Adhesion molecules and differentiation syndrome: phenotypic and functional analysis of the effect of ATRA, As_2O_3, phenylbutyrate, and G-CSF in acute promyelocytic leukemia

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ABSTRACT

Background and Objectives

Differentiation Syndrome (DS) is a treatment complication which can occur in patients treated with acute promyelocytic leukemia (APL) with all trans-retinoic acid (ATRA) or As_2O_3, and is characterized by enhanced leukocyte transmigration. As_2O_3, Phenylbutyrate (PB) and G-CSF are known to potentiate ATRA effects. Our aim was to analyze the changes in expression and function of adhesion molecules induced by ATRA, As_2O_3, G-CSF and PB, and their association.

Design and Methods

APL blasts and NB4 cells were treated with ATRA, As_2O_3, PB, G-CSF or their association and the expression of adhesion molecules was determined by flow cytometry. Cell adhesion was evaluated *in vitro* using Matrigel and for the in vivo analysis, Balb-c mice were injected with NB4 cells pre-treated with ATRA, As_2O_3, ATRA+G-CSF or ATRA+As_2O_3. In addition, CD54 and CD18 knock-out mice were injected with NB4 cells and concomitantly treated with ATRA. In both models, the MPO activity in the lungs was determined 6 hours after the injection of the cells.

Results

In NB4 and APL blasts, ATRA and As_2O_3 increased CD54 expression, but no synergism was detected. CD11b and CD18 were also up-regulated by ATRA in primary cells. PB and G-CSF had no effect, but the latter potentiated ATRA-induced CD18 up-regulation. These changes were accompanied by increased adhesion to Matrigel and to lung microvasculature, and reversed by anti-CD54, anti-CD18 antibodies. In CD54 and CD18 *knock-out* mice the ATRA effect was canceled.

Interpretation and Conclusions

The use of As_2O_3, PB and G-CSF in association with ATRA should not aggravate DS in APL.

Key words: acute promyelocytic leukemia, adhesion molecules, histone deacetylase inhibitor, G-CSF.

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Acute promyelocytic leukemia (APL) is a specific subtype of acute myelogenous leukemia (AML), characterized by its frequent association with reciprocal translocations between chromosomes 17 and 15 [t(15;17)] leading to the fusion of the retinoic acid receptor α and promyelocytic leukemia (PML) genes located on chromosomes 17 and 15 respectively. The PML/RARα fusion protein acts as a transcription repressor and results in blocking the differentiation of APL blasts at the stage of promyelocytes.[1,2] Pharmacological doses of all trans-retinoic acid (ATRA) reverse this blockage, induce clinical remission and are the mainstay of the treatment.[3] However, approximately one fourth of the patients treated with ATRA present a potentially fatal complication called APL differentiation syndrome (DS), formerly known as Retinoid Acid Syndrome. This is characterized by fever, weight gain and pulmonary infiltrates. It is sometimes accompanied by respiratory failure, pleural and pericardial effusions and, less frequently, renal failure.[4-6] DS mortality has decreased from approximately 30% to less than 10% with the early institution of anthracyclines associated with ATRA therapy.[7]

With early diagnosis, DS responds well to dexamethasone treatment. The results from the European APL Group trials suggested that, besides corticosteroids, chemotherapy concomitant to ATRA reduced the incidence of the syndrome, although there was no clear reduction in the related mortality rate.[4] Previous studies in vitro suggest that ATRA induced changes in adhesion molecules are involved in DS physiopathology. Brown *et al.*[8] have demonstrated that ATRA induced rolling of NB4 cells (an APL cell line) on endothelium through the modulation of E-selectin, without the participation of α_4 integrin and P-selectin. In addition, Zang *et al.*[9] demonstrated that the expression of the β2-integrins LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) was induced by ATRA. This modulation may further contribute to DS development since the stable arrest and transmigration of neutrophils are dependent on β2-integrins and the intercellular adhesion molecule-1 (ICAM-1).

The current treatment protocols for APL based on the association of ATRA and anthracyclines successfully induce prolonged remissions in over 80% of patients.[10] However, relapses are frequently accompanied by ATRA resistance and has a poor prognosis.[11] Arsenic trioxide (As_2O_3) has been shown to be an effective agent in patients with relapsed APL, inducing hematologic and molecular complete remission (CR) rates in 85% and 79% of the patients respectively.[12] Nevertheless, DS was observed in 23% of APL relapsed patients treated with As_2O_3 in the US multicenter trial.[13] This side effect could be explained by the partial differentiation induced by this drug at low doses.[14]

Among the strategies under development to treat relapsed/ATRA-resistant patients is the use of histone acetylases inhibitors (HDACis), such as phenylbutyrate (PB), which had been shown to reverse PML/RARα aberrant transcription repression and to potentiate ATRA-induced granulocytic differentiation.[15] Clinical trials using HDACis have yielded some promising preliminary results.[16] Nevertheless, the ability of HDAC inhibitors to restore ATRA sensitivity in resistant cells may depend on their specific mutation in the ligand binding domain of PML/RARα.[11]

Another potential therapeutic agent for ATRA-resistant APL is the granulocyte colony-stimulating factor (G-CSF, filgrastim). This cytokine was shown to bind to hematopoietic progenitors leading to proliferation and inhibiting apoptosis, committing these cells to differentiation into the myeloid lineage.[17] G-CSF is also active on mature neutrophils, enhancing chemotaxis, motility, phagocytosis and degradation of microorganisms.[18] Acute myeloid blasts express the G-CSF receptor, and both *in vivo* and *in vitro* studies have shown that G-CSF reduced their proliferation as a result of increased commitment to terminal differentiation.[19] In APL cells, G-CSF was shown to enhance the pro-differentiating effect of ATRA *in vitro* and when associated with ATRA was able to induce remission in an ATRA-resistant APL patient harboring the t(11;17)/PLZF-RARα rearrangement.[20]

Nevertheless, the *in vivo* effect of G-CSF and HDACis, as well as that of their association with ATRA, on the expression of adhesion molecules and on the adhering proprieties of APL cells has never been determined. To address this issue, we used flow cytometry to analyze the expression of adhesion molecules from the β-integrin (CD11a, CD11b, CD18 and CD29), immunoglobulin (CD54) and selectin (CD62L and CD162) families. Furthermore, we analyzed the role of leukocytic and endothelial cell CD54 and CD18 antigens in transmigration *in vivo*, using mutant mice in which the genes encoding for these proteins were inactivated by homologous recombination (CD54 and CD18 *knock-outs*).[21,22]

Design and Methods

Cells and culture conditions

After obtaining consent, bone marrow or peripheral blood cells were collected from 18 patients with newly diagnosed APL. Diagnosis was based on morphologic and cytochemical analysis of bone marrow smears and confirmed by identification of the fusion gene PML-RARα rearrangement by RT-PCR as previously described.[23] Mononuclear cells were separated by density centrifugation using Ficoll Isopaque (Pharmacia, Uppsala, Sweden) and cell suspensions containing

more than 90% leukemic cells (primary cells) were obtained. Both primary cells and the human promyelocytic cell line NB4 were seeded at a density of 5×10^5/mL and maintained in RPMI-1640 medium (GIBCO Invitrogen Co., Grans Island, USA) supplemented with 10% fetal calf serum, 2 mM of L-glutamine and 40 mg/mL of gentamicine at 37°C in a 5% CO2 humidified atmosphere. Samples were incubated for 18 hrs. with DMSO (vehicle), ATRA (1 μM; Sigma, St Louis, MO, USA), PB (1 mM; Sigma), G-CSF (200 U/mL; Filgrastim, Roche, Basel, Switzerland), dexamethasone (300 ng/mL; Sigma), or with the indicated associations at the same doses. Alternatively, cells were incubated with each pharmacologic agent in the presence of mouse anti-human CD11b or CD18 (0.1 mg/mL; BD Biosciences Pharmingen, San Diego, CA, USA). Non-binding mouse isotypic IgG1 and IgG2a antibodies were used as controls (BD Biosciences Pharmingen). The study was approved by the Ethics Commitee of the University Hospital of the Ribeirão Preto Medical School, University of São Paulo (process 3615/2004) and complies with the 1975 Helsinki Declaration (revised 2000).

Flow cytometric analysis

Cell surface expression of adhesion molecules on NB4 and primary leukemic cells was assessed by direct immunofluorescence. The panel of monoclonal antibodies (mAbs) directly conjugated with phycoerythrin (PE) used was: CD11a, CD11b, CD18, CD29, CD54, CD62L and CD162 (BD Biosciences Pharmingen). After treatment, cells were washed twice with PBS, and 100 μL of the cell suspension were protected from light and incubated with 10 μL with specific mAb for 60 mins. at 4°C. Isotype-matched antibodies of irrelevant specificity were used as negative controls. A *FACScan* flow cytometer with CellQuest software (Becton-Dickinson, San José, CA, USA) was used for analysis. The mean fluorescence intensity (MFI) was calculated by subtracting the value of the mean fluorescence channel of the respective isotypic control from that value obtained from the sample incubated with adhesion molecule specific MAb.

In vivo analysis of pulmonary infiltration by APL cells

Whether anti-CD54 or anti-CD18 MAbs were present or not, NB4 cells were treated in vitro with ATRA, G-CSF or ATRA+G-CSF as described above, harvested after 18 hours, washed twice in PBS, and a cell suspension containing $5x10^7$ cells/mL was prepared. One hundred microliters of this suspension were inoculated via the retro-orbital plexus of Balb-c/cBy mice (n=10 per group). Control mice were inoculated with PBS (C) or untreated NB4 cells. After 6 hrs., mice were sacrificed and pulmonary MPO activity was determined. In another set of independent experiments, CD54 or CD18 knock out mice generated as previously described[21,22] (n=12 per group) and their wild-type controls (C57-Bl6 and sv129 respectively) were injected i.p. with ATRA at the dose 1.5 μg/g of body weight. After 1.5 hrs., 5×10^6 NB4 cells were injected via the retro-orbital plexus. Two further ATRA injections followed at 3 hr. intervals. Control animals were injected with PBS (C), ATRA or NB4 cells only. Mice were sacrificed 6 hrs. after the 3rd ATRA injetion and pulmonary MPO activity was determined.

Pulmonary myeloperoxidase (MPO) activity

The extent of promyelocytic blast accumulation in the lungs was measured by assaying organ MPO content. The superior lobes of the right lungs of the mice were removed and homogenated in 2 volumes of ice-cold pH 4.7 buffer (NaCl 0.1 M, NaPO4 0.02 M, NaEDTA 0.015 M), and centrifuged at 3,000 rpm for 15 mins. The pellet was subjected to hypotonic lysis (900 μL of 0.2% NaCl solution followed 30 secs. later by the addition of an equal volume of a solution containing NaCl 1.6% and glucose 5%). After a further centrifugation, the pellet was resuspended in 0.05 M NaPO4 buffer (ph 5.4) containing 0.5% hexadecyltrimethylammonium (HTAB) and re-homogenized. The homogenate was then frozen and thawed three times and centrifuged again at 10,000 rpm for 15 mins. at 4°C. MPO activity in the resuspended pellet was assayed by measuring the change in optical density (OD) at 450 nm using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.5 mM).

Statistical methods

Differences between groups were compared by one-way ANOVA with Dunnet's post-test when data presented normal distribution, and by the Kruskall-Wallis test followed by Dunn's multiple comparisons test when data had non-Gaussian distribution. A value of $p<0.05$ was considered statistically significant. All analysis was performed using the SPSS 9.0 (SPSS Inc., Chicago, IL, USA) or the GraphPad Instat softwares (GraphPad Software, Inc., San Diego, CA, USA).

Results

Effect of ATRA, PB, G-CSF and their association on the expression of adhesion molecules

In NB4 cells, ATRA significantly upregulated CD54 and CD11b expression (Figure 1A and B). Changes in the former adhesion molecule were most evident with an approximately 13-fold increase in the median of MFI values. As_2O_3 (0.1 and 1 μM) also upregulated CD54, but no synergism was detected between the two drugs. When used as single agents, PB and G-CSF did not cause changes in the expression of any of the

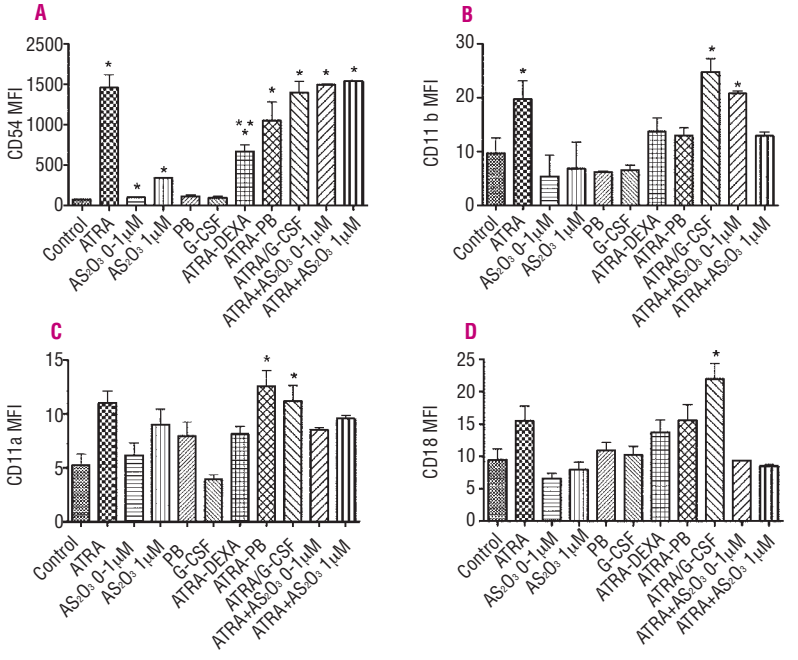

Figure 1. Effect of ATRA, As_2O_3, PB, G-CSF, ATRA+Dexamethasone, ATRA+PB, ATRA+G-CSF and ATRA+ As_2O_3 on the expression of adhesion molecules of NB4 cells. Bars show the median (+ 75th percentile) of the mean fluorescence intensity (MFI) for CD54 (A), CD11b (B), CD11a (C) and CD18 (D) of NB4 cells cultured for 18 hrs. in absence (C) or presence of ATRA (1 μM), As_2O_3 (0.1 or 1 μM), PB (1 mM), G-CSF (200U/mL), dexamethasone (Dexa, 300 ng/mL) or the indicated associations at the same concentrations. Eight independent experiments were performed. *p<0.05 compared to control; **p<0.05 compared to ATRA.

tested markers. Nevertheless, when used in association with ATRA they increased CD11a expression (Figure 1C). A similar effect on CD18 expression was observed with ATRA+PB (Figure 1D). Dexamethasone inhibited the ATRA-induced increase in CD54 MFI (Figure 1A). The expression of the remaining markers (CD29, CD62L and CD162) was not affected by any of the drugs, whether used alone or in combination (*data not shown*).

Due to limited cell numbers, in APL primary cells we only analyzed the effect of ATRA, PB and G-CSF and their association. ATRA significantly increased the expression of CD54, CD18 and CD11b (Figure 2 A-C). Similar to the effect observed in NB4 cells, ATRA/induced a particularly high increase in CD54 expression which was partially reversed by dexamethasone. G-CSF increased CD11b expression without significantly changing the intensity of fluorescence of the remaining markers. Furthermore, treatment with ATRA+G-CSF resulted in a significantly higher expression of CD18 and CD11b compared to ATRA alone. PB did not change MFIs of any of the adhesion molecules or modify the ATRA effect. The expression of CD29, CD62L and CD162 in primary cells was not affected by any treatment (*data not shown*). Along with the higher intensity of expression, there was an

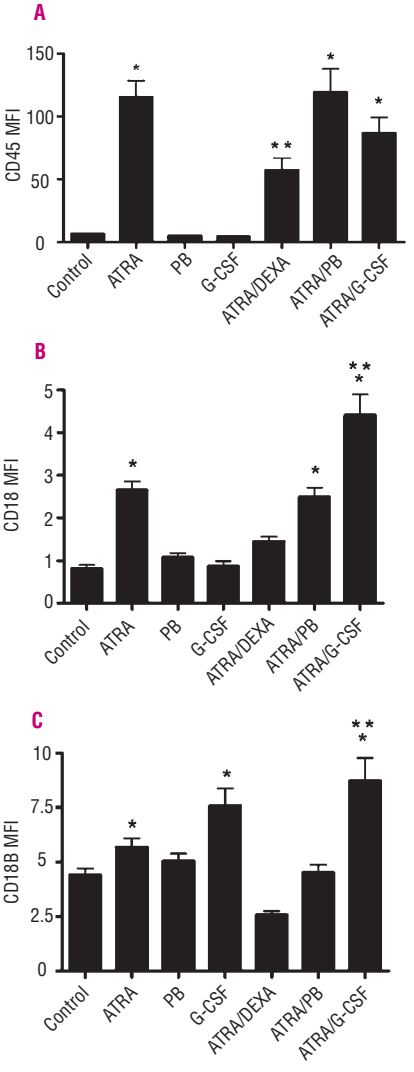

Figure 2. Effect of ATRA, PB, G-CSF, ATRA+dexamethasone, ATRA+PB and ATRA+G-CSF on the expression of adhesion molecules of APL primary cells. Bars show the median (+75th percentile) of the mean fluorescence intensity (MFI) for CD54 (A), CD18 (B) and CD11b (C) of blasts from patients with APL cultured for 18 hrs. in absence (C) or presence of ATRA (1 μM), PB (1 mM), G-CSF (200U/mL), dexamethasone (Dexa, 300 ng/mL) or the indicated associations at the same concentrations. Samples from 18 patients were analyzed. *p<0.05 compared to control; **p<0.05 compared to ATRA.

increase in the percentage of CD54⁺ cells following ATRA treatment in NB4 [95.7% (87.1-99%) *vs* 90% (74.8-95.2%)] and in primary cells [75.1% (22.8-90.3%) *vs* 5.1% (0.5-28.7%)]. In addition, the percentage of CD11b⁺ cells was higher in NB4 cells treated with ATRA [31.2% (24.8-78.1%) *vs* 8.5% (6.5-39.9%)]. The percentage of positive cells for the remaining markers was unchanged. As observed in the analysis of expression intensity, dexamethasone partially reversed the ATRA effect.

Furthermore, treatment with PB or G-CSF did not change the percentage of positive cells for the different markers or modify the ATRA effect. The morphologic analysis of cytospin preparations of untreated and treated samples showed no difference in the percentage of mature and immature myeloid cells. These results demonstrate that ATRA induces up-regulation of ICAM-1 and β2-integrin prior to morphologically identifiable granulocytic differentiation. Furthermore, the antagonistic effect of the dexamethasone was not associated with blocking ATRA-induced differentiation.

Effect of ATRA, PB, G-CSF and their association on the adhesion of NB4 cells to Matrigel

In order to determine whether the changes in the expression of adhesion molecules were accompanied by an increase in the adhesion proprieties of APL cells to the extracellular matrix, we treated NB4 cells with ATRA, PB, G-CSF and their association and tested for cell attachment to Matrigel. Compared to vehicle-treated controls, ATRA significantly increased the number of adhered cells (Figure 3). On the other hand, PB and G-CSF did not increase adhesion to Matrigel or modify the ATRA effect. Confirming the hypothesis that the increased adhesion to Matrigel was at least in part due to the increase in CD18 and CD54 expression, the co-incubation with anti-CD18 and/or anti-CD54 MAbs was able to reverse the ATRA effect. However, this was not achieved with an antibody of irrelevant specificity. Dexamethasone also blocked the ATRA effect (Figure 3).

In vivo effect of ATRA, ATRA+G-CSF on the adhesion of APL cells

Since significant synergism was only detected between G-CSF and ATRA regarding adhesion molecule expression, only these agents were used for *in vivo* assays. Figure 4 shows the MPO activity in the lungs of Balb-C mice after I.V. injection of saline (C) or NB4 cells pre-treated *ex vivo* with ATRA or ATRA+G-CSF. The increased MPO activity in mice injected with cells pre-treated with ATRA compared with those injected with saline or untreated cells reflects the increased NB4 cells infiltration. However, the treatment with the association of G-CSF and ATRA did not significantly increase MPO activity in the lungs compared with

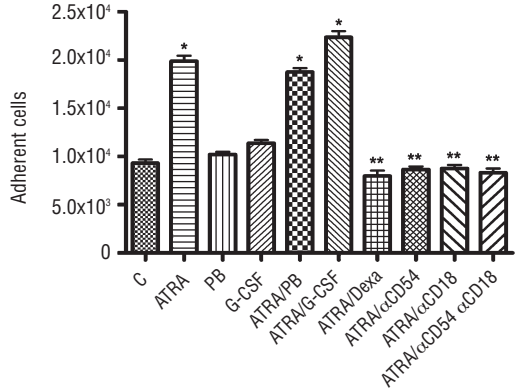

Figure 3. Cell adhesion to Matrigel. NB4 cells (10⁵ cells) were cultured for 18 hrs. in absence (C) or presence of ATRA (1 μM), PB (1mM), G-CSF (200U/mL), dexametasone (Dexa, 300 ng/mL) or the indicated associations at the same concentrations. After incubation, cells were added to Matrigel containing wells and allowed to adhere for 2 hrs. at 37°C, then lysed and meloperoxidase activity quantified by colorimetry. Wherever indicated, cells were treated with ATRA and 30 mins. before being added to Matrigel, incubated with MAbs anti-CD18 and/or anti-CD54 or with mouse immunoglobulin of irrelevant specificity (mIg). The data are presented as fold change of the untreated controls, and the bars represent the mean values (± SD) of 13 independent experiments. *p<0.05 compared to control; **p<0.05 compared to ATRA.

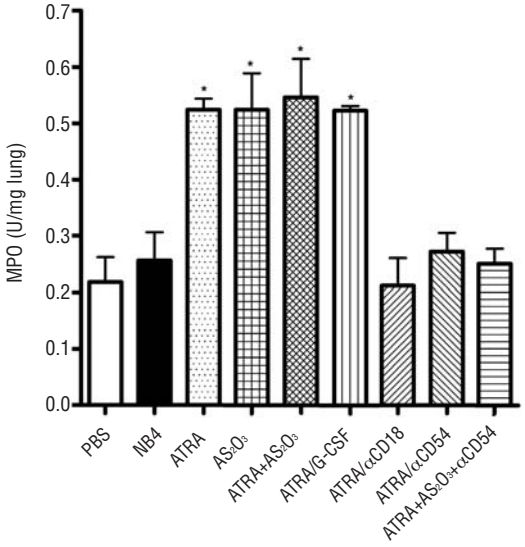

Figure 4. Adhesion to lung microvasculature. NB4 cells were cultured in absence or presence of ATRA, As_2O_3, ATRA+G-CSF, ATRA⁺ As_2O_3 for 18 hrs. Wherever indicated, ATRA-treated samples were then incubated with MAbs anti-CD18 (αCD18) or anti-CD54 (αCD54) for 30 mins. After incubation, five million cells were inoculated via the retro-orbital plexus of Balb-c mice (n=10 per group). Control mice were inoculated with PBS (C) or untreated NB4 cells. After 6 hours, mice were sacrificed and the pulmonary MPO activity was determined. Values represent number of myeloid cells per 100 g of lung. Horizontal bars represent the mean. *p<0.05 compared to PBS and untreated NB4 cells.

ATRA alone. When NB4 cells were co-incubated with ATRA and anti-CD18 or anti-CD54 MAbs, MPO activity decreased to levels similar to those of the control groups (Figure 4).

Analysis of ATRA-induced cell adhesion in CD54 and CD18 K.O. mice

To test in vivo the role of endothelial CD54 and CD18 in DS genesis, we treated CD54 and CD18 *k.o.* mice, and their wild-type controls with ATRA, and inoculated them with NB4 cells. In the wild-type group, a significant increase in MPO activity was detected in mice injected with ATRA+NB4 cells compared to controls that received ATRA or cells only (Figure 5). By contrast, no significant difference was observed in MPO activity in the lungs of CD54 and CD18 *k.o.* mice receiving saline, ATRA, NB4 cells or ATRA+NB4 cells. These results suggest that cell adhesion to pulmonary capillaries characteristic of the differentiation syndrome depends on the up-regulation of adhesion molecules by APL cells.

Discussion

Leukocyte emigration from blood is a key event in the development of DS. It depends on a sequential cascade of leukocyte-endothelium interactions which are regulated by adhesion molecules. Pathologic findings of DS resemble other medical conditions related to increased granulocyte adhesiveness, especially to the lung microvasculature, like transfusion-related lung injury (TRALI).[24] Following primary attachment, firm adhesion and emigration of leukocytes from blood depend on the interaction of β2-integrins on the surface of leukocytes with adhesion molecules of the immunoglobulin family on the endothelium.[25] We detected a significant increase in the expression of CD11b and CD54 in ATRA-treated NB4 cells, while in APL primary cells, in addition to these two markers, CD18 was also up-regulated by ATRA. As_2O_3 is probably the most effective single agent in APL therapy.[26] It induces apoptosis at high doses and cell differentiation at low concentrations.[14,27] Arsenic can induce the phosphorylation of SMRT/N-CoR through the MAPK pathway, and similar to ATRA, induces differentiation through disruption of the PML-RARα repressive activity.[28] In the present study, As_2O_3 up-regulated CD54 in NB4 cells both at 0.1 and 1 μM concentration. Importantly, no synergism between ATRA and As_2O_3 was detected. This observation is relevant in face of the recent results of clinical trials using the combination of As_2O_3 and ATRA as front line therapy for APL.[29] Based on our results, the combined treatment is not expected to lead to an increase in DS incidence.

PB as single agent did not significantly affect the expression of adhesion molecules or modify the ATRA effect. On the other hand, G-CSF treatment up-regulated CD11b expression and potentiated ATRA-induced CD18 and CD11b expression on APL primary cells. The synergism between HDACis or G-CSF and ATRA in the induction of granulocytic differentiation has been well demonstrated but can only be seen after 3 to 4 days of incubation.[30,31] In the present study, samples were treated for shorter periods, therefore the data was not affected by differences in cell subpopulation, as shown by the morphologic analysis.

The increase in expression was accompanied by a higher adhesion to Matrigel and to pulmonary endothelium, and was blocked by pre-incubation with dexamethasone, anti-CD54 or anti-CD18. The beneficial effect of dexamethasone in the treatment of patients with DS has been well demonstrated.[4,6] Our results suggest that at least part of its effects may be caused by blocking ATRA-induced up-regulation of adhesion molecules. Interestingly, despite the synergism between G-CSF and ATRA in the induction of CD18 expression, no significant difference in adhesion *in vitro* and *in vivo* was found.

The results obtained in the *ex vivo* experiments using cells pre-incubated with anti-CD54/anti-CD18 were confirmed by those with CD54 and CD18 *k.o.* mice. Together they suggest that both leukocytic and endothelial adhesion molecules are essential for DS development. Previous studies demonstrated that ATRA enhanced the interaction between NB4 cells

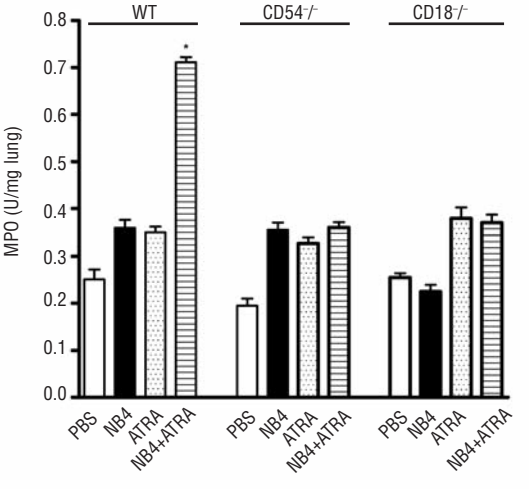

Figure 5. ***In vivo*** **analysis of ATRA-induced adhesion of APL cells to the lung.** ***Knock out*** **mice for CD54 or for CD18 and their wild-type controls (C57Bl-6 and sv129 respectively) (n=12 per group) were injected i.p. with ATRA (1.5 μg/g of body weight), and 1.5 hrs. later injected via the retro-orbital plexus with NB4 cells (5×10^6). Two other ATRA injections followed at 3 hr. intervals from the first. Control animals were injected with PBS (C), ATRA or NB4 cells only. Six hours after the third ATRA injection, mice were sacrificed and the pulmonary MPO activity was determined. Bars represent the mean values (± SD). *$p<0.05$ compared to PBS and untreated NB4 cells.**

(aggregation) and facilitated transmigration, an effect that was inhibited by anti LFA-1 (CD11a/CD18) and ICAM-2 (CD102) MAbs.[32] Therefore, DS may depend on several different levels of cellular interaction.

Leukocyte adhesion and transmigration has been extensively studied in models of infection or tissue-injury, the former frequently using LPS or sepsis as a stimulus and the latter, drug and mechanical trauma.[33,34] However, these are not precipitating factors for DS, and despite the many physiopathological similarities, the mechanism of leukocyte emigration may differ according to the stimulus and target organ. In the present study, we used both *ex vivo* and *in vivo* models without inflammatory stimulus, but we chose to use immuno-competent mice. Immuno-deficient strains, such as NOD or NOD/SCID mice are frequently used in the study of transplant biology. However, our interest lay in drug-induced changes of cell adhesion and not in engraftment. Our results underline the relevance of the up-regulation leukocyte ICAM-1 by ATRA, even in the absence of inflammatory stimulus of the endothelium.

In conclusion, our results suggest that the use of As_2O_3, G-CSF or PB concomitant with ATRA should not increase the frequency or aggravate the clinical course of DS in APL patients. They also emphasize the relevance of leukocytic CD54 and CD18 in the pathogenesis of this potentially fatal complication.

Authors' Contributions

GCudS, MBT, RS, SEM and DS have performed experiments, acquired and analyzed data; they have also drafted the manuscript and approved its final version; ABG and ASGL have performed experiments and acquired data; they have also drafted the manuscript and approved its final version; LHF, RPF, FQC and EMR have contributed to the concept and design of the study, have analyzed and interpretated of data, and revised the drafted manuscript and improving significantly its intellectual content. Finally they have read and approved its final version.

Conflict of Interest

The authors reported no potential conflicts of interest.

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