; D.C. and C.C. designed research and wrote the paper; and M.P., H.M.-T., and R.A.P. designed and performed research and wrote the paper.

Conflict-of-interest disclosure: R.A.P., C.C., and D.C. have patents pending through Inserm related to technology employed in this present study. R.A.P. and C.C. are founding members of and have financial interest in a company (Vivavacs) and are members of the executive and scientific boards negotiating rights to these same patents. The remaining authors declare no competing financial interests.

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