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Novel use of All-*trans*-Retinoic Acid in a model of Lipopolysaccharide-immunosuppression to decrease the generation of myeloid-derived suppressor cells by reducing the proliferation of CD34+ precursor cells.

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Running head: ATRA effects on LPS-induced myelopoiesis

ABSTRACT

All-*trans*-Retinoic Acid (ATRA) is a derivative of vitamin A with anti-proliferative properties. Endotoxin shock and subsequent immunosuppression (IS) by lipopolysaccharide (LPS) stimulates myelopoiesis with expansion of myeloid-derived suppressor cells (MDSC). Since we have previously shown that ATRA reverses the IS state by decreasing functional MDSC, our aim was to investigate if ATRA was able to modulate MDSC generation by regulating myelopoiesis in murine hematopoietic organs. We found that ATRA administration *in vivo* and *in vitro* decreased the number of CD34+ precursor cells that were increased in IS mice. When we studied the cellular mechanisms involved, we did not find any differences in apoptosis of CD34+ precursors or in the differentiation of these cells to their mature counterparts. Surprisingly, ATRA decreased precursor proliferation, *in vitro* and *in vivo,* as assessed by a reduction in the size and number of colony forming units (CFU) generated from CD34+ cells and by a decreased incorporation of ^{3}H-thymidine. Moreover, ATRA administration to IS mice decreased the number of MDSC in the spleen, with a restoration of T lymphocyte proliferation and a restitution of the histological architecture. Our results indicate, for the first time, a new use of ATRA to abolish LPS-induced myelopoiesis, affecting the proliferation of precursor cells, and in consequence, decreasing MDSC generation, having a direct impact on the improvement of immune competence. Administration of ATRA could overcome the immunosuppressive state generated by sepsis that often leads to opportunistic life-threatening infections. Therefore, ATRA could be considered a complementary treatment to enhance immune responses.

Keywords: sepsis; LPS; immunosuppression; MDSC; ATRA

ABBREVIATIONS

LPS: lipopolysaccharide

ATRA: All-*trans* Retinoic Acid

MDSC: Myeloid-Derived Suppressor Cells

IS: Immunosuppressed group

BM: Bone Marrow

CFU: Colony Forming Units

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

HSC: Hematopoietic Stem Cells

Con A: Concanavalin A

AnV: Annexin V

INTRODUCTION

Retinoids are natural derivatives, or synthetic analogs, of vitamin A involved in many important biological processes. Aberrant retinoid signaling mechanisms have been associated with cancer development in humans and animals (1). Retinoids are being increasingly included in both chemopreventive and therapeutic schemes of various tumoral diseases (2). In general, they are believed to inhibit carcinogenesis by blocking the promotion of already initiated or transformed cells by three mechanisms: induction of apoptosis, irreversible cell cycle arrest (cellular senescence), and induction of abnormal cells to differentiate back to normal (2, 3). In particular, all-*trans*-retinoic acid (ATRA), the active derivative of vitamin A, has been extensively studied as an anti-cancer agent (2). It has been shown that ATRA inhibits the growth of tumor cells by blocking the cell cycle (3). However, the effect of ATRA on normal myeloid cells is much less clear.

Sepsis is one of the oldest and most elusive syndromes in medical history. The mortality of sepsis still remains at about 20% to 30% in the USA (4). Patients who survive the initial shock, mainly the pro-inflammatory, phase of sepsis are at risk of developing a state of immune suppression and become susceptible to nosocomial infections with organisms that are not typically pathogenic in immunocompetent hosts. In fact, secondary infections that develop due to post-sepsis immunosuppression are a major cause of death in patients with sepsis (5). In this sense, the effects of ATRA on an immunosuppressed scenario due to infections have been less studied.

Immature myeloid-derived suppressor cells (MDSC) are a heterogeneous cell population consisting of myeloid progenitor cells, considered to be one of the major components of the immune suppressive network responsible for suppressing T-cell responses in pathological conditions such as tumors (6). More recently, expansion of MDSC has been described in acute and chronic inflammatory diseases including trauma, inflammation, burn, and autoimmune diseases (7). In mouse, MDSC are identified by a Gr-1+ CD11b+ CD31+ phenotype and their expansion has been recently reported in spleen and lymph nodes from septic animals (8), and in BM and lymph nodes from lipopolysaccharide (LPS)-induced immunosuppressed mice (9, 10), as well as in blood from septic patients (11).

Hematopoiesis is a hierarchical process in which hematopoietic stem cells (HSC) differentiate into progenitor cells (common lymphoid or common myeloid progenitors) that are capable of exponential proliferation as well as continuing the process of differentiation, accounting for repopulation of myeloid, lymphoid and megakaryocyte/erythroid cells during normal replenishment (12). CD34 has served as the most important marker for hematopoietic precursors and is present all along progenitors that give rise to myeloid cells (13). Moreover, only CD34+ cells have the ability to form hematopoietic colonies *in vitro*, whereas no colonies are generated from CD34- cells at any developmental stage (14).

After infections, in several inflammatory reactions and after LPS administration, there is an increased myelopoiesis in bone marrow (BM) and a subsequent cell precursor mobilization to peripheral blood in order to repopulate myeloid populations (12). In this sense, an increase of CD34+ cells has been reported in septic patients and in cecum ligation and puncture (CLP) mice (15). As part of this CD34+ cell expansion, there is also an increase in MDSC population. Possibly developed as a normal regulatory mechanism to counteract a triggered immune response, MDSC generation in a dysregulated pathological scenario, such as sepsis, may be involved in the etiology of LPS-induced immunosuppression. In this sense, the reduction of this suppressive population has been shown to be beneficial in different experimental settings (7). Moreover, direct suppressive activity of CD34+ cells has been reported as well. In sepsis, the presence of granulocyte colony stimulating factor (G-CSF) is reported to mobilize a murine population of CD34+ cells endowed with potent immunosuppressive activity (16). This population was shown to inhibit the alloreactive response *in vitro* and *in vivo*. Moreover, in patients with head and neck cancer, the presence of CD34+ cells suppressed functional competence of lymphocytes within the squamous cell carcinoma and inhibited their capacity to secrete interleukin-2 (17). Altogether, these results support the idea that eliminating or reducing the increase of the CD34+ cell precursor population, may contribute to an improved immunosuppressive state in sepsis.

Experimentally, repetitive inoculation of increasing doses of LPS to mice induces derangements in the inflammatory response and a state of adaptive immunosuppression (9, 18), mimicking several clinical signs found in patients who develop immunosuppression after a septic episode (19). Exposure to LPS has been considered the initial phase and one of the causes of the immunosuppression frequently observed in

late sepsis. Using this model we and others have shown that mice develop a clear state of immunosuppression, as evidenced by an impaired antigen-specific antibody production, decreased T cell proliferation, lymphocyte depletion, increased of immature myeloid cells with suppressive functions, reduced ability to produce TNF-α and other cytokines *in vitro*, and induction of anti-inflammatory mediators such as IL-10, transforming growth factor-beta and glucocorticoids (9, 10, 18, 20).

In a previous work, we have shown that ATRA administration to LPS-immunosuppressed mice was able to restore the number of different lymphocyte populations and reversed the inhibited MDSC-mediated T cell proliferation in lymph nodes (9). Therefore, in this work our aim was to address the question whether ATRA can affect LPS-induced myelopoiesis, regulating the number of immature MDSC and probably their generation, in order to reestablish the immunologic unbalance in immunosuppression. Elucidating this mechanism is critical not only for understanding the biology of ATRA effects on myeloid cells but also for assessing its potential use, together with other therapies, in post-sepsis immunosuppression.

MATERIALS AND METHODS

Reagents

LPS: *E. coli* O111:B4 (Sigma, St. Louis, USA), ATRA for *in vivo* experiments (Vesanoid®, F. Hoffman-La Roche Ltd, Basel, Switzerland), RPMI-1640 (Gibco, MA, USA), FCS (Gibco, MA, USA), 2-mercapoethanol (Gibco, MA, USA), L-glutamine (Gibco, MA, USA), antibiotic-antimycotic (Gibco, MA, USA), GM-CSF (Sigma, St Louis, MO, USA), ATRA for *in vitro* experiments (Sigma, St. Louis, USA), Antibodies: Phycoerythrin (PE)-CD34, -F4/80, -Gr-1, -DHR; fluorescein isothiocyanate (FITC)-CD11c, -Annexin V (AnV), -iNOS; Phycoerythrin-Cyanine 5 (PE-Cy5)-Gr-1,-CD3,-CD31 (All antibodies were purchased from BD Bioscience, San Jose, CA, USA), paraformaldehyde (Anedra, Buenos Aires, Argentina), Bacto agar (Difco Laboratories, USA), Iscove's modified Dulbecco's medium (IMDM; HyClone, Logan, UT, USA), ^{3}H-thymidine (Dupont Nen, Boston, USA), scintillation fluid (Optiphase Hisafe 2, PerkinElmer, Waltham, MA, USA), Concanavalin A: (Sigma, St. Louis, USA), magnetic beads: (Invitrogen Dynal AS, Oslo, Norway), gentian Violet (Merck, Darmstadt, Germany), glacial acetic

(Merck, Darmstadt, Germany), triton X-100 (Merck, Darmstadt, Germany), GraphPad Prism 5.0 software (San Diego, CA, USA).

Experimental model of LPS-induced immunosuppression

BALB/c mice were bred in the animal facility of IMEX, Academia Nacional de Medicina, Buenos Aires. Male mice aged 2–3 month and weighing 20–25 g were used throughout the experiments. They were maintained under a 12-h light–dark cycle at 22±2 °C and fed with standard diet and water *ad libitum.* For all experiments, LPS-induced immunosuppression was developed by daily subcutaneous (s.c.) inoculation of increasing doses of LPS for a period of 15 days, as follows: two doses of 5, two doses of 10, two doses of 20, three doses of 50 and six doses of 100 μg/day/mouse. In *in vivo* experiments, concomitantly to LPS administration, another group of mice was daily inoculated via intraperitoneal (i.p.) either with vaseline (IS group), or ATRA (500 μg/day/mouse, IS+A group). ATRA was obtained from commercial supply. The capsule content was removed in sterile conditions and the concentration adjusted with vaseline immediately before administration. Other groups of animals were given every day s.c. saline solution and i.p. vaseline (Control group), or s.c. saline solution and ATRA i.p. (ATRA group). The total number of animals per group was 8 or 12 according to each experiment. For experiments that the total number per group was 8, we performed 2 experimental repetitions with 4 animals in each group and for experiments that the total number per group was 12, we performed 3 experimental repetitions with 4 animals in each group (see corresponding Legend to Figure).

The experiments were conducted according to principles set forth in the Guide for the Care and Use of Laboratory Animals (21), and the study was approved by the Animal Care and Use Committee of our Institution.

Cell tissue collection

The spleen and bone marrow (BM) were removed from mice under sterile conditions, and single-cell suspensions were prepared by homogenization through a sterile stainless steel mesh. To perform the experiments, the spleens were processed in 2 ml of medium RPMI-1640, 10 % FCS, 0.1 % 2-

mercapoethanol, 1 % L-glutamine and 1 % antibiotic-antimycotic (complete medium). After centrifugation, the cell suspension was collected. Femurs and tibiae of mice were removed and freed of muscles and tendons. The bones were placed in 70 % ethanol for 2 min and subsequently washed in PBS. Both bone ends were cut and cells were flushed from the bones under sterile conditions with 2 ml of complete medium. Cell suspensions were washed and resuspended in complete medium. Absolute leukocyte counts were obtained using a Neubauer chamber using Turk´s solution (0.01 % gentian violet in 3 % glacial acetic acid) by optical microscopy.

Cell cultures with ATRA in vitro

Cells suspensions obtained from immunosuppressed (IS) spleens and BM were supplemented with low concentrations of GM-CSF (10 ng/ml) in order to stimulate granulocyte and monocyte precursors to proliferate and survive in culture. Splenic and BM cell cultures from IS mice were treated with ATRA (10 μM, IS/+A) or were left untreated (IS/-A).

Flow cytometric studies

The main different populations present in the spleen and BM were studied using flow cytometry. 1 x 10^6 cells were incubated with specific rat anti-mouse antibodies conjugated with different fluorochromes: Phycoerythrin (PE)-CD34, -F4/80, -Gr-1; fluorescein isothiocyanate (FITC)-CD11c; Phycoerythrin-Cyanine 5 (PE-Cy5)-Gr-1, -CD31. Cells were washed and resuspended in paraformaldehyde 0.5 %. The percentage of positive cells was determined among 70.000 events. The absolute number of the specific subpopulations was calculated as follows: (Absolute total leukocyte cell count x Percentage obtained by flow cytometry)/100.

Apoptosis

The percentage of hematopoietic precursors (CD34+) undergoing apoptosis was measured by flow cytometry using FITC-Annexin V (AnV), and anti-CD34-PE in cell suspensions obtained from the spleen and BM after 24 h of ATRA addition. AnV staining was performed using a commercial kit according to

manufacturer instructions. The absolute number of the apoptotic cells was calculated as follows: (Absolute total leukocyte cell count x Percentage obtained by flow cytometry)/100.

Colony formation unit (CFU) assay

BM and splenic cells were plated in 0,1% Bacto agar in Iscove's modified Dulbecco's medium containing penicillin–streptomycin, 20 % FCS, 20% glutamine and 100 ng/ml of recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF). They were added to six-well plates with 8 x 10^5 BM cells and 1 x 10^6 spleen cells per well. The plates were incubated at 37 °C in 5 % CO_2 for 7 days in order to form a semi-solid agar allowing granulocyte-monocytes colonies to grow and differentiate. Colonies (>40 cells) were scored by scanning the whole Petri dishes using an inverted microscope with phase contrast with 20 x magnification. In order to obtain the colonies area, pictures were taken with 40 x magnification and the areas calculated using the Fiji software program (22).

Proliferation assays

5 x 10^5 splenic and BM cells were seeded in 96 well plates with GM-CSF (10 ng/ml) for 48 h at 37 ºC in 5 % CO_2. Then 0.5 μCi/well of ^{3}H-thymidine was added and incubated for another 20 h. After that cells were harvest and scintillation fluid was added to the vials (2 ml/disc) and the radioactivity was measured using a beta-counter.

For T lymphocyte proliferation assays, suspensions from spleen were obtained and resuspended in RPMI-1640, 10 % FCS, 0.1 % 2-mercapoethanol, 1 % L-glutamine and 1 % antibiotic-antimycotic. Lymphocyte counts were determined with a Neubauer chamber using Turk´s solution. Moreover, $CD3^+$ lymphocytes were determined by flow cytometry and the same initial number of $CD3^+$ cells (2 x 10^5) for all groups were seeded in 96 well plates in triplicates in the presence or absence of Concanavalin A (Con A, 5 μg/ml) for 48 h at 37 ºC in 5 % CO_2. Then 0.5 μCi/well of ^{3}H-thymidine was added and incubated for another 20 h and proceeded as previously described to measure the incorporation of ^{3}H-thymidine.

Gr-1 depletion

Spleen suspensions (5 x 10^6 cells) were incubated with an anti-Gr-1 mAb for 30 min at 4 ºC and then washed. Depletion was subsequently performed by incubating with magnetic beads according to manufacturer instructions. The cell depleted fraction showed less than 2 % of Gr-1+ cells as assessed by flow cytometry.

Intracellular inducible NO synthase (iNOS) determination

24 h after Con A addition to the cultures, cells were collected with trypsin-0.5 % EDTA, washed and counted with Turk's solution by optic microscopy. Then, cells were incubated with PE-CD11b and PE-Cy5-Gr-1 antibodies for 30 min, washed and fixed with paraformaldehyde 0.05 %. Cells were subsequently treated with triton X-100 (0.25 %) for 15 min, blocked with 3 % BSA for 1 h and stained with an anti-iNOS antibody conjugated to FITC for 30 min. In all cases, isotype-matched antibodies were assayed in parallel and fluorescence was determined by flow cytometry on 100,000 events of each sample. The % of iNOS+ cells within the Gr-1+ CD11b+ population was determined and the absolute number of the iNOS+ Gr-1+ CD11b+ populations was calculated as follows: (absolute cell count x percentage obtained by flow cytometry)/100.

Reactive oxygen species (ROS) determination

After 24 h of Con A addition to the cultures, cells were collected with trypsin-0.5 % EDTA, washed and counted using Turk's solution by optic microscopy. To determine the production of ROS, dihydrorhodamine (DHR)-123 (Sigma) was used. Cells were incubated with a PE-Cy5-Gr-1 antibody and 1 μM of DHR-123 for 15 min at 37 °C. Upon ROS generation a brightly fluorescent FL-1 product is produced, which was detected by flow cytometry. The % of DHR+ cells within the Gr-1+ population was determined and the absolute number of the DHR+ Gr-1+ populations was calculated as follows: (absolute cell count x percentage obtained by flow cytometry)/100.

Histology

The spleens of all sacrificed animals were fixed in 10 % buffered formalin and embedded in paraffin. Semi-serial cuts were performed for hematoxylin-eosin staining (H&E).

Statistics

For *in vivo* experiments, comparisons between multiple groups (Control, IS, and IS+A) were performed using one-way ANOVA, applying the correction of Bonferroni with the GraphPad Prism 5.0 software. For *in vitro* experiments, comparisons between cells obtained from IS mice left untreated (IS/-A) or treated with ATRA (IS/+A) were performed using a two-tailed paired *t*-test. In all cases, $p<0.05$ was considered significant.

RESULTS

ATRA decreases the number of hematopoietic CD34+ precursors in a murine model of LPS-immunosuppression

To explore the capacity of ATRA to modify the number of CD34+ progenitors in LPS-induced immunosuppression, mice were daily inoculated with LPS (IS), and ATRA was administered concomitantly with LPS (IS+A). Cells obtained from the BM and the spleens were used because the spleen in adult mice, in contrast to humans, is both a hematopoietic and a secondary lymphoid organ (23). The percentage of CD34+ cells was determined by flow cytometry and the absolute number was calculated considering the total leukocyte count as detailed in material and methods. As Figure 1 shows, the IS group presented an increased number of hematopoietic CD34+ precursors both in the spleen and BM compared to the Control group. The treatment with ATRA in IS mice (IS+A) decreased the number of this population in both organs compared to the IS group. Administration of ATRA alone had no effect on the number of CD34+ cells compared to the Control group (data not shown). These results show the ability of ATRA to decrease the expansion of hematopoietic precursors in LPS-immunosuppressed mice.

ATRA decreases hematopoietic precursor proliferation in LPS-immunosuppression

Taking into account the decrease in CD34+ cells observed in IS mice treated with ATRA, we performed *in vitro* cultures incubating splenic and BM cells from IS mice with or without ATRA in order to study the cellular mechanisms by which ATRA decreased the expansion of hematopoietic CD34+ precursors in LPS-induced immunosuppression.

As reported by other authors (24 and own unpublished results), we observed that cells, especially myeloid cells, do not survive more than 3 days without the presence of cytokines in culture (data not shown). Therefore, for all *in vitro* approaches we supplemented cell cultures with low concentrations of recombinant mouse granulocyte-monocyte colony stimulating factor (GM-CSF) in order to prevent unspecific cell death and, additionally, to favor granulocytic-macrophage myeloid precursor growth and proliferation.

Figure 2 A, shows that the numbers of CD34+ cells in IS BM and splenic cultures treated with ATRA (IS/+A) were decreased compared to untreated cells (IS/-A) after 72 hs of culture. To elucidate the mechanisms of this reduction, we determined if CD34+ precursors were able to undergo further maturation into differentiated cells such as neutrophils (Gr-1+ CD11b+), macrophages (F4/80+) or dendritic cells (CD11c+). For this purpose, we incubated IS BM and splenic cells for 72 h with ATRA and analyzed the resulting cell populations by flow cytometry. As Figure 2 B shows, ATRA decreased the number of Gr-1+ CD11b+ and CD11c+, while F4/80+ cells remained unchanged compared to untreated cells in both spleen and BM. This result indicates that the decrease of CD34+ cells observed by treatment with ATRA is not a consequence of an increased differentiation of these precursor cells to mature lineages.

Then, we determined if the lower number of CD34+ cells observed by treatment with ATRA *in vivo* and in *vitro* was due to apoptosis measuring AnV+ CD34+ cells by flow cytometry after addition of ATRA. However, as depicted in the Supplementary Figure 1, http://links.lww.com/SHK/A514, ATRA did not affected the apoptotic rate in IS cell cultures.

Altogether, these results indicate that in an LPS-induced immunosuppressed context ATRA does not decrease the level of immature CD34+ progenitors by increasing their differentiation to mature myeloid lineages (Gr-1+ CD11b+, F4/80+, CD11c+) or by inducing their apoptosis.

This led us to explore whether ATRA could modulate the growth/proliferation of CD34+ myeloid progenitors. First, we performed a colony forming unit (CFU) assay using semi-solid agar with GM-CSF, that allows the evaluation of the quantity and characteristics of colonies generated exclusively by CD34+ cells after 7 days (14). As shown in Figure 3 A, the number of CFU decreased when IS cells from BM and spleen were treated with ATRA. Moreover, the area of the colonies was also lower in the presence of ATRA (Figure 3 B). These results suggest that ATRA is modulating not only the number of myeloid precursors capable of generating colonies but also their ability to proliferate. To further confirm if ATRA is affecting the proliferation of hematopoietic precursors, a proliferation assay was performed measuring the incorporation of ^{3}H-thymidine after 72 h of ATRA addition in splenic and BM cells in suspension obtained from IS mice. Supporting the CFU assay results, the addition of ATRA decreased the incorporation of ^{3}H-thymidine in both hematopoietic organs (Figure 3 C).

ATRA administration to LPS-immunosuppressed mice affects hematopoietic precursor proliferation

In order to extrapolate the effects observed in IS BM and splenic cultures treated with ATRA, mice were given ATRA concomitantly with LPS inoculation and CFU assays in the presence of GM-CSF were performed with cells obtained from BM and spleen of mice. As depicted in Figure 4 A, administration of ATRA to mice (IS+A group) was able to reduce the number of CFU compared to the IS group, reaching values similar to those observed in the Control group, both in spleen and BM. Furthermore, the area of the colonies of IS+A mice was lower compared to IS mice (Figure 4 B). Moreover, incorporation of ^{3}H-thymidine in cells obtained from the spleen and BM from IS+A mice supplemented with GM-CSF in culture decreased compared to the IS group (Figure 4 C).

Altogether, the *in vitro* and *in vivo* results indicate that the mechanism by which ATRA decreases the number of hematopoietic CD34+ precursors is by reducing their cellular proliferation.

Administration of ATRA to IS mice decreased the generation of immature myeloid-derived suppressor cells (MDSC) and restored T lymphocyte proliferation

MDSC are an immature myeloid cell population that are increased in late phases of sepsis playing a key role in the inhibition the T lymphocyte proliferation observed in IS mice (9). Moreover, based on the effects of ATRA in the decrease of immature cell proliferation, our next objective was to study if this drug was able to decrease the number of MDSC in IS mice and, as a result, modulate the impaired T cell proliferation in LPS-derived immunosuppression. Mouse MDSCs are identified by the co-expression of CD11b, Gr-1 and the immaturity marker CD31 on their cell surface (25). As the spleen in adult mice is both a hematopoietic and a secondary immune organ, where immunological responses are initiated, we analyzed the presence of MDSC in the spleen of IS mice and their modulation by ATRA. Figure 5 A shows that IS mice had an increment in the number of MDSC (Gr-1+ CD11b+ CD31+) compared to the Control group and that the administration of ATRA (IS+A) was able to decrease this number. In order to evaluate whether this decrease was associated with a lower MDSC-mediated T cell suppressive function, we performed T-cell proliferation assays from splenic cells measuring the incorporation of ^{3}H-thymidine using Concanavalin A (Con A) as a specific T-cell mitogen. As shown in Figure 5B, T cell proliferation was decreased in IS mice compared to the Control group. Depletion of Gr-1+ cells from IS cell suspensions restored T cell proliferation to Control levels, indicating that MDSC in IS mice are responsible for the observed T cell proliferation inhibition. Additionally, treatment with ATRA to IS mice was able to restore the inhibited T cell proliferation, indicating that the lower number of MSDC observed by treatment with ATRA in IS mice is related to a reduced inhibitory function. Mice treated only with ATRA showed numbers of MDSC and levels of T cell proliferation similar to Control mice (data not shown).

It is known that the correct architecture of secondary lymphoid organs is necessary for an efficient initiation of adaptive immune responses (26). Therefore, we examined the spleen's histology in order to study the effect of ATRA on the histological structure of this organ in the different groups. Figure 5 C shows that the white pulp from spleens from Control mice had normal periarteriolar lymphoid sheaths (PALS), follicles and marginal zones. Splenic cords and venous sinuses were also seen in the red pulp

normal. However, the white pulp structures from IS mice were smaller and with an irregular shape; this shrinkage was mainly due to the depletion of lymphocyte populations in the outer PALS, the follicle, and the marginal zone. Additionally, the red pulp was relatively enlarged due to a decrease in all white pulp cellular components. Surprisingly, the spleens of IS animals treated with ATRA showed a partially restored histology. The white pulp structures reassumed their size and shape in an irregular fashion; some of the animals showed complete and normal cell populations while others showed an incomplete cell population lacking, mainly, the marginal zone. The red pulp seemed to be restored and the white/red pulp relationship was partially restored. Spleens from animals treated only with ATRA were similar to those of the Control group (data not shown). Altogether, these results indicate that ATRA is able to improve T cell functions in IS mice by decreasing MDCS cells and restoring the architecture of the spleen.

ATRA *in vitro* restored T cell proliferation and reduces the number of MDSC with suppressor activity from IS mice

In order to determine if ATRA directly affects the number of MDSC and in consequence, impacts on T cell proliferation, we performed *in vitro* assays adding ATRA to the cultures of splenic cells from IS mice. MDSC have been described to inhibit T cell proliferation by different mechanisms: causing an increase in the inducible form of Nitric Oxide Synthase (iNOS) that produces NO (Nitric Oxide) and releasing Reactive Oxygen Species (ROS) (10). Figure 6 A shows that the addition of ATRA to IS cells *in vitro* caused an increase in T cell proliferation. This effect was accompanied by a decrease in the number of Gr-1+ iNOS+ and Gr-1+ DHR+ cell populations (Figure 6 B and C), indicating that ATRA can directly suppress the generation of MDSC and in consequence, improve T cell proliferation.

DISCUSSION

Vitamin A (retinol) is known to play an essential role in immunity and may be important for the optimal functioning of the innate and adaptive immune systems. Most of the effects of vitamin A are mediated by ATRA. Vitamin A deficiency has been associated with exacerbated immunodeficiency, reduced or unbalanced lymphocyte counts, and deregulated antibody production (1). Moreover, clinical

trials have shown that vitamin A supplementation reduces morbidity and mortality from various infectious diseases, and numerous studies in animal models have confirmed the ability of vitamin A to prevent infections and to strengthen the immune system (27). Secondary infections that develop due to post-sepsis immunosuppression are a major cause of death in septic patients (19). In fact, in recent years, this loss of immune competence has been considered to be one of the main problems in late sepsis (28). For that reason, many investigators consider that the effort to recover or preserve host immune functions will be the next major advance in the management of patients with sepsis.

The clinical importance of our study relies in finding strategies to restore the immune competence in patients that developed an immunosuppressive phase after sepsis and succumb to secondary opportunistic infections. Our work is focused on the study of this immunosuppressive phase, and our model mimics quite well this stage. Repetitive inoculation of LPS has been widely used to study the refractory to subsequent stimulation with LPS or bacteria associated to sepsis found at the cellular level. A similar loss of LPS reactivity, such as the one observed in LPS-immunosuppressed mice, has been repeatedly reported in circulating leukocytes of septic patients and in patients with non-infectious systemic inflammatory response syndrome (SIRS). Studies on cellular signaling within leukocytes from septic and SIRS patients revealed numerous alterations reminiscent of those observed in mice or cells treated with repetitive doses of LPS (Reviewed in (29). Persistent exposure to LPS in patients is possible, as evidenced by Torgersen *et al*., who reviewed postmortem findings in 235 surgical intensive care unit (ICU) patients admitted with a diagnosis of sepsis and found that, at death, a continuous septic focus was observed in 88.7 % of patients who were treated for longer than 7 days (30). All experimental models have limitations, and even though no model reproduces all aspects of human sepsis, each experimental model allows studying at least those aspects of human sepsis that the model reproduces. Therefore, and considering the aspects presented above, we believe that our model is adequate to investigate and intervened on the immunosuppressive phase that patients may develop after a septic episode.

We have previously reported that administration of ATRA to LPS-immunosuppressed mice was able to restore immune competence, and this was in part mediated by the modulation of the inhibitory population of MDSC, which were inhibiting T cell proliferation in lymph nodes. Moreover, ATRA

reverses the impaired LPS-induced primary humoral immune response (9). In this work, our hypothesis was that ATRA could reduce MDSC generation by modulating myeloid lineage expansion, and therefore improve immunological competence. Our results are relevant to better understand the etiology of sepsis-induced immunosuppression. We found that ATRA abolished LPS-induced myelopoiesis, affecting the proliferation of CD34+ precursor cells, and in consequence, decreasing MDSC generation, having a direct impact on the improvement of immune competence. In this sense, we observed a restored T lymphocyte proliferation in IS mice treated with ATRA in concordance with a reestablished histological architecture. This would be necessary for creating a supportive environment that facilitates the initiation of an adaptive immune response which is impaired in immunosuppressed septic patients. Moreover, we have previously shown that administration of ATRA to IS mice also restored the impaired antigen-specific humoral response (9).

As ATRA has been reported to decrease the number of cell precursors by induction of apoptosis (31), differentiation to their mature counterparts (32), or inhibiting their expansion (33), we also conducted experiments to discern the cellular mechanisms by which ATRA could be affecting MDSC generation. We found that ATRA interferes with myelopoiesis by decreasing the number of CD34+ myeloid precursor cells. This was also associated with a decrease in downstream populations derived from them, such as MDSC (Gr-1+ CD11b+ CD31+). The decrease of CD34+ precursor cells was not mediated by apoptosis or by an increase in differentiation to more mature forms, such as macrophages (F4/80+), neutrophils (Gr-1+ CD11b+) or dendritic (CD11c+) cells, as the numbers of these populations were similar or even lower in IS animals treated with ATRA compared to the IS alone. However, there was a consistent decrease in the proliferation of hematopoietic myeloid-committed precursors observed by the incorporation of ^{3}H-thymidine in the cultures supplemented with GM-CSF, and in the colony formation units (CFU) that were decreased both in number and size. As this last assay implicates specific clone expansion of CD34+ cells (14), the fact that the size of the colonies was reduced indicates that ATRA is lowering the proliferative rate of CD34+ precursors, resulting, in consequence, in a decreased number of daughter cells, including immature MDSC. Moreover, as ATRA is decreasing the number of early progenitor cells that will generate cell populations with suppressive activities in a pathological

context, ATRA administration might be an interesting and fast strategy to reduce MDSC generation in their place of origin (hematopoietic organs), avoiding their expansion in later stages when the immunosuppressed scenario is highly developed. Additionally, as ATRA caused the same effect *in vitro*, when precursors were partially isolated from the IS context, this indicates that ATRA is directly affecting the capacity of cells to proliferate. The decreased proliferation in immature precursors results in a decreased downstream myeloid progeny.

The anti-proliferative effects of ATRA could also be relevant for other immunosuppressive contexts, such as tumors, as it has also been reported that mouse granulocyte-macrophage progenitors isolated from tumor-bearing mice display strong suppressive properties, being capable of inhibiting polyclonal stimuli- and alloantigen-induced T cell proliferation (34).

The results presented in this work showed for the first time the anti-proliferative effects of ATRA on myeloid precursor cells in an IS-derived context. The importance of ATRA in an immunosuppressive scenario is related to MDSC and the regulation of their generation. In our model of immunosuppression, LPS inoculation caused an augmented demand for myeloid cells leading to increased proliferation of their precursors. This also brings about an increase in MDSC cells that negatively regulate lymphocyte responses. In this sense, the regulation of MDSC generation/differentiation has been shown to improve the immune function against tumors in animal models and patients (7). MDSC were recently described in septic patients (11), and although their presence may imply a normal negative feedback to the huge pro-inflammatory response that is triggered in sepsis, it is also possible that these cells may also be involved, in part, in the susceptibility to infections that some patients develop (immunosuppressive phase).

Additional potential advantages of ATRA administration to post-sepsis immunosuppressed patients is supported by several observations. Reduced levels in serum and increased urinary excretion of retinol have been reported in critically ill patients with sepsis or septic shock (35). As vitamin A deficiency damages the immunological response, it is possible that this deficiency renders patients more susceptible to secondary infections and contributes to immunosuppression after sepsis. Therefore, administration of ATRA may restore vitamin A deficiency, and controlling MDSC generation/expansion will improve the immunological state of these patients. The results of this study point to ATRA as a

possible complementary treatment to reduce the early generation of MDSC, enabling septic patients to overcome the immunosuppressive phase.

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Author Contribution

Daiana Martire-Greco, Veronica Landoni, and Gabriela Fernandez conceived and designed the experiments. Daiana Martire-Greco, Nahuel Rodriguez-Rodrigues, Luis A. Castillo, María Belén Vecchione, Marcelo de Campos-Nebel, Córdoba Moreno, Marlina, Roberto Meiss and Mónica Vermeulen performed the experiments. Daiana Martire-Greco, Veronica Landoni, and Gabriela Fernandez analyzed the data. Daiana Martire-Greco and Gabriela Fernandez wrote the manuscript.

REFERENCES

1. Chambon P: A decade of molecular biology of retinoic acid receptors. *FASEB J* 10(9):940-54, 1996.

2. Hansen LA, Sigman CC, Andreola F, Ross SA, Kelloff GJ and De Luca LM: Retinoids in chemoprevention and differentiation therapy. *Carcinogenesis* 21(7):1271-9, 2000.

3. Campisi J: Suppressing cancer: the importance of being senescent. *Science* 309(5736):886-7, 2005.

4. Angus DC and van der Poll T: Severe sepsis and septic shock. *N Engl J Med* 369(21):2063, 2013.

5. Otto GP, Sossdorf M, Claus RA, Rodel J, Menge K, Reinhart K, Bauer M and Riedemann NC: The late phase of sepsis is characterized by an increased microbiological burden and death rate. *Crit Care* 15(4):R183, 2011.

6. Gabrilovich DI, Ostrand-Rosenberg S and Bronte V: Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol* 12(4):253-68, 2012.

7. Waldron TJ, Quatromoni JG, Karakasheva TA, Singhal S and Rustgi AK: Myeloid derived suppressor cells: Targets for therapy. *Oncoimmunology* 2(4):e24117, 2013.

8. Derive M, Bouazza Y, Alauzet C and Gibot S: Myeloid-derived suppressor cells control microbial sepsis. *Intensive Care Med* 38(6):1040-9, 2012.

9. Martire-Greco D, Landoni VI, Chiarella P, Rodriguez-Rodrigues N, Schierloh P, Rearte B, Isturiz MA and Fernandez GC: all-trans-retinoic acid improves immunocompetence in a murine model of lipopolysaccharide-induced immunosuppression. *Clin Sci (Lond)* 126(5):355-65, 2014.

10. Landoni VI, Martire-Greco D, Rodriguez-Rodrigues N, Chiarella P, Schierloh P, Isturiz MA and Fernandez GC: Immature myeloid Gr-1+ CD11b+ cells from lipopolysaccharide-immunosuppressed mice acquire inhibitory activity in the bone marrow and migrate to lymph nodes to exert their suppressive function. *Clin Sci (Lond)* 130(4):259-71, 2016.

11. Darcy CJ, Minigo G, Piera KA, Davis JS, McNeil YR, Chen Y, Volkheimer AD, Weinberg JB, Anstey NM and Woodberry T: Neutrophils with myeloid derived suppressor function deplete arginine and constrain T cell function in septic shock patients. *Crit Care* 18(4):R163, 2014.

12. Nagai Y, Garrett KP, Ohta S, Bahrun U, Kouro T, Akira S, Takatsu K and Kincade PW: Toll-like receptors on hematopoietic progenitor cells stimulate innate immune system replenishment. *Immunity* 24(6):801-12, 2006.

13. Krause DS, Fackler MJ, Civin CI and May WS: CD34: structure, biology, and clinical utility. *Blood* 87(1):1-13, 1996.

14. Matsuoka S, Ebihara Y, Xu M, Ishii T, Sugiyama D, Yoshino H, Ueda T, Manabe A, Tanaka R, Ikeda Y, Nakahata T and Tsuji K: CD34 expression on long-term repopulating hematopoietic stem cells changes during developmental stages. *Blood* 97(2):419-25, 2001.

15. Skirecki T, Zielińska-Borkowska U, Kawiak J and Hoser G: Sepsis modulates the human hematopoietic stem cell compartment in peripheral blood and bone marrow. *Crit Care* 18 (Suppl 2):P33, 2014.

16. D'Aveni M, Rossignol J, Coman T, Sivakumaran S, Henderson S, Manzo T, Santos e Sousa P, Bruneau J, Fouquet G, Zavala F, Alegria-Prevot O, Garfa-Traore M, Suarez F, Trebeden-Negre H, Mohty M, Bennett CL, Chakraverty R, Hermine O and Rubio MT: G-CSF mobilizes CD34+ regulatory monocytes that inhibit graft-versus-host disease. *Sci Transl Med* 7(281):281ra42, 2015.

17. Pak AS, Wright MA, Matthews JP, Collins SL, Petruzzelli GJ and Young MR: Mechanisms of immune suppression in patients with head and neck cancer: presence of CD34(+) cells which suppress immune functions within cancers that secrete granulocyte-macrophage colony-stimulating factor. *Clin Cancer Res* 1(1):95-103, 1995.

18. Rearte B, Maglioco A, Balboa L, Bruzzo J, Landoni VI, Laborde EA, Chiarella P, Ruggiero RA, Fernandez GC and Isturiz MA: Mifepristone (RU486) restores humoral and T cell-mediated immune response in endotoxin immunosuppressed mice. *Clin Exp Immunol* 162(3):568-77, 2010.

19. Boomer JS, To K, Chang KC, Takasu O, Osborne DF, Walton AH, Bricker TL, Jarman SD, 2nd, Kreisel D, Krupnick AS, Srivastava A, Swanson PE, Green JM and Hotchkiss RS: Immunosuppression in patients who die of sepsis and multiple organ failure. *JAMA* 306(23):2594-605, 2011.

20. Rearte B, Landoni V, Laborde E, Fernandez G and Isturiz M: Differential effects of glucocorticoids in the establishment and maintenance of endotoxin tolerance. *Clin Exp Immunol* 159(2):208-16, 2010.

21. Institute of Laboratory Animal Resources (U.S.). Committee on Care and Use of Laboratory Animals. and National Institutes of Health (U.S.). Division of Research Resources.: *Guide for the care and use of laboratory animals*. U.S. Dept. of Health and Human Services, Public Health Service, National Insititutes of Health, Bethesda, Md., 1985.

22. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P and Cardona A: Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9(7):676-82, 2012.

23. Kiel MJ, Yilmaz OH, Iwashita T, Terhorst C and Morrison SJ: SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* 121(7):1109-21, 2005.

24. Kusmartsev S and Gabrilovich DI: Inhibition of myeloid cell differentiation in cancer: the role of reactive oxygen species. *J Leukoc Biol* 74(2):186-96, 2003.

25. Serafini P, Borrello I and Bronte V: Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. *Semin Cancer Biol* 16(1):53-65, 2006.

26. Kain MJ and Owens BM: Stromal cell regulation of homeostatic and inflammatory lymphoid organogenesis. *Immunology* 140(1):12-21, 2013.

27. Stephensen CB: Vitamin A, infection, and immune function. *Annu Rev Nutr* 21:167-92, 2001.

28. Hotchkiss RS, Coopersmith CM, McDunn JE and Ferguson TA: The sepsis seesaw: tilting toward immunosuppression. *Nat Med* 15(5):496-7, 2009.

29. Biswas SK and Lopez-Collazo E: Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends Immunol* 30(10):475-87, 2009.

30. Torgersen C, Moser P, Luckner G, Mayr V, Jochberger S, Hasibeder WR and Dunser MW: Macroscopic postmortem findings in 235 surgical intensive care patients with sepsis. *Anesth Analg* 108(6):1841-7, 2009.

31. Jiang M, Zhu K, Grenet J and Lahti JM: Retinoic acid induces caspase-8 transcription via phospho-CREB and increases apoptotic responses to death stimuli in neuroblastoma cells. *Biochim Biophys Acta* 1783(6):1055-67, 2008.

32. Kusmartsev S, Cheng F, Yu B, Nefedova Y, Sotomayor E, Lush R and Gabrilovich D: All-trans-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination. *Cancer Res* 63(15):4441-9, 2003.

33. Noy N: Between death and survival: retinoic acid in regulation of apoptosis. *Annu Rev Nutr* 30:201-17, 2010.

34. Pu S, Qin B, He H, Zhan J, Wu Q, Zhang X, Yang L, Qu C and Zhou Z: Identification of early myeloid progenitors as immunosuppressive cells. *Sci Rep* 6:23115, 2016.

35. Ribeiro Nogueira C, Ramalho A, Lameu E, Da Silva Franca CA, David C and Accioly E: Serum concentrations of vitamin A and oxidative stress in critically ill patients with sepsis. *Nutr Hosp* 24(3):312-7, 2009.

LEGENDS TO FIGURES

Figure 1. ATRA reduced *in vivo* the number of hematopoietic CD34+ precursors in LPS-immunosuppressed mice. Number of CD34+ cells in spleen and BM from Control, IS, and IS+A mice and a representative flow cytometry chart of total CD34+ cells from spleen and BM of each group is shown. Results are expressed as the mean ± SE. Total n=8 per group (2 experimental repetitions with n=4 per group).* p<0.05 vs. Control and # p<0.05 vs. IS.

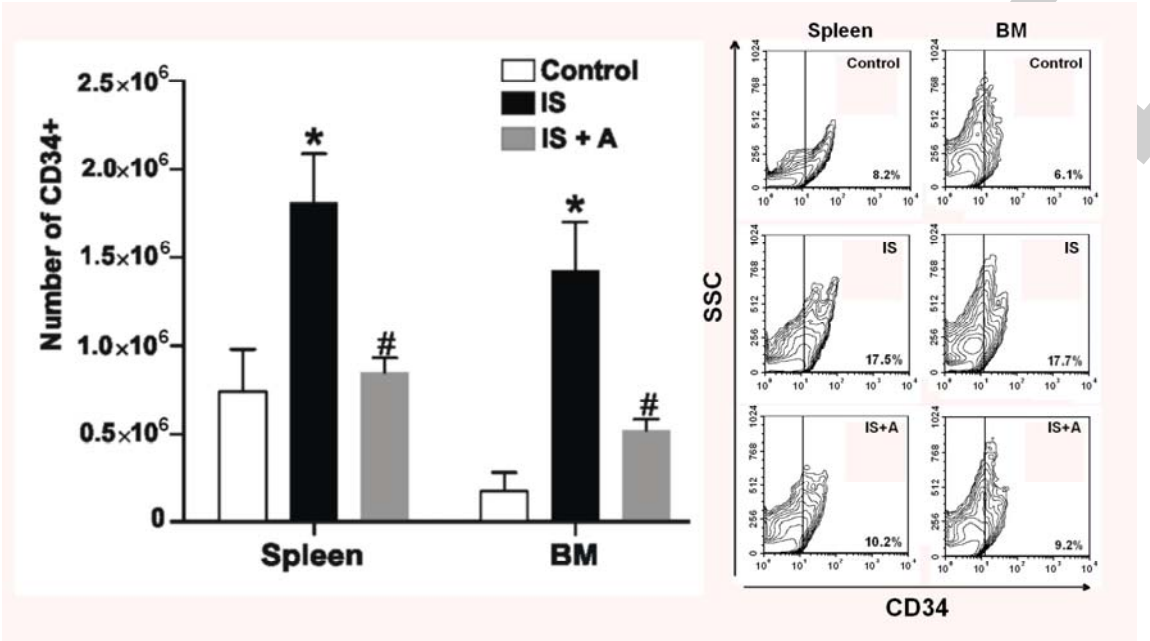


Figure 2. The effect of ATRA on hematopoietic precursors is not mediated by differentiation to mature lineages. A- Number of CD34+ cells observed in IS splenic and BM cell suspensions alone (IS/-A) or supplemented with ATRA (IS/+A) *in vitro* and low concentration of GM-CSF in the culture media after 72 h. A representative flow cytometry chart of total CD34+ from spleen and BM cells is shown for both groups. B- Number of neutrophils (Gr-1+ CD11b+), macrophages (F4/80+) or dendritic (CD11c+) cells measured in cell suspensions from IS spleens and BM alone (IS/-A) or supplemented with ATRA (IS/+A) and GM-CSF *in vitro*. A representative flow cytometry chart showing the mature cell populations present in spleen and BM is shown. Results are expressed as the mean ± SE. Total n=12 per group (3 experimental repetitions with n=4 per group). # $p<0.05$ vs. IS/-A.

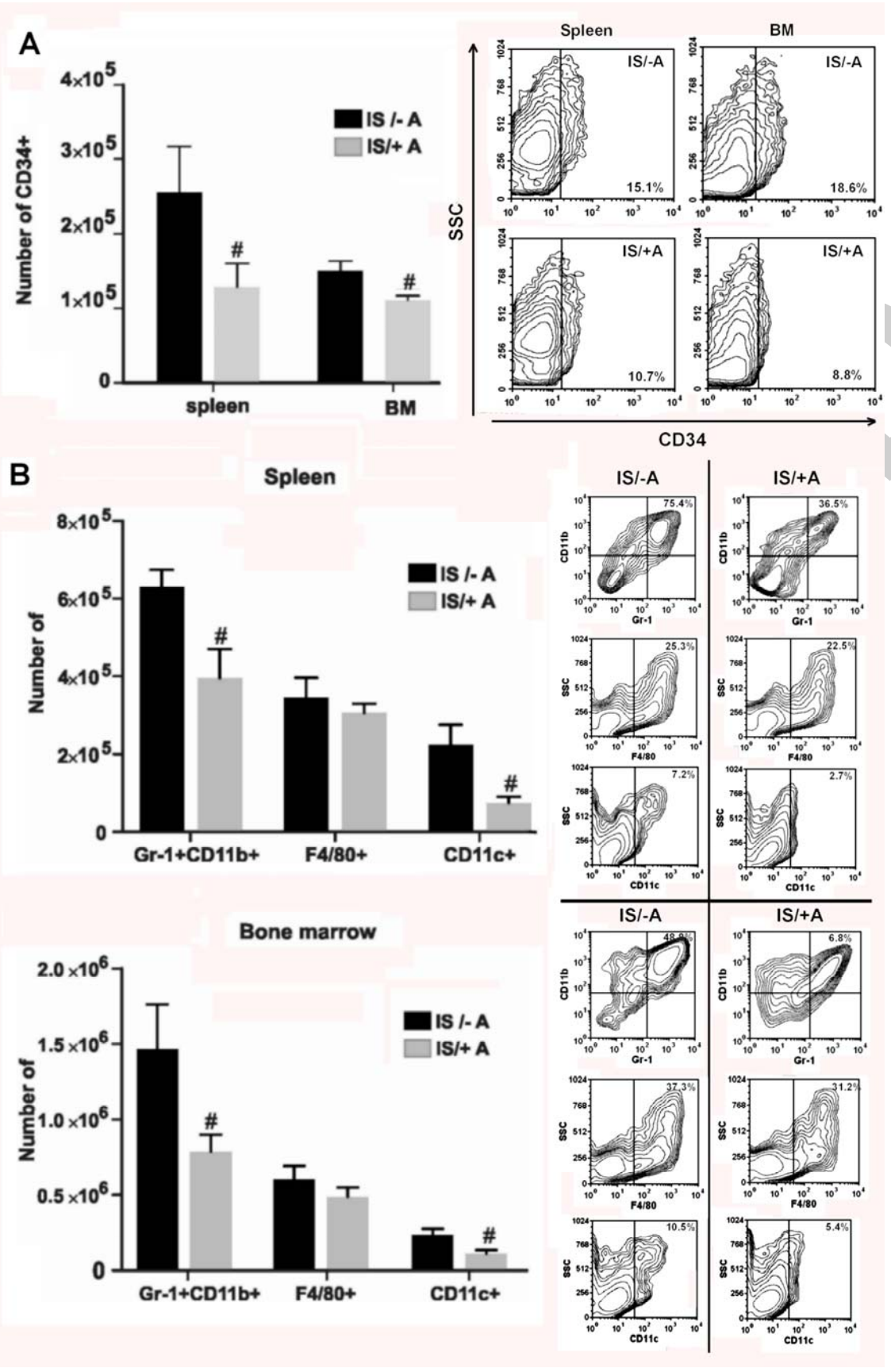


Figure 3. Addition of ATRA *in vitro* to IS cell suspensions decreased the proliferation of hematopoietic precursors. A-Number of GM-CFU grown during 7 days in semi-solid agar with GM-CSF (GM-CFU assay), from splenic and BM IS cell suspensions alone (IS/-A) or after adding ATRA (IS/+A) *in vitro.* B- Colony area from the GM-CFU obtained from spleen and BM and corresponding pictures (x 40) of a representative colony from each group (IS/-A and IS/+A). C- Incorporation of ^{3}H-thymidine measured as counts per minute (CPM) obtained from proliferation assays with cell suspensions from IS spleen and BM supplemented with ATRA (IS/+A) or left untreated (IS/-A) and GM-CSF after 72 h in culture. Results are expressed as the mean ± SE. Total n=12 per group (3 experimental repetitions with n=4 per group). # $p<0.05$ vs. IS/-A.

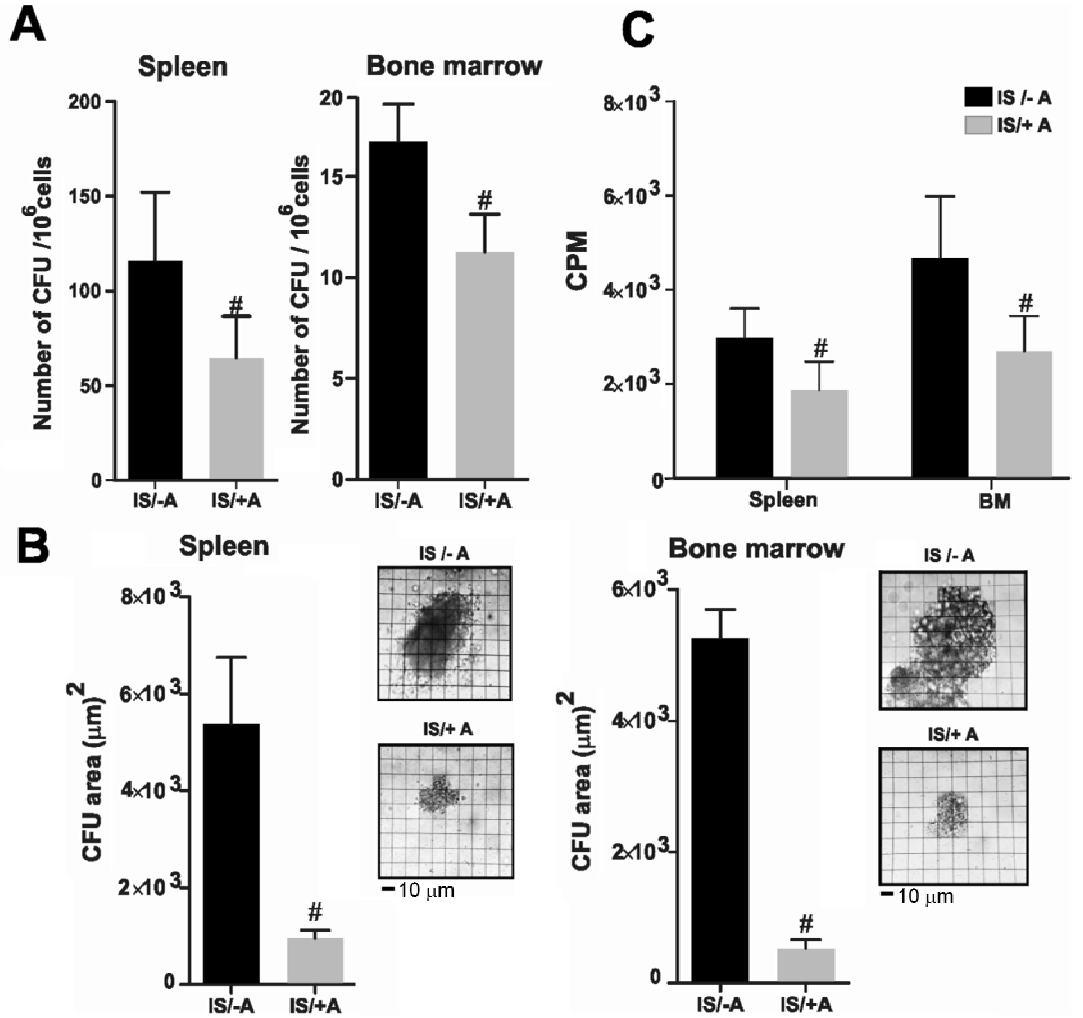


Figure 4. Administration of ATRA *in vivo* to IS mice decreased the proliferation of hematopoietic precursors. A-Number of GM-CFU colonies grown 7 days in semi-solid agar with GM-CSF (GM-CFU assay), from splenic and BM cell suspensions of Control, IS, and IS+A groups. B- Colony area of the GM-CFU obtained from spleen and BM, and representative pictures (x 40) of one colony from each group (Control, IS, and IS+A). C- Incorporation of ^{3}H-thymidine measured as counts per minute (CPM) obtained from proliferation assays with splenic and BM cell suspensions from Control, IS and IS+A supplemented with GM-CSF after 72 h in culture. Results are expressed as the mean ± SE. Total n=12 per group (3 experimental repetitions with n=4 per group). * $p<0.05$ vs. Control and # $p<0.05$ vs. IS.

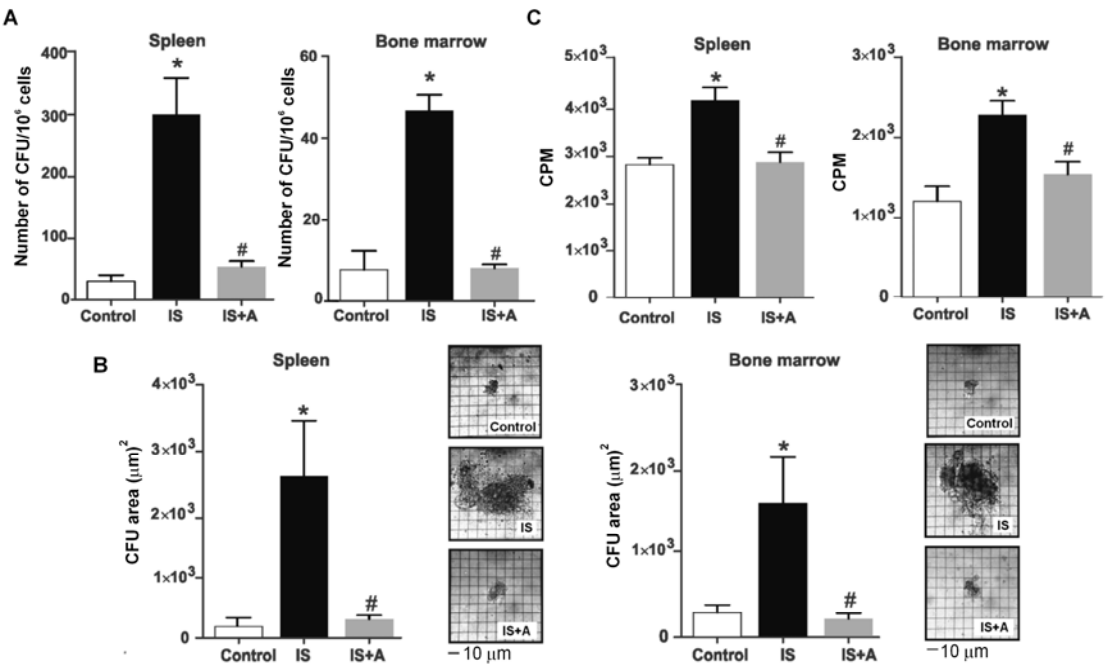


Figure 5. ATRA *in vivo* reduced the number of immature MDSC (Gr-1+ CD11b+ CD31+) and restored T cell proliferation. A- Number of Gr-1+ CD11b+ CD31+ population obtained from spleens of Control, IS and IS+A groups. A representative flow cytometry chart is shown for all groups. Gr-1+ cells were first gated, and the analysis of CD11b+ CD31+ cells within the Gr-1+ gated population was performed. B- Counts per minute (CPM) obtained from T lymphocyte proliferation assays, using Con A as a specific T-cell mitogen in splenic cell suspensions from Control, IS, and IS+A groups after 72 h of Con A addition. The IS depleted Gr-1 bar represents the depletion of the Gr-1 population using an anti-Gr-1 antibody and magnetic beads. C- Histological examination of the spleen of the different experimental groups (hematoxylin and eosin staining, x 100). Results are expressed as the mean ± SE. Total n=8 per group (2 experimental repetitions with n=4 per group), * $p<0.05$ vs. Control and # $p<0.05$ vs. IS.

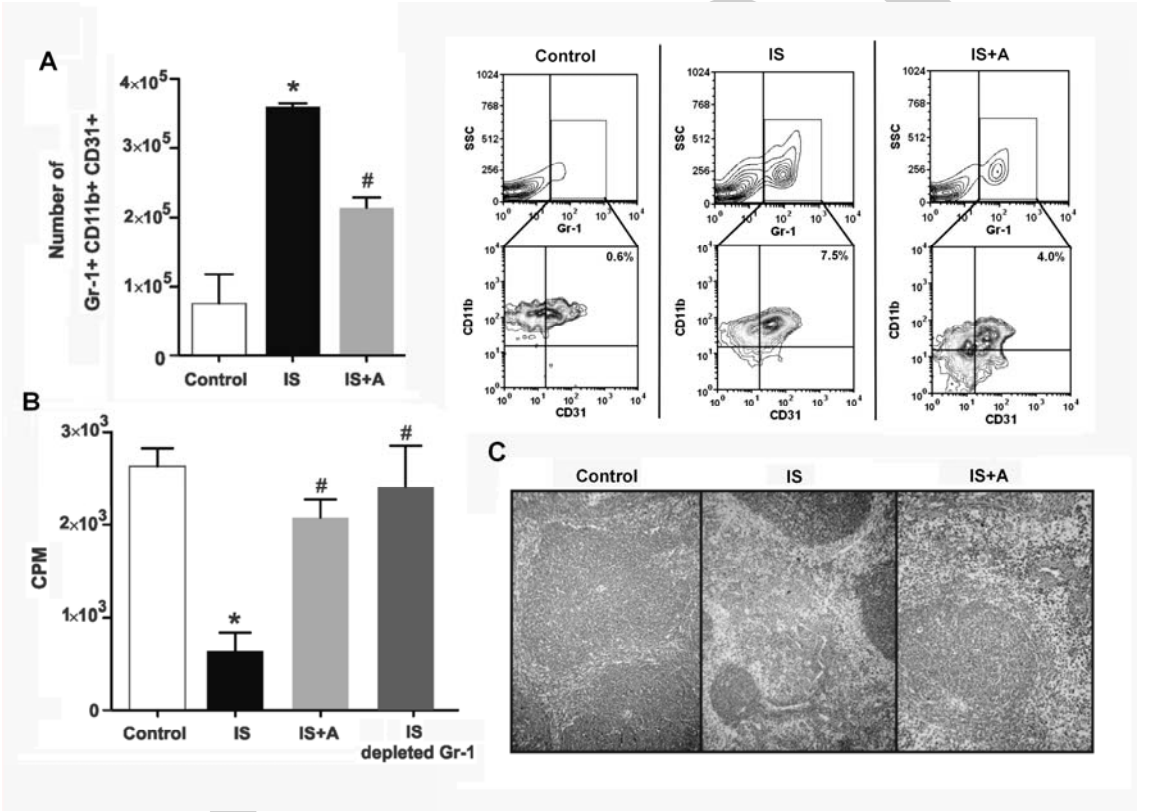
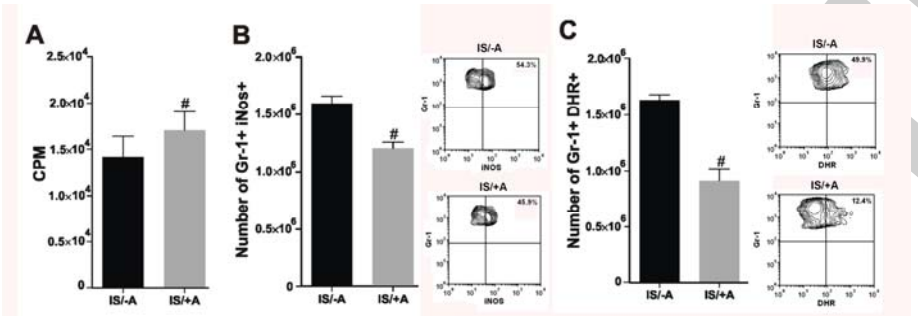


Figure 6. ATRA *in vitro* directly decreased MDSC and its T-cell suppressive function. A- CPM obtained from proliferation T lymphocyte assays using cell suspensions from IS spleens supplemented with ATRA (IS/+A) *in vitro* or left untreated (IS/-A) at 72 h after Con A addition. B- Number of Gr-1+ iNOS+ cells obtained in culture after 24 h of Con A addition from IS splenic cell suspensions alone (IS/-A) or supplemented with ATRA (IS/+A). C- Number of Gr-1+ DHR+ cells obtained in culture after 24 h of Con A addition from IS splenic cell suspensions alone (IS/-A) or supplemented with ATRA (IS/+A). A representative flow cytometry chart is shown showing the gate of cells corresponding to the total Gr-1+ iNOS+ (B) or Gr-1+ DHR+ (C) cells for the different experimental groups. Results are expressed as the mean ± SE. Total n=8 per group (2 experimental repetitions with n=4 per group),# $p<0.05$ vs. IS/-A.



Supplementary Figure 1. ATRA *in vitro* did not induce apoptosis of CD34+ cell. A- Percentage of AnV+ cells within the CD34+ population measured in cell suspensions from IS spleens and BM alone (IS/-A) or supplemented with ATRA (IS/+A) and GM-CSF *in vitro*. B- Number of CD34+ AnV+ population measured in cell suspensions as described previously. C- A representative flow cytometry chart is shown showing the CD34+ AnV+ cell population. Total n=12 per group (3 experimental repetitions with n=4 per group). # $p<0.05$ vs. IS/-A.