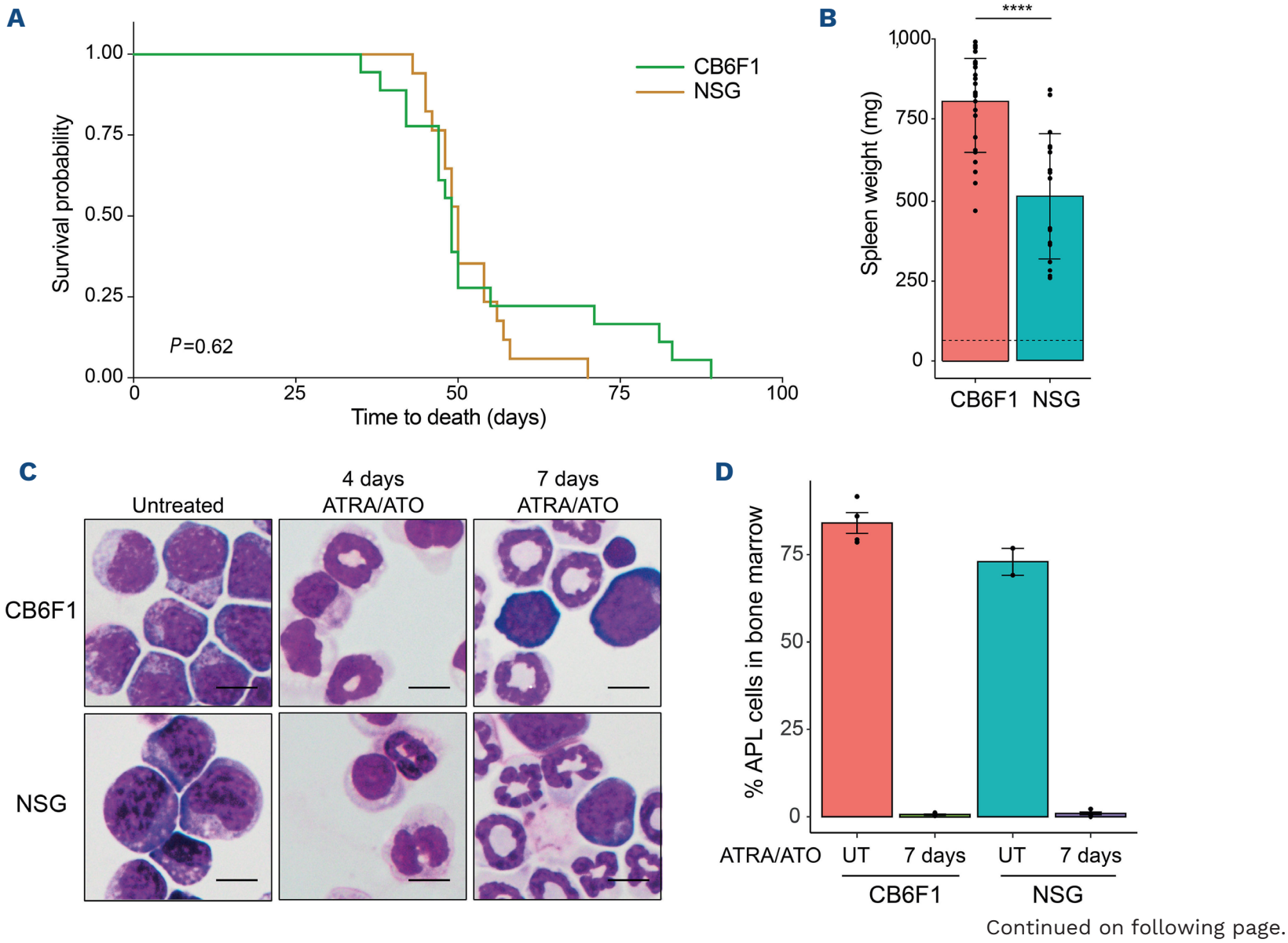
Immune intervention is dispensable for retinoic acid/arsenic therapy of murine acute promyelocytic leukemia

Acute promyelocytic leukemia (APL) is a paradigm for cure by targeted therapies: the combination of all-*trans* retinoic acid (ATRA) and arsenic trioxide (ATO) cures the large majority of APL patients.[1] Both ATO and ATRA target PML::RARA for proteolytic degradation, by directly binding its PML and RARA moieties. Murine studies have demonstrated that drug-induced degradation of PML::RARA drives restoration of retinoic acid target gene expression and reformation of PML nuclear bodies.[2] The immune system plays an important role in the basal control of many malignancies and their subsequent clearance upon exposure to many therapies. Yet, human APL cells are not expected to be intrinsically strongly immunogenic, because their genome is very stable.[3] Nevertheless, the ATRA/ATO combination induces interferon signaling in APL cells, suggesting that therapy could prime them for immune clearance.[4] Some studies in murine APL models have suggested that the immune system controls ATRA response duration and that vaccination against PML::RARA enhances survival.[5-7] In this setting, the human *PML::RARA* transgene could become immunogenic in immunocompetent mice.[7] Indeed, antibodies against human RARA were detected following ATRA therapy in APL mice, but unexpectedly also in ATRA-treated APL patients.[8] In some mice models, the combination of ATRA and ATO clears the disease within a week and definitively eradicates it.[9] How this dramatic clearance is achieved and subsequently maintained remains poorly understood. The APL setting, in which cure is the most common outcome, offers a unique system to explore any role of immune cells in leukemia eradication.

We compared the clinical efficacy of the ATRA/ATO combination in primary murine APL cells transplanted into syngenic CB6F1 immunocompetent mice or into mice with the most profoundly immunodeficient available background, NSG mice, using protocols approved by the Comité d'Ethique en Expérimentation Animale Paris-Nord N. 121 (projects N. 23796 and 23921.05). NSG mice are deficient in B, T and natural killer (NK) cells, so that adaptive and, to a large extent, innate immunities are essentially absent.[10] The kinetics of leukemia development, spleen invasion and recipient time to death were



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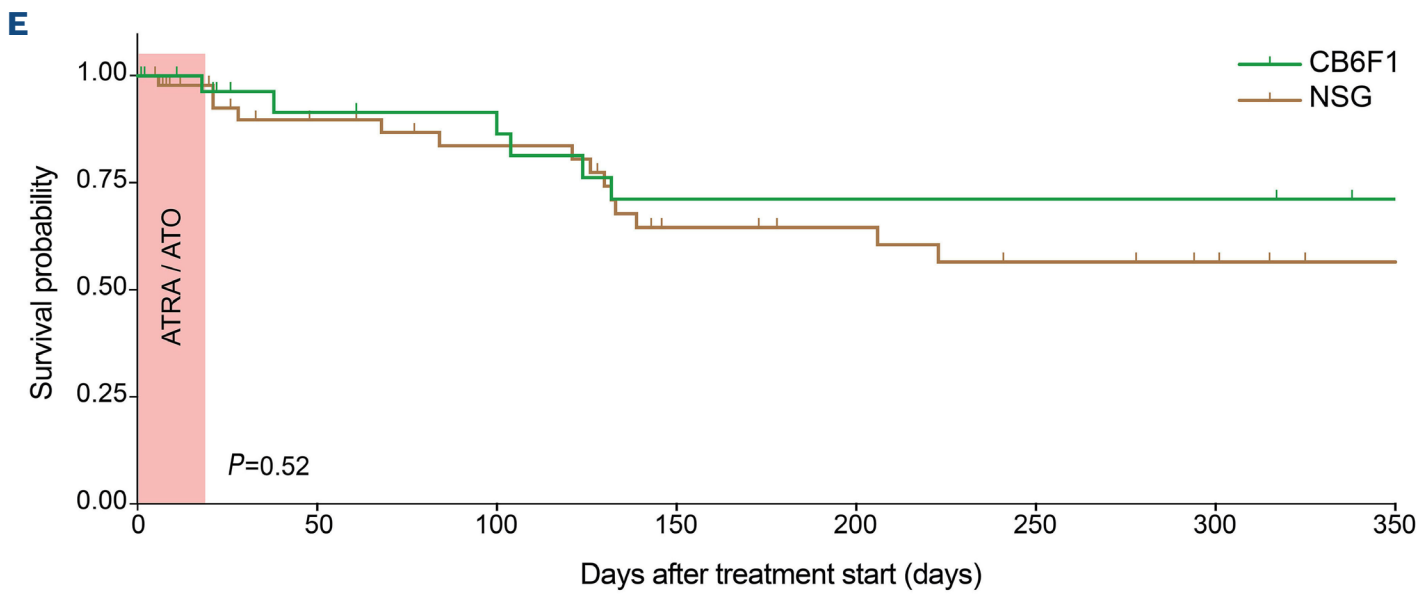

Figure 1. All-*trans* retinoic acid/arsenic trioxide therapy can cure acute promyelocytic leukemia in NSG recipient mice. Acute promyelocytic leukemia (APL) cells were obtained by crossing mCG-PML::RARA mice[14] into C57BL/6J (kind gift from T. Ley) and BALB/cByJ animals and maintained in syngenic CB6F1/OlaHsd mice. Transplantable APL blasts were then transduced with green fluorescent protein (GFP)-expressing retroviruses obtained by transient transfection of Plat-E cells with pMSCV IRES eGFP. (A) Time to death of mice implanted with 10^5 APL cells (GFP^+ sorted) in syngenic CB6F1 or NSG recipients. This experiment was repeated four times; results for all mice (CB6F1, N=18; NSG, N=17) are reported here. (B) Comparison of spleen weights in the two types of recipients. (C) Representative May-Grünwald Giemsa staining of bone marrow cells from syngenic CB6F1 or NSG murine APL recipients treated with all-*trans* retinoic acid (ATRA)/arsenic trioxide (ATO). ATRA/ATO therapy consisted of subcutaneous implantation of 21-day-release 10 mg pellets of ATRA and daily intravenous injection of 5 µg/g body weight of ATO (1 mg/mL). Scale bars (5 µm) are indicated. Samples are from total bone marrow, except for the day 4 sample which consists of GFP-sorted APL cells undergoing differentiation. Normal bone marrow recovery is observed at day 7. (D) ATRA/ATO therapy-induced clearance in the two types of recipients. Representative experiment with four mice per treated group. (E) Survival analysis of APL mice developing in syngenic or NSG recipients after 21 days of treatment with ATRA/ATO. Mice dying of causes other than APL were censored, as were those which died early, during or immediately after induction therapy. This experiment was repeated five times and the results for all mice (CB6F1, N=40; NSG, N=46) are reported here. Kaplan-Meier representation of survival, non-significant log-rank test. ****P<0.0001 by the Mann-Whitney test. UT: untreated.

similar in both mice with immunocompetent and immunodeficient backgrounds (Figure 1A,B). ATRA/ATO treatment was initiated when the bone marrow was invaded and the spleen showed clinically detectable enlargement. Following therapy with the ATRA/ATO combination, induction of differentiation (Figure 1C) and kinetics of APL regression (Figure 1D) were similar in both types of recipients, with a complete morphological clearance of the bone marrow in 7 days (Figure 1C), as reported in other models.[9] Treatment was discontinued after 21 days and survival monitored. Mice deaths during induction therapy (reflecting in part the differentiation syndrome[11]) or unrelated to APL relapse were censored. Remarkably, in several independent experiments, survival was similar in mice with the immunocompetent (CB6F1) and the immunodeficient (NSG) backgrounds (Figure 1E). In the NSG mice, we ruled out the transplantation of some GFP-labeled activated immune cells together with APL blasts (*data not shown*) which might have confused interpretation of our findings. Collectively, our results imply that APL cure by ATRA/ATO is essentially a cell-autonomous process with no obligatory immune intervention.

ATRA or ATO induces terminal granulocytic differentiation and senescence of APL cells *in vivo*.[2] The resulting granulocytes may be eliminated by macrophages,[12] while senescent APL cells may also be eliminated by NK cells. Yet, NK cells are severely depleted and macrophage functions are impaired in NSG mice, raising the question of how leukemic cells are actually eliminated. Following therapy with ATRA or, even more, with ATRA/ATO, we first observed a very rapid reappearance of megakaryocytes and their progenitors, as detected by CD61 staining (Figure 2A, C) in both models. Unexpectedly, we consistently observed massive emperipolesis, the engulfment of intact differentiating APL cells by megakaryocytes[13] (Figure 2B, C). Electron microscopy unveiled large megakaryocytes ingesting cells with granulocytic features or cellular debris (Figure 3A-D). A similar occurrence of therapy-induced emperipolesis was noted in both CB6F1 and NSG APL models, while we could not detect it in bone marrows from normal, unstressed animals, as previously reported. Why differentiating APL cells are so prone to emperipolesis remains to be explored.

The ATRA/ATO regimen is definitively curative for the majority of APL patients. We unexpectedly found that APL can be cured by ATRA/ATO even in the most profoundly immunodeficient mice models, demonstrating that immune clearance is not a prerequisite for leukemia eradication. While ATRA/ATO cures some murine APL,[1,9] the specific APL knock-in model[14] or different genetic background used here may have allowed some persistent cells to survive. Nevertheless, the

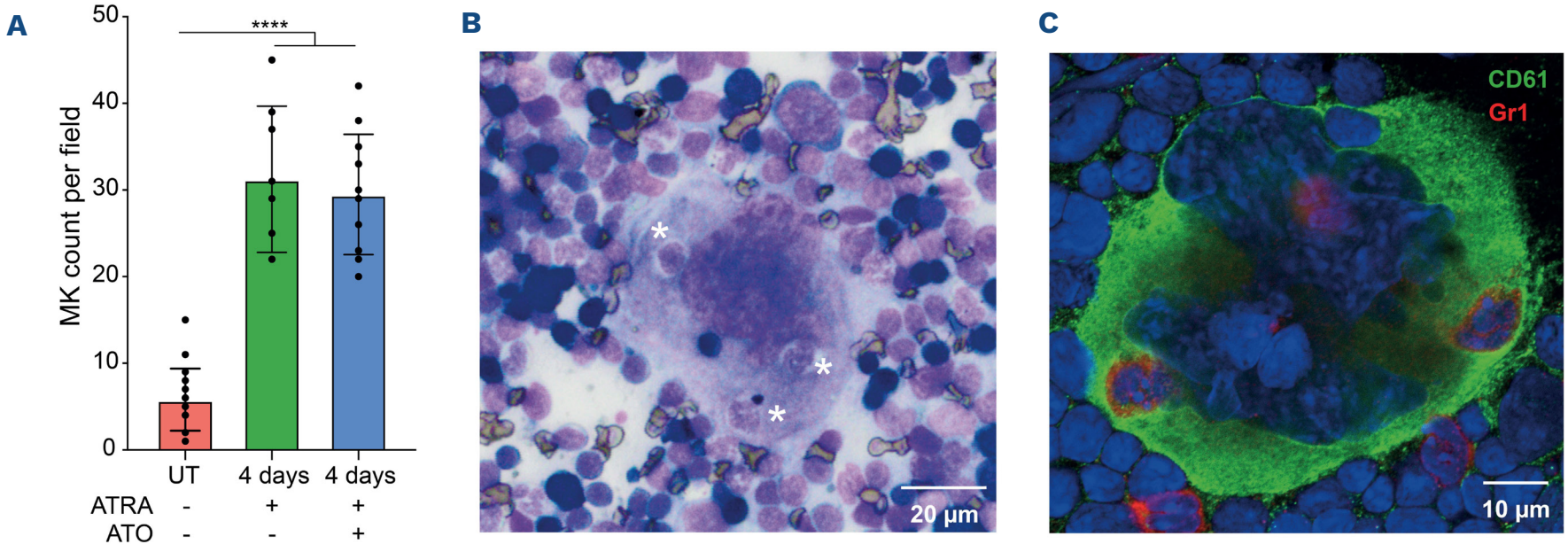

Figure 2. Differentiated granulocytes undergo emperipolesis upon treatment with all-*trans* retinoic acid/arsenic trioxide therapy. (A) Quantification of megakaryocytes in the syngenic acute promyelocytic leukemia model on bone marrows sections (cryo-cut to 6 or 8 µm), as detected by CD61 labeling. (B, C) Megakaryocyte engulfment of differentiating myeloid cells (indicated with *). (B) May-Grünwald Giemsa staining of spleen cells obtained by apposition and (C) confocal analysis of these cells labeled for CD61 (megakaryocytes) and Gr-1 (differentiated myeloid cells). 4′,6-Diamidino-2-phenylindole (DAPI) is in blue. Scale bars are indicated. ****P<0.0001 by the Mann-Whitney test. MK: megakaryocytes; UT: untreated; ATRA: all-*trans* retinoic acid; ATO: arsenic trioxide.

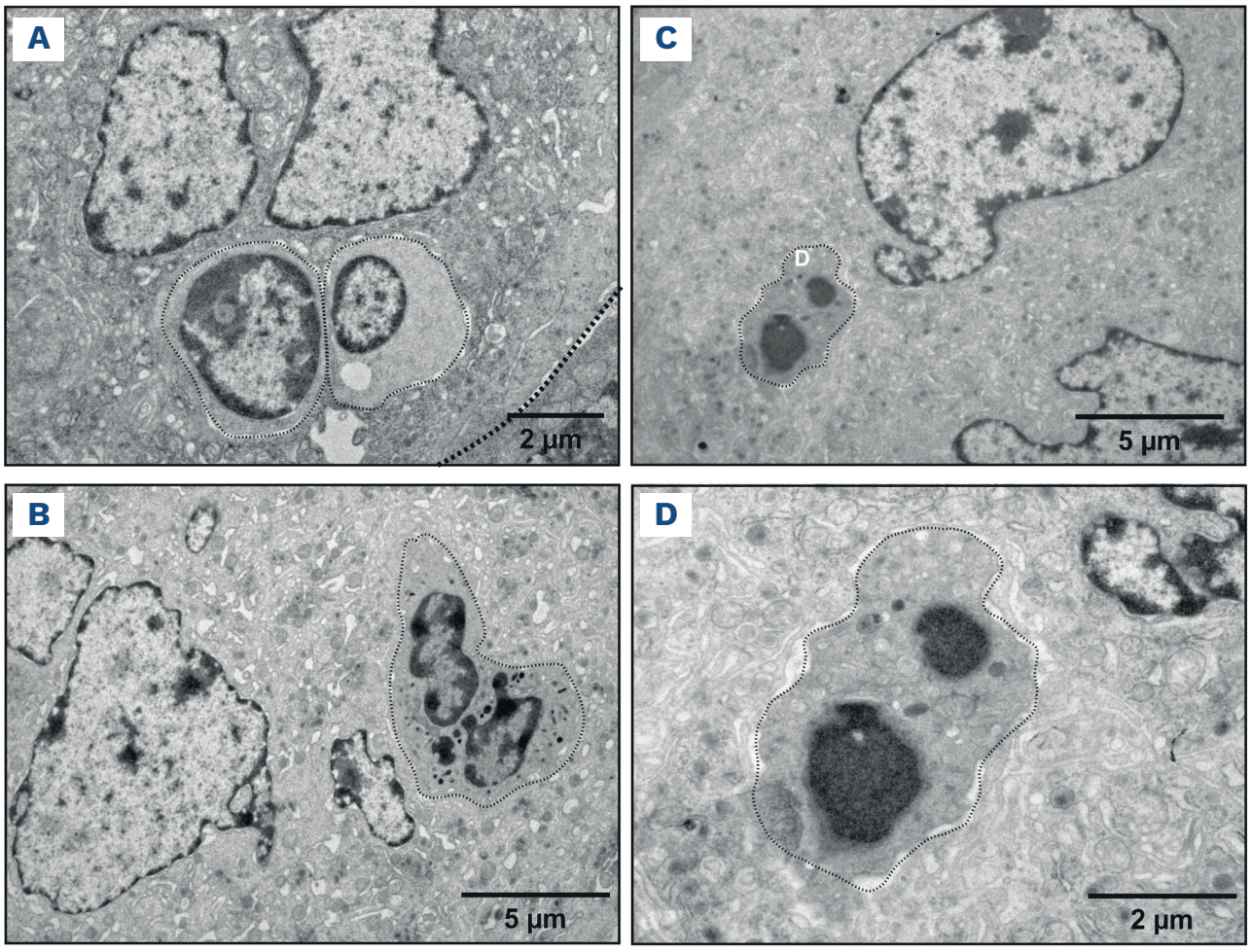

Figure 3. Electron microscopy analysis of emperipolesis upon treatment with all-*trans* retinoic acid/arsenic trioxide therapy. (A) A megakaryocyte, delineated by a line of large dots, has engulfed cells, delineated by lines of small dots, in its cytoplasm. (B) A terminally differentiated granulocyte (dotted line) internalized within a megakaryocyte. (C) High magnifications of regions of apoptotic remnants (dotted lines, D in white font), enlarged in (D). Scale bars are indicated. Electron microscopy analysis from pooled bone marrows from the femurs of three to five mice after Epon embedding on ultrathin sections.

therapeutic efficacy of ATRA/ATO was not modulated by the immune system. While some studies found that the immune system did play a significant role in some murine APL treated by ATRA, these studies used different APL transgenic models, mouse strain backgrounds and, critically, very different doses of ATRA. With liposomal ATRA enforcing very high intracellular concentrations, single-agent cures were obtained, and B or T cells facilitated eradication.[5] Using suboptimal ATRA dose scheduling (similar to the one used here), single-agent ATRA therapy was boosted by vaccination directed against human PML::RARA.[6] The drastic antileukemic effect of the ATRA/ATO combination, which turns off all oncogenic signaling through PML::RARA degradation and activation of PML-driven senescence, drives a pure cell-autonomous antileukemic effect, thus circumventing the need for immune intervention. Macrophage phagocytosis of cellular remnants (proposed to be APL cells) were reported in APL patients in complete remission following ATRA/ATO therapy.[15] Collectively, these experiments, exploring one of the rare examples of cure by targeted therapies, demonstrate that the contribution of the

immune system to enforcement of tumor clearance is not an absolute prerequisite to leukemia eradication.

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https://doi.org/10.3324/haematol.2025.288562

Received: June 26, 2025.
Accepted: September 1, 2025.
Early view: September 11, 2025.

Disclosures
No conflicts of interest to disclose.

Contributions
CE, GF, FQ, HS, SS, KL, TD and MN-K performed experiments. CE, GF, FQ, GP and HdT designed experiments, interpreted data and contributed to writing the manuscript. All authors reviewed the manuscript.

Acknowledgments
We thank T. Ley (Washington University, St. Louis, MO, USA) for his generous gift of CatG-*PML::RARA*$^{+/+}$ C57BL/6 mice.[14]

Funding
Work in the laboratory is supported by the ERC (PML-Therapy, ADG-785917), as well as Institut National du Cancer (PLBio). GF was supported by a PhD grant from Université Paris Cité-MESRI. The animal facility was supported in part by ANR, through the France 2030 program, ANR-23-IAHU-0005, Paris St. Louis Leukemia Institute.

Data-sharing statement
Reasonable requests for reagents and data should be addressed to the corresponding author.

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