Murine acute promyelocytic leukemia cells can be recognized and cleared in vivo by adaptive immune mechanisms

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Murine acute promyelocytic leukemia cells can be recognized and cleared \textit{in vivo} by adaptive immune mechanisms

A large number of recent studies have investigated the potential for enhancing the immune response to detect and eliminate malignant cells in the host.\textsuperscript{1,2} Exploiting the molecular specificity of cellular immune recognition represents an attractive method for developing tumor-specific therapies for cancer. The identification of T lymphocytes invading and/or surrounding solid tumors \textit{in vivo} suggested that T cells recognize cancer cells, although little evidence exists that these T cells are capable of killing cells within the tumor. In the case of leukemia, the evidence for T-cell detection and elimination has been provided by clinical responses to donor lymphocyte infusions (DLI) in patients following allogeneic bone marrow transplantation. DLI can provide curative allogeneic graft-versus-leukemia (GvL) responses, but they also cause graft-versus-host disease (GvHD).\textsuperscript{3,4} In some leukemia patients, cytotoxic T lymphocytes (CTL) specific for the leukemic clones are capable of killing the leukemic cells \textit{in vitro}.\textsuperscript{5} Despite the potential for T cells to detect and eliminate leukemia cells, the majority of AML patients have, by definition, failed to mount effective immune responses against their tumors. The reasons for this failure remain unclear, but may include inaccessibility of the tumor cells, evasion of the immune response by the malignant cells, and/or inefficient stimulation of the immune system by the neoantigens of the tumor cell.\textsuperscript{6}

Acute promyelocytic leukemia (APL) is primarily associated with the t(15;17) (q22;q11.2) translocation, which leads to the expression of novel fusion proteins; part of the promyelocytic leukemia (PML) gene is fused in frame with exons 3-9 of the retinoic acid receptor alpha (RARx) gene.\textsuperscript{7}
We have previously generated transgenic mice that express human PML-RARα and RARα-PML (the forward and reciprocal gene products of the translocation) in early myeloid cells under the control of a human cathepsin G transgene.9,10 Approximately 60% of these mice develop APL after a long latent period; the APL cells from spleens of leukemic mice can be cryopreserved, and thawed for subsequent in vitro and in vivo study. These cells can cause fatal APL after transfer into immunocompetent, genetically compatible secondary hosts.9,10

A recent report has shown that a human PML-RARα breakpoint-derived DNA vaccine can prevent APL in a secondary murine transplant model.11 Here, we show that murine APL cells are capable of being immunologically recognized, and that the adaptive immune system is important for the clearance of murine APL cells in vivo. We confirm that protective immunity can be generated by DNA immunization with full-length PML-RARα, however, equal protection was provided by the PML portion of the human fusion gene, which differs significantly from murine PML. Our results also suggest that the immune response against APL cells probably does not involve fusion breakpoint sequences. Thus, while APL cells can be recognized and controlled by the immune system in vivo, the efficacy of DNA vaccination in murine APL models may involve xeno-specific responses against the human PML portion of the transgenic fusion protein. Regardless, our data strongly suggest that effective vaccination approaches should be possible when appropriate tumor-specific antigens are identified for this disease.

**Design and Methods**

**Mice**

Recipient mice for APL tumor challenge included male C57Bl/6xC3H F1 (Taconic, Germantown, NY, USA), male C3H SCID (Taconic), and female C57Bl/6 (Jackson Labs, Bar Harbor, ME, USA) animals. All mice were 8-12 weeks of age at the time of tumor challenge.

**Cryopreserved APL cells and tumor challenge**

The tumor cells used in secondary transfer experiments were derived from cryopreserved splenocytes from leukemic hCG-PML-RARαxchg-C-RARα-PML transgenic mice in a C57Bl/6xC3H F1 (B6C3H) background.9 Multiple doses of each APL sample were administered intraperitoneally to B6C3H animals to determine the minimum dose that reproducibly led to death from APL in 5/5 recipients (LD50). The LD50 in immunocompetent animals ranged from 10^4-10^6 APL cells. For allogeneic primary challenge and re-challenge, cell numbers 3-10 fold above the LD50 were used. Doses of 10^6 APL cells were injected for tumor challenges following DNA immunization. Recipient animals were monitored for health and physical appearance. Peripheral blood from moribund animals was sampled via the retro-orbital plexus for blood counts, and animals with elevated WBC counts (>30,000/μL) were sacrificed for histopathologic confirmation of disease in the spleen and liver. Statistical calculations for all APL cell transfer experiments were performed by Kaplan-Meier survival analysis, comparing pairs of curves by the log-rank test, using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

**Flow cytometry**

APL cells and B6C3H F1 splenocytes were stained with 1 μg of each antibody or the appropriate isotype control antibody according to the manufacturer's instructions. Conjugated antibodies included CD34-PE, CD45-PerCP, and PE-conjugated Gr-1, K-1, I-A^k, and I-A^b (BD Biosciences Pharmingen, San Diego, CA, USA).

**Peptide binding and proliferation assays**

Ten 10-mer peptides spanning the bcr-1 PML-RARα fusion breakpoint, and positive (OVA peptide) and negative control peptides, were synthesized by standard methods using an ABI 433A peptide synthesizer (Biomolecules Midwest Inc., St. Louis, MO, USA). RMA-S Class I stabilization assays were performed as described elsewhere.12 3H-thymidine incorporation assays were performed according to standard procedures using splenocytes from DNA-immunized survivors of APL cell challenges as responders.13 Stimulator cells were irradiated wild type B6C3H splenocytes that were or were not precultured overnight with 100 μM concentrations of the indicated 10-mer peptides.

**DNA immunization**

The vector pcDNA3.1 (Invitrogen) was used for all DNA immunizations. The following cDNA were subcloned into pcDNA3.1: bcr-1 PML-RARα, bcr-3 RARα-PML, human PML (exons 1-6), human RARα (exons 3-9), murine GM-CSF, and murine CD40 ligand (CD40L or CD154). The GM-CSF and CD40L cDNA were generously provided by Dr. Thomas Kipps. Animals were vaccinated four times at weekly intervals by intramuscular injection in the hind flank with 50 μg of immunizing plasmid DNA, with or without 50 μg each of the adjuvant GM-CSF and CD40L plasmid DNA. One week after the final DNA boost, animals were challenged with 10^6 live APL cells intraperitoneally and monitored for development of APL as described above. Three independent cryopreserved APL samples were used in these experiments (10552, 10759, and 10822).

**Western blotting**

Transient transfection of K562 cells by electroporation and Western blotting of RIPA lysates, harvested
24 hours after electroporation, was performed as previously described. Antibodies included anti-RARα (C-20), anti-PML (PG-M3), and anti-actin (C-11) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

**Table 1. MHC class I and II surface staining in the CD34⁺ APL cell fraction.**

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<th>MHC class I</th>
<th>MHC class II</th>
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**Results**

**Murine APL cells express MHC class I and II molecules**

T-cell immune responses occur when T lymphocytes detect differences in MHC class I or II molecules, or peptides presented within these molecules. Therefore, a primary requirement for T-cell recognition and elimination of APL tumor cells is MHC class I and/or class II cell surface expression. Zheng et al. reported that expression of PML-RARα in NB4 cells was associated with down-regulation of MHC class I molecules via inhibition of TAP transporters and LMP proteins. Treatment of NB4 cells with either retinoic acid or interferon resulted in degradation of PML-RARα, morphologic differentiation, and an increase in expression of TAP and LMP mRNA. We therefore tested whether transgenic murine APL cells express MHC molecules. Since our cryopreserved APL cells are on a mixed C57Bl/6 (H-2b) and C3H (H-2k) background (B6C3H), we tested for expression of both H-2b and H-2k haplotypes.

We previously identified CD34 as an abnormally expressed surface protein on transgenic APL cells. Cell sorting analysis of CD34⁺/Gr-1⁻ cells identified this distinct, abnormal population of myeloid cells. Two-color flow cytometric analysis was therefore conducted to determine whether the CD34⁺-expressing population of early APL cells expresses MHC cell surface markers. As shown, all APL samples evaluated express MHC class I molecules on cells in the CD34⁺ population (Table 1). The average individual class I molecule positivity within the CD34⁺ population was 65%±22% (range of positivity, 1.0-97.9%). APL cells were also evaluated for expression of MHC class II molecules. Approximately 15-25% of human AML M5 (APL) samples express MHC class II molecules. Using two-color flow cytometry analysis, 27 of 28 APL samples expressed MHC class II antigens on >2% of cells within the CD34⁺ population (Table 1). The range of positivity varied from 1.0-41.3%, with an average of 14%±11%. Using separate fluorophores, all MHC class II positive cells were also positive for MHC class I antigens (data not shown).

**APL cells are efficiently eliminated in allogeneic recipients, but not in genetically compatible mice**

In an allogeneic immune response, foreign cells are recognized by allo-specific antigenic differences in MHC class I and II on the cell surface, which trigger a strong activating response in allogeneic T cells. Cryopreserved APL cells (H-2b×H-2k) were injected into C57Bl/6 animals (H-2b), or into genetically compatible C57Bl/6×C3H F1 mice (H-2b×H-2k), to test the ability of the recipient allogeneic T cells to detect and eliminate the donor APL cells. We predicted that the APL cells would only be rejected if: (i) H-2b antigens are expressed on the cell surface, and (ii) the APL cells are susceptible to in vivo recognition and clearance by the recipient immune system. Five allogeneic C57Bl/6 and five control B6C3H F1 recipients were injected intraperitoneally with 3-10 times the LD₁₀₀ (determined in genetically compatible B6C3H F1 animals) of three independent APL tumors (total number of recipient mice=30). All of the B6C3H mice developed APL and died within 100 days (Figure 1), which reveals that APL initiating cells within all samples were capable of engrafting and expanding in recipient mice if the tumor cells were not controlled immunologically. In contrast, all C57Bl/6 animals survived to four months post-challenge, had normal peripheral blood counts and differentials, and were negative by polymerase chain reaction monitoring for minimal residual disease in the peripheral blood. Four months after the initial challenge, the C57Bl/6 mice were rechallenged with the identical dose of the same APL tumor sample and again, no animal developed disease. Animals were sacrificed three months after the second challenge and were found to be disease-free by analysis of peripheral blood counts and by histopathologic evaluation of liver and spleen.
Since the PML-RAR model is relevant for clearance, and to define the contribution of the adaptive immune system to protection, we injected wild type, genetically compatible, immunocompetent mice (B6C3H F1), and C3H SCID mice (which lack functional T and B cells) with the same APL tumors. A total of 150 recipient animals were evaluated in this study (*n* = 5 B6C3H F1 or SCID mice per group injected with one of three tumor samples, each transferred at five different doses). At high tumor cell doses (10⁶ cells), wild type and SCID animals appeared to be equally susceptible to leukemia development (Figure 2E and F). However, we observed a differential susceptibility when smaller doses of APL cells were injected (Figure 2). The lethal tumor cell dose for 50% of the recipients (LD₅₀) was approximately 100-fold lower in SCID recipients than in wild type B6C3H F1 animals. These data suggest that the adaptive immune system can clear APL cells via the recognition of tumor-specific antigens *in vivo*. These results also imply that approximately 1 in 200 cells from cryopreserved leukemic spleen samples contain leukemia-initiating activity (i.e. 100 cells cause approximately 50% of animals to develop APL).

**DNA immunization with human PML-RARα or the PML portion of PML-RARα protects against fatal APL**

Injection of naked DNA vectors encoding for eukaryotic expression of immune antigens has been shown to elicit robust host responses. Additionally, DNA immunization against tumor-specific antigens can lead to immunologic detection and eradication of previously tolerated tumors. Since the PML-RARα and RARα-PML proteins contain tumor-specific antigens, plasmids were generated to express these cDNA under the control of the cytomegalovirus promoter in the pcDNA3.1 expression vector (Invitrogen). Additionally, to test whether the potential immune response was directed against the component parts of the fusion protein, vectors were generated to express the PML or RARα portions of the bcr-1 derived PML-

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**SCID mice are more susceptible to lethal APL than and genetically compatible, immunocompetent mice**

To determine whether tumor-specific antigen recognition is relevant for clearance, and to define the contribution of the adaptive immune system to protection, we injected wild type, genetically compatible immunocompetent mice (B6C3H F1), and C3H SCID mice (which lack functional T and B cells) with the same APL tumors. A total of 150 recipient animals were evaluated in this study (*n* = 5 B6C3H F1 or SCID

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**Figure 1.** Secondary transfer of APL cells into genetically compatible or allogeneic recipients. The Kaplan-Meier probability of leukemia-free survival of C57Bl/6xC3H F1 (B6C3H) or C57Bl/6 (B6) animals is plotted against time after intraperitoneal injection of the indicated dose of one of three APL tumors (10822, 10759, and 10292). Open symbols represent B6C3H recipients, and closed symbols represent B6 recipients (5 mice per group). The arrow represents the time at which surviving B6 animals were rechallenged with the same dose of the same APL cells.

**Figure 2.** Dose response to APL cell challenge in immunocompetent or immunodeficient secondary hosts. A-E. The Kaplan-Meier probability of leukemia-free survival of C57Bl/6xC3H F1 (B6C3H) or C3H SCID animals is plotted against time after intraperitoneal injection of APL cells at the following doses: (A) 10⁶ cells per recipient, (B) 10⁵, (C) 10⁴, (D) 10³, (E) 10². Triangles indicate B6C3H recipients, and squares indicate C3H SCID recipients (5 mice per group). F. Percent mortality is plotted as a function of cell dose in B6C3H (filled symbols) or C3H SCID (open symbols) recipients of the three indicated APL tumor samples (total number of recipient mice in this experiment *n* = 150).
RARα fusion gene (Figure 3A). The expression of the cloned cDNA in this expression vector was verified by transient transfection of K562 cells with the indicated plasmids, followed by Western blotting using antibodies directed against the PML or RARα domains (Figure 3B). The antibodies used do not recognize domains contained in the reciprocal RARα-PML protein (lane 6), but this vector produced a protein of the expected size using 35S-methionine-labeled in vitro transcription and translation (data not shown).

Wild type B6C3H F1 animals were vaccinated by intramuscular injection of 50 μg of purified plasmid DNA weekly for four weeks, followed one week later by challenge with the LD100 dose of intraperitoneally injected live APL cells (Figure 3C). In some cases, the immunizations included pcDNA3.1-based constructs that expressed murine GM-CSF and/or CD40L cDNA; these adjuvants have been shown to enhance immune responses in some model systems.22,23 All results were similar whether or not the adjuvant constructs were included, and thus all data are combined here. In total, 162 mice were vaccinated four times each with plasmid DNA, then injected with live APL cells. Three different APL tumor samples were used to challenge the immunized mice, with similar results. Animals that were immunized with the PML-RARα cDNA, or the PML-portion only, were relatively protected from APL challenge (Figure 3D, p<0.0001 for PML-RARα or PML compared pairwise with all other vaccinations by Kaplan-Meier survival analysis). Vaccination with the RARα portion of the fusion gene alone, or with a construct expressing 9 amino acids of the PML portion fused to the entire RARα portion (fus-RARα, see below), did not result in protection. Similarly, vaccination with the reciprocal RARα-PML fusion gene did not confer protection.

**PML-RARα breakpoint-derived peptides do not induce T-cell proliferation in vitro**

Despite the observations from DNA immunizations described above, the amino acids that span the PML-RARα junction could yield immunogenic peptides not found in the endogenous PML or RARα proteins; these peptides could potentially elicit a tumor-specific
immune response. We conducted several studies to address whether the human bcr-1 PML-RARα breakpoint region, present in our transgenic tumor cells, could be a candidate tumor antigen in the murine immune response against APL. First, ten 10-mer peptides spanning the PML-RARα bcr-1 junction were tested for their ability to bind and stabilize MHC class I antigens on the surface of the murine RMA-S cell line. This cell line expresses H-2b MHC class I molecules, but is defective in loading of peptides onto MHC molecules in the endoplasmic reticulum, and thus displays a very low level of stable surface class I expression.

When RMA-S cells are incubated with peptides that bind to H-2b MHC class I molecules, surface expression is stabilized, and can be observed by fluorescence-mediated antibody staining. None of the ten breakpoint-spanning peptides was able to support MHC class I expression, whereas a control ovalbumin peptide (OVA) did stabilize expression (Table 2). These findings show that these ten 10-mers are unlikely to bind MHC H-2b molecules in intact cells in vivo.

Since binding assays may not be sensitive to all peptides presented in vivo, DNA immunizations were also conducted with constructs expressing the nine amino acids in PML upstream of the breakpoint, fused to the entire RARα sequence. This vector should stimulate class I immune responses directed against breakpoint sequences. Vaccination with this construct (Fusion-RARα) did not result in protection from APL challenge (Figure 3D). Finally, we tested whether splenocytes from full-length PML-RARα cDNA immunized animals that survived APL challenge would proliferate in response to the ten 10-mer breakpoint-derived peptides in a standard one way mixed lymphocyte reaction in vitro. Spleen cells from survivor animals proliferated in response to PMA and ionomycin as predicted, but did not proliferate when co-cultured with immunologically compatible splenocytes alone, or splenocytes precultured in the presence of any of the ten junction peptides (Table 2). Thus, while it remains possible that a processed peptide of a slightly different length may bind to MHC class I molecules, it is unlikely that processing of the breakpoint region of PML-RARα yields peptides that are immunologically recognized in the mouse.

**Discussion**

In this report, we have shown that transgenic murine APL cells are capable of being recognized and eliminated in immunocompetent murine hosts. SCID mice (which lack T and B cells) were much more susceptible to APL challenges, suggesting that the adaptive immune system is important for the clearance of APL cells in vivo. DNA immunization and peptide studies suggested that the enhanced immune clearance of transgenic APL cells in vaccinated animals is probably not due to recognition of the unique PML-RARα fusion protein breakpoint region. Rather, recognition of
species-specific epitopes of human PML (from the PML-RARα transgene) may account for the protective effect of plasmid DNA immunization in mice. These results indicate that promise exists for utilizing immune responses against APL cells in vivo, but also suggests that the optimal antigens for vaccination and/or immune recognition are still unknown.

One goal of this work was to determine whether murine APL cells are capable of being recognized and killed by the immune system. A previous study reported that PML-RARα may interfere with the normal function of endogenous PML in regulating the TAP and LMP components of the endoplasmic reticulum class I peptide-loading pathway.15 It was suggested that because of that interference, PML-RARα may cause downregulation of MHC molecules in APL cells. However, several studies have questioned this finding by showing the expected levels of HLA molecules on human APL cells.17,25,26 Similarly, we have shown here that most murine APL cells also express MHC class I and class II. Since murine APL cells are effectively eliminated in allogeneic hosts, they likely also express these surface molecules in vivo, which would make these cells susceptible to allogeneic recognition and elimination by the host immune system.

Since the t(15;17) creates a potential tumor-specific novel antigen, several studies have investigated the binding and presentation of PML-RARα breakpoint peptides by HLA molecules. One initial study found that a human CD4+ T-cell clone could be generated in vitro that recognized antigen-presenting cells presenting a PML-RARα junction 25-mer peptide in the context of HLA-DR. However, a subsequent study found that the same peptide did not stimulate immune responses in lymphocytes from APL patients in remission, and no cloned T cells from the same patients reacted with antigen-presenting cells presenting the breakpoint peptide. When PML-RARα breakpoint peptides were tested for binding to human HLA class I molecules, no binding (or only low affinity binding) was observed, although peptides from other translocation breakpoints (e.g. the t(9;22) associated BCR-ABL fusion) bound with high affinity to multiple HLA alleles. Thus, several studies have suggested that peptides in the PML-RARα breakpoint region do not provide the basis for tumor-specific immune responses against APL cells.

Nonetheless, alternative antigens presented on APL cells may exist, since our studies clearly demonstrate a role for the adaptive immune system in APL cell elimination in vivo. We previously showed that treatment with all-trans retinoic acid (ATRA) cooperated with endogenous T and B cells to eliminate APL cells in vivo.10 Interestingly, a recent report using a different murine APL model showed the efficacy of a vaccine expressing a 34 amino acid PML-RARα breakpoint peptide fused to the tetanus toxin fragment C.17 In vaccinated mice given transgenic APL cells, protection was only observed when the mice were concurrently treated with ATRA. The marker for increased survival in those mice was the presence of a serum antibody against RARα, but this antibody was also induced by treatment with ATRA alone, in the absence of any DNA vaccine. It is not clear whether this antibody is involved in protective immunity, or whether it serves only as a marker of anti-leukemic responses. ATRA treatment of mice bearing transplanted APL cells may enhance the presentation or processing of alternative tumor-specific antigens. As such, the cloning of tumor-specific T cells, or the identification of presented antigens in APL cells, may reveal more potent and efficacious targets for vaccination.

DNA vaccination with a PML-RARα cDNA induces protective immunity against APL in our mouse model, and this finding is similar to that reported by Padua et al.14 In that report, effective vaccination was achieved using a PML-RARα breakpoint fragment fused to tetanus toxin fragment C, but full length PML-RARα did not confer protection. Differences in mouse strains, schedules and routes of vaccination, and specific APL cells used, could account for minor differences in the findings presented here compared with those in Padua’s report. Most significantly, in contrast to the experiments presented here, Padua et al. intravenously transferred APL cells to recipients one week prior to initiating DNA vaccination. Nonetheless, both reports convincingly demonstrate that DNA vaccination with human PML-RARα is protective against transgenic murine APL tumors. However, in an additional experiment presented here, we found that equally effective immunity was induced by immunization with only the PML portion of human PML-RARα. In both studies, the tumor cells used were obtained from mice genetically engineered to express a human bcr-1 PML-RARα cDNA in myeloid cells. Human and mouse PML are only 67% similar at the amino acid level (while human and mouse RARα are 99% similar), and thus, the immunogenicity of PML-RARα vaccination in the mouse may be attributable to xeno-specific differences within the PML portion of the fusion gene. To illustrate, of the 34 amino acids in the PML-RARα fragment used by Padua et al., 8 of 17 amino acids in the PML portion of that peptide differ between human and mouse sequences; all 17 amino acids in the RARα portion are identical in humans and mice. These data suggest that immunization with human PML-RARα DNA or peptides in human patients with APL will not be as effective as that observed in these mouse systems. We have shown that murine APL cells are not immunologically cloaked, and that they can be recognized by the adaptive immune system in both allogeneic and genetically compatible settings. Further, we have shown
that the anti-APL immune response in mice can be stimulated by DNA vaccination. Recent work with chronic myeloid leukemia has shown that immune responses can be stimulated to recognize either fusion protein-derived antigens (BCR-ABL)\(^1\) or a myeloid specific antigen (proteinase 3)\(^2\) that is expressed in chronic myeloid leukemia cells. However, in light of the data presented here, and the poor binding of PML-RAR\(^\alpha\) breakpoint-derived peptides to human HLA molecules, clinical trials of vaccination in patients with APL will almost certainly require identification of as yet unknown APL cell immune antigens. Nonetheless, these results highlight the potential benefit of sparing immune function during therapy for leukemia; with or without vaccination against a tumor-specific antigen, adaptive immunity may aid the clearance of tumor cells in vivo.

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