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

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Authors contribution

CE, GF, FQ, HS, SS, KL, TD, MK performed experiments; CE, GF, FQ, GP, HdT designed experiments, interpreted data and contributed to the writing of the manuscript. All authors reviewed the manuscript.

Authors Dislcosures

None of the authors declares any conflict of interest related to this work.

Data sharing

Reasonable requests for reagents and data should be addressed to the corresponding author.

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]. Accordingly, 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, where 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 in syngenic CB6F1 immunocompetent mice or in 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 n° 23921.05). NSG mice are deficient in B, T and 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 similar in both immuno-competent and immuno-deficient 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 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 the immunocompetent (CB6F1) and the immunodeficient (NSG) background (Figure 1E). In the NSG background, we ruled out the transplantation of some GFP-labelled activated immune cells together with APL blasts (data not shown) which might have confused interpretation of our findings. Collectively, these results imply that APL cure by ATRA/ATO is essentially a cell- autonomous process with no obligatory immune intervention.

ATRA or ATO induce 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 Natural Killer (NK) cells. Yet, NK cell are severely depleted and macrophages functions impaired in NSG mice, questioning how leukemic cells are actually eliminated. Following therapy with ATRA or furthermore ATRA/ATO, we first observed a very rapid reappearance of megakaryocytes and their progenitors, as detected by CD61 staining (Figure 2A and C)

in either model. 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). Similar occurrence of therapy-induced emperipolesis was noted in both CB6F1 or 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 APLs [1, 9], the specific APL knock-in model [14] or different genetic background used here may have allowed some persister cells to survive. Yet, the therapeutic efficacy of ATRA/ATO was not modulated by the immune system. While some studies reported that in murine APLs treated by ATRA, the immune system did play a significant role, these studies used different APL transgenic models, mouse strain background 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 sub-optimal ATRA 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, that 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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Figure legends

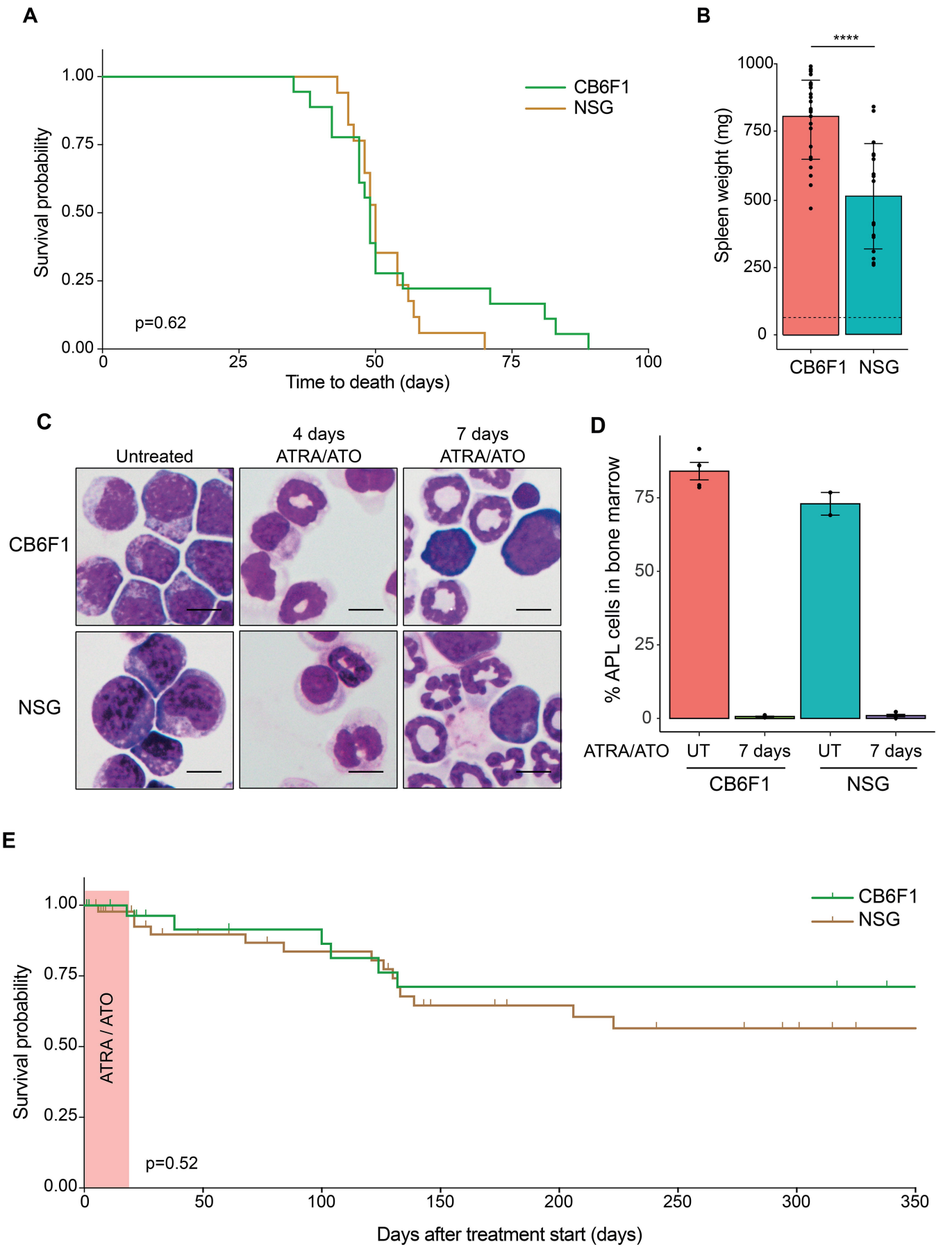
Figure 1: **ATRA/ATO therapy can cure APL in NSG recipients.** APL cells were obtained by crossing mCG-PML::RARA [14] mice into C57BL/6J (kind gift of T. Ley) and BALB/cByJ and maintained into syngenic CB6F1/OlaHsd mice. Transplantable APL blasts were then transduced with a GFP-expressing retroviruses obtained by transient transfection of Plat-E cells with pMSCV IRES eGFP (**A**) Time to death of mice implanted with 10^5 APLs (GFP+ sorted) in syngenic CB6F1 or NSG recipients. This experiment was repeated four times, all mice (18 CB6F1 – 17 NSG) were reported here. (**B**) Comparison of spleen weights in the two types of recipients. (**C**) Representative May Grünwald Giemsa staining of bone marrow cells from ATRA/ATO-treated murine APLs in syngenic CB6F1 or NSG recipients. ATRA/ATO therapy consists of subcutaneous implantation of 21-day-release 10-mg pellets of ATRA and daily intravenous injection of 5 µg/g body weight of ATO (1mg/ml). Scale bars (5µm) are indicated. Samples are from total bone marrow, except for the day 4 sample which consist 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 a 21 days treatment with ATRA/ATO. Mice dying of other cause were censored, as well as the early death during or immediately after induction therapy. This experiment was repeated five times, all mice (40 CB6F1 – 46 NSG) were reported here. Kaplan-Meier representation of survival, non-significant log-rank test.

Figure 2: Differentiated granulocytes undergo emperipolesis upon

ATRA/ATO treatment. (**A**) Quantification of megakaryocytes in the syngenic APL model on bone marrows sections (cryo-cut to 6 or 8µm), as detected by CD61 labelling. **** denotes p<0.0001 by Mann-Whitney test. **B-C** Megakaryocyte engulfment of differentiating myeloid cells (indicated with *). (**B**) MGG staining of spleen cells obtained by apposition and (**C**) confocal analysis of these cells with CD61 (megakaryocyte) and Gr-1 (differentiated myeloid) labelling. DAPI is in blue. Scale bars are indicated.

Figure 3: Electron microscopy analysis of emperipolesis upon ATRA/ATO treatment. (A) A megakaryocyte, delineated by a (large dotted line), has engulfed cells in its cytoplasm delineated by small dotted lines. (**B**) terminally differentiated granulocyte (dotted lines) internalized within 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 3-5 mice after Epon embedding on ultrathin sections.

Figure 1


A
Survival probability
1.00
0.75
0.50
0.25
0.00
p=0.62
0
25
50
75
100
Time to death (days)
CB6F1
NSG
B
****
Spleen weight (mg)
1000
750
500
250
0
CB6F1
NSG
C
Untreated
4 days ATRA/ATO
7 days ATRA/ATO
CB6F1
NSG
D
% APL cells in bone marrow
75
50
25
0
ATRA/ATO
UT
7 days
UT
7 days
CB6F1
NSG
E
Survival probability
1.00
0.75
0.50
0.25
0.00
ATRA / ATO
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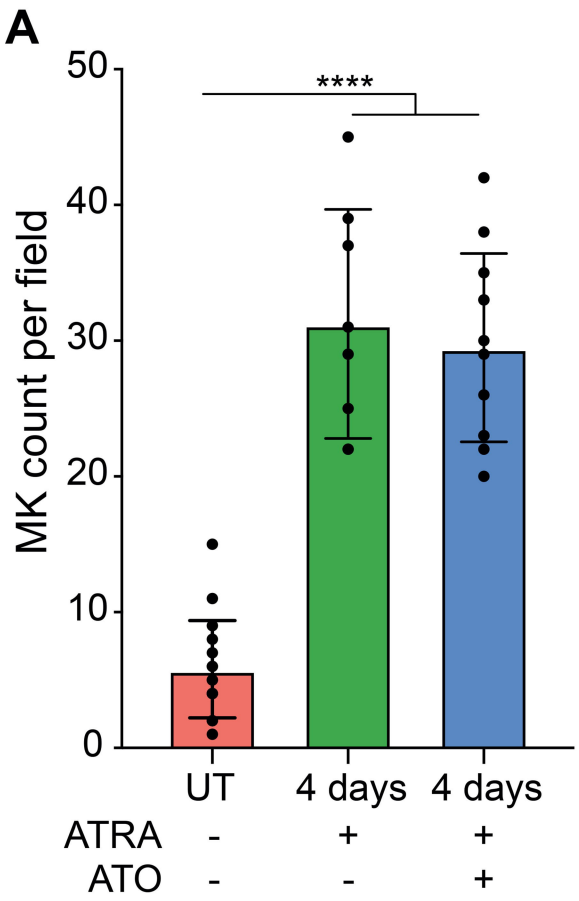
50
100
150
200
250
300
350
Days after treatment start (days)
CB6F1
NSG

Figure 2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MK count per field
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UT
4 days
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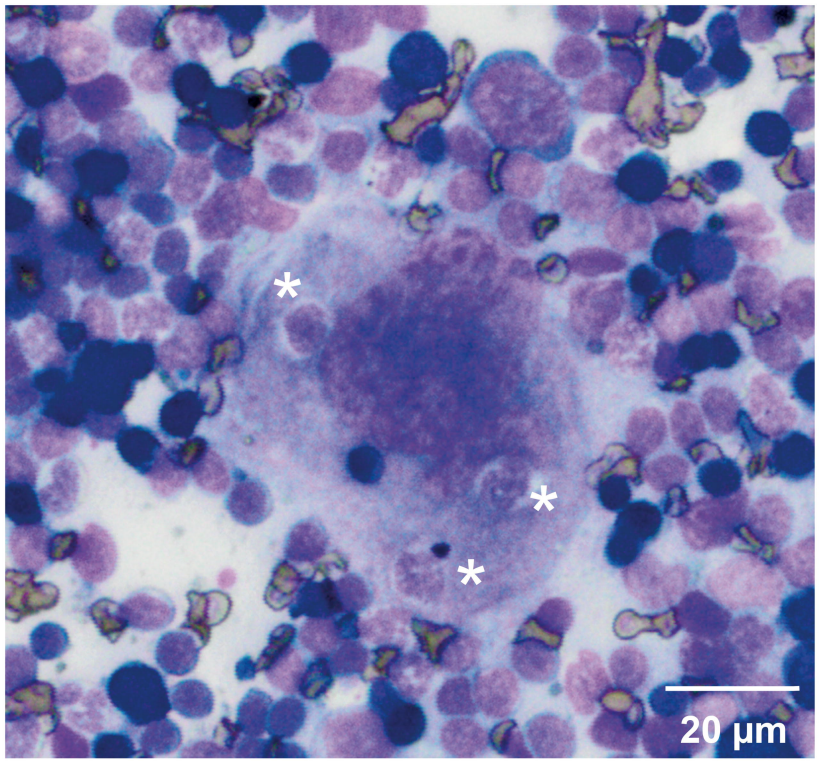
B
20 µm

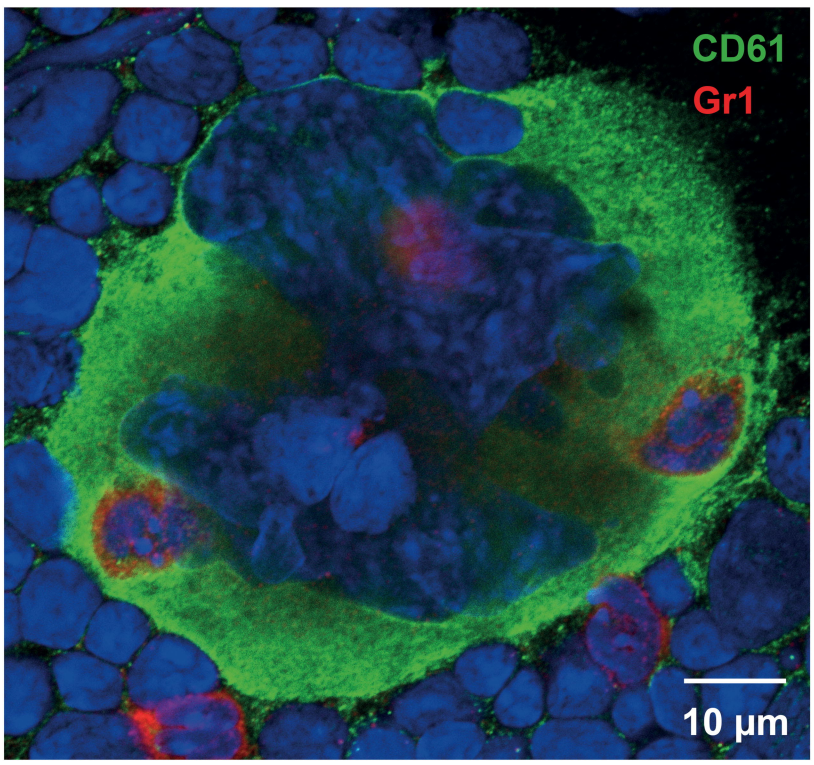
C
CD61
Gr1
10 µm

Figure 3