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Original Paper

all-*trans*-Retinoic acid improves immunocompetence in a murine model of lipopolysaccharide-induced immunosuppression

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Abstract

Secondary infections due to post-sepsis immunosuppression are a major cause of death in patients with sepsis. Strategies aimed at restoring immune functions offer a new perspective in the treatment of sepsis. In the present study, we used LPS (lipopolysaccharide)-immunosuppressed mice to analyse the effects of ATRA (all-*trans* retinoic acid) on different immune parameters. The IS (immunocompromised) group had decreased lymphocyte and increased MDSC (myeloid-derived suppressor cell) counts in lymph nodes. They also had an impaired *in vitro* T-cell proliferation, mediated by MDSCs. ATRA administration restored T-cell proliferation, which was associated with a decreased number of live MDSCs. The IS group treated with ATRA had an increased number of $CD4^+$ and $CD8^+$ T-cells. ATRA partially improved the primary humoral immune response, even when immunosuppression was established first and ATRA was administered subsequently. Our results demonstrate that ATRA restores immunocompetence by modulating the number of leucocytes and the survival of MDSCs, and thus represents an additional potential strategy in the treatment of the immunosuppressive state of sepsis.

Key words: all-*trans*-retinoic acid (ATRA), immunosuppression, lipopolysaccharide (LPS), myeloid-derived suppressor cell (MDSC), sepsis

INTRODUCTION

The sepsis syndrome constitutes the leading cause of death in non-coronary ICUs (Intensive Care Units). As most of the clinical trials testing anti-inflammatory treatments have failed, the hypothesis that death after sepsis is due to an overwhelming pro-inflammatory state is being revised. In this regard, it is now agreed that an anti-inflammatory response is concomitantly triggered as a negative feedback, which, if not appropriately balanced, may contribute to an inability to kill invading micro-organisms, increasing the risk of acquiring nosocomial infections [1]. In fact, secondary infections that develop due to post-sepsis immunosuppression are a major cause of death in patients with sepsis [2,3]. Exposure to LPS (lipopolysaccharide), the main component of the membrane of Gram-negative bacteria, has been considered the initial phase and one of the causes of the immunosuppression frequently observed in late sepsis [4,5]. The response to LPS involves a rapid secretion of pro-inflammatory cytokines [TNF (tumour necrosis factor)-α, IFN (interferon)-γ, IL (interleukin)-1, IL-6 and IL-8] and the simultaneous induction of anti-inflammatory mediators such as IL-10 and TGF (transforming growth factor)-β, which render the host temporarily refractory to subsequent lethal doses of LPS challenge in a process known as LPS or endotoxin tolerance [6,7]. This process is considered to be one of the main causes of immunosuppression reported in patients with sepsis due to Gram-negative infections [4,8]. Experimentally, repetitive inoculation of increasing doses of LPS in mice induces derangements in the inflammatory response and a state of adaptive immunosuppression, which is characterized by deactivated monocyte function, lymphocyte dysfunction and decreased primary

Abbreviations: AnV, annexin V; ATRA, all-*trans*-retinoic acid; ConA, concanavalin A; Cy5, indodicarbocyanine; DHR, dihydrorhodamine 123; Foxp3,forkhead box p3; i.p., intraperitoneally; ICU, Intensive Care Unit; IL, interleukin; iNOS, inducible NO synthase; LN, lymph node; LPS, lipopolysaccharide; MDSC, myeloid-derived suppressor cell; PE, phycoerythrin; ROS, reactive oxygen species; SRBC, sheep red blood cell; TNF, tumour necrosis factor; T_{reg}-cell, regulatory T-cell.

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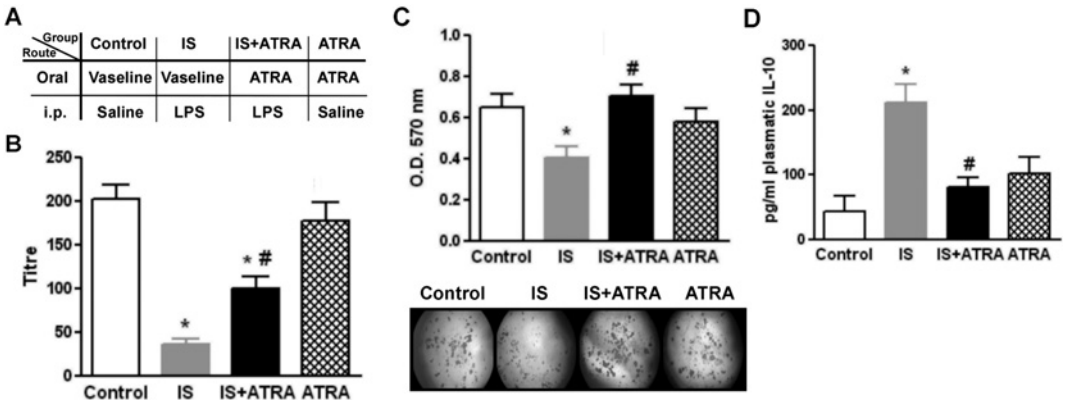

Figure 1 **ATRA restores decreased functional parameters and plasma IL-10 levels in LPS-immunosuppressed mice**
(**A**) Summary of the experimental groups used in the present study. (**B**) Primary humoral immune response. Results are expressed as means ± S.E.M., $n = 20$ per group. (**C**) Upper panel, T-cell proliferation. Results are expressed as means ± S.E.M., $n = 20$ per group. O.D., absorbance. Lower panel, representative microphotographs of wells showing proliferative foci in the experimental groups. (**D**) Plasma IL-10 levels. Results are expressed as means ± S.E.M., $n = 10$–12 per group. $^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the IS group.

humoral immune responses, mimicking several aspects of the immunosuppressive phase observed in patients with sepsis [4,9].

A population of myeloid precursors, characterized as Gr-1^{+}CD11b^{+}, has been described to suppress the immune response in different experimental and clinical pathologies [10,11]. The accumulation of Gr-1^{+}CD11b^{+} MDSCs (myeloid-derived suppressor cells) in secondary lymphoid organs and blood can be detected under a variety of conditions associated with impaired immunity, such as cancer, overwhelming microbial infection, cyclophosphamide treatment, total lymphoid irradiation and others [12]. It has been demonstrated that MDSCs are able to inhibit antigen-specific T-cell responses [13,14]. One of the mechanisms associated with this inhibition is mediated by two enzymes that use arginine as a substrate: the inducible form of NO synthase (iNOS) and arginase-1 [15]. The induction or activation of these enzymes upon T-cell activation depletes the levels of arginine, which is an amino acid essential for T-cell activation [16,17]. Additionally, NO produced by iNOS blocks T-cell function by interfering with the signal transduction pathway of the IL-2 receptor [18], and high levels of NO can also induce T-cell apoptosis [19]. Moreover, it has been shown that MDSCs can also suppress antigen-specific T-cell responses via the release of ROS (reactive oxygen species) [20].

ATRA (all-*trans*-retinoic acid) is a derivative of vitamin A commonly used, together with other drugs, in the treatment of acute promyelocytic leukaemia, and causes immature highly proliferative promyelocytes to differentiate into normal functioning mature blood cells [21,22]. Because of the important role of MDSCs in tumour-associated immunosuppression, intensive studies in recent years have been focused on the identification of therapeutic means to eliminate these cells. It has been shown that ATRA *in vitro* has a potent activity in differentiating MDSCs to dendritic cells, macrophages and neutrophils [23,24]. As the transcriptional activity of ATRA can lead to differentiation, cell-cycle arrest and apoptosis, this compound often inhibits cell growth [25]. Treatment of cancer patients and tumour-bearing mice with ATRA has resulted in substantial decreases in MDSCs and in the improvement of immune responses [26,27].

Additionally, many functions of retinoic acid have been identified in the regulation of immune responses. ATRA is required for optimal production of neutrophils, NK (natural killer) cells and B-cells [28], and it can promote humoral immune responses to pathogens [29,30].

As restoration of the adaptive immune response is crucial in the fight against secondary infections associated with sepsis-derived immunosuppression, we inoculated mice with increasing doses of LPS daily (LPS-induced immunosuppression) as a model that mimics the immunosuppressive phase that patients may develop after sepsis, and explored the ability of ATRA to improve immunocompetence in these animals.

MATERIALS AND METHODS

Experimental model

To induce LPS-induced immunosuppression, Balb/c mice, aged 2–3 months, were daily inoculated i.p. (intraperitoneally) with increasing doses of LPS (*Escherichia coli* O111:B4; Sigma) for 15 days as follows: two doses of 5 μg/day per mouse, two doses of 10 μg/day per mouse, two doses of 20 μg/day per mouse, three doses of 50 μg/day per mouse and six doses of 100 μg/day per mouse. Concomitantly with these LPS doses, a group of mice were daily administered Vaseline orally (IS group), and another group received daily oral doses of ATRA (Vesanoid®; Hoffman-La Roche) dissolved in Vaseline (500 μg/day per mouse; IS + ATRA group). The capsule content was removed in sterile conditions and the concentration adjusted with Vaseline immediately before administration. Another group of animals were given saline solution i.p. and Vaseline orally (control group) or saline solution i.p. and ATRA orally every day (ATRA group) (see Figure 1A).

In other set of experiments, two different LPS/ATRA administration protocols were used. In Protocol 1, increasing doses of LPS alone (5, 10, 20, 50 and 100 μg/day per mouse) were given for 1 week and then LPS (100 μg/day per mouse; IS group) or LPS + ATRA (100 μg of LPS /day per mouse and 500 μg of ATRA/day per mouse; IS + ATRA group) were administered for an additional 2 weeks. Protocol 2 consisted of administration of increasing doses of LPS alone (5, 10, 20, 50 and 100 μg/day per mouse) for 1 week and then animals were left untreated (IS group) or given ATRA alone (500 μg/day per mouse; IS + ATRA group) for an additional 2 weeks.

The experiments performed were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals [31], and the study was approved by the Animal Care and Use Committee of our Institution.

Cell tissue collection

To have a representative sampling of LNs (lymph nodes), both inguinal and axillary LNs were removed from mice under sterile conditions, and single-cell suspensions were prepared by homogenization through a sterile stainless steel mesh. Absolute leucocyte counts were obtained with a Neubauer chamber using Turk's solution.

Haemagglutination test

To induce a primary humoral immune response, 0.2 ml of 25% (v/v) washed SRBCs (sheep red blood cells) were inoculated i.p., and, 1 week later, blood was collected and serum was obtained. Serial 2-fold dilutions of the serum were made and mixed with an equal volume of a 0.35% solution of SRBCs in a round-bottomed 96 well-plate. Plates were left at room temperature (20 °C) for 24 h. The antibody titres were considered as the inverse of the greatest dilution that inhibits haemagglutination.

T-cell proliferation

Cell suspensions from LNs were obtained and resuspended in RPMI-1640, 10% (v/v) FCS, 0.1% 2-mercapoethanol, 1% L-glutamine and 1% (v/v) antibiotic/antimycotic solution. A differential count of the lymphocytes was performed with a Neubauer chamber using Turk's solution. In addition, $CD3^+$ lymphocytes were determined by flow cytometry (see below) and the same initial number of $CD3^+$ cells (0.2×10^6) for all groups were seeded into 96 well plates in triplicate in the presence or absence of 5 μg/ml ConA (concanavalin A; Sigma) for 72 h at 37 °C in 5% CO_2. Then, cells were incubated with 0.5 mg/ml MTT (Sigma) and the plate was left at 37 °C in 5% CO_2 for an additional period of 4 h. MTT is reduced to a purple formazan in living cells [32]. To dissolve the insoluble purple formazan product, a solution of 20% (w/v) SDS in 50% (v/v) dimethylformamide was added and left overnight at 37 °C. The absorbance of the coloured solution was quantified at 570 nm. The mean absorbance in the absence of ConA was subtracted from the mean absorbance in its presence for each sample.

Determination of IL-10

IL-10 was determined using a commercial ELISA kit (eBiosciences), according to the manufacturer's instructions.

Flow cytometric studies

The main cell populations present in LNs were studied using flow cytometry. Cells (10^6) were incubated with specific rat anti-mouse antibodies conjugated with different fluorochromes: PE (phycoerythrin)–CD19, FITC–CD4, FITC–CD11b and FITC–CD3, PE-Cy5 (indodicarbocyanine)–Gr-1 and PE-Cy5–CD8. All antibodies were from BD Bioscience. For the determination of T_{reg}-cells (regulatory T-cells), a commercial kit (eBiosciences) was used following the manufacturer's instructions. Cells were washed and resuspended in 0.5% paraformaldehyde. The percentage of positive cells was determined in 70000 events. The absolute number of the specific subpopulations was calculated as follows: (absolute total leucocyte cell count × percentage obtained by flow cytometry)/100.

Determination of NO

NO production was determined by measurement of nitrite (in cell-free-supernatants) using the Griess reaction. Briefly, 50 μl of sample was mixed with 50 μl of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2% phosphoric acid) in 96-well plates and incubated at 25 °C for 10 min. The absorbance at 540 nm was measured with a microplate reader. $NaNO_2$ was used as a standard to calculate nitrite concentrations.

Measurement of arginase-1 activity

Arginase-1 activity was measured in cell lysates of 0.6×10^5 cells as described by Munder et al. [33], with slight modifications. Briefly, cells were lysed with 50 μl of lysis buffer (0.1% Triton X-100 and protease inhibitor cocktail; Sigma). After 30 min of agitation on a shaker at 37 °C, 60 μl of 25 mM Tris/HCl and 2 mM $MnCl_2$ was added. Arginase-1 was activated by heating the solution for 10 min at 55 °C. L-Arginine (Sigma) was hydrolysed by incubating the lysate with 100 μl of 500 mM L-arginine (pH 9.7) at 37 °C for 60 min. The reaction was stopped with 400 μl of 96% H_2SO_4/85% H_3PO_4/water (1:3:7, by vol.). The urea concentration was measured at 540 nm after addition of 40 μl of α-isonitrosopropiophenone (Sigma) dissolved in 100% ethanol, followed by heating at 95 °C for 30 min. Data are given as units of arginase-1, where 1 unit of arginase-1 is defined as the amount of enzyme that catalyses the formation of 1 μg of urea in 60 min.

Determination of ROS production

To determine the production of ROS in T-cell proliferation cultures, DHR (dihydrorhodamine 123; Sigma) was used. Briefly, cells were collected from the culture wells, stained with an anti-(mouse Gr-1) antibody and incubated for 15 min at 37 °C with 1 μM DHR. ROS production induces a brightly fluorescent FL-1 product, which was detected by flow cytometry. The percentage of $Gr\text{-}1^+ DHR^+$ cells was determined.

Apoptosis

The percentage of MDSCs and lymphocytes undergoing apoptosis was measured by flow cytometry using AnV (annexin V)–FITC, and the following anti-mouse antibodies: PE-Cy5–Gr-1, PE–CD11b and PE–CD3 in cell suspensions obtained from LNs. AnV staining was performed using a commercial kit (BD

Biosciences), according to the manufacturer's instructions. The absolute number of the apoptotic and live cells was calculated as follows: (absolute total leucocyte cell count × percentage obtained by flow cytometry)/100

Statistical analysis

Comparisons between multiple groups were made by ANOVA, applying the Bonferroni correction. $P < 0.05$ was considered significant.

RESULTS

ATRA improves functional immune responses in LPS-treated mice

LPS exposure has been considered the initial phase and one of the causes of immunosuppression frequently observed in late sepsis [4,5,8,34]. The IS group showed signs of immunosuppression as demonstrated by an almost complete absence of a primary humoral immune response against a T-cell-dependent antigen (SRBCs) (Figure 1B). Moreover, *ex vivo* T-cell proliferation assays using ConA (a T-cell-specific stimulus) were performed with total LN homogenates using the same number of T-cells ($CD3^+$ cells) in all of the groups to avoid differences in initial numbers of potentially proliferating cells that may lead to a biased result. After 72 h, we found a statistically significant decrease in T-cell proliferation in the IS group (Figure 1C). The concomitant treatment with oral daily doses of ATRA (IS + ATRA group) restored these responses, although for the primary humoral immune response the restoration was partial. The responses observed in a group of mice that received ATRA alone were similar to those observed in the control group. Another parameter that can be elevated in the circulation during immunosuppression is the anti-inflammatory cytokine IL-10. We measured plasma IL-10 levels and found an increase in the IS group. The concomitant treatment with ATRA was able to decrease these levels (Figure 1D).

ATRA modulates the number of lymphocytes in LPS-treated mice

In order to understand the mechanisms by which ATRA restored the functional responses in LPS-treated mice, we first analysed the different lymphocyte populations present in LNs. In contrast with humans, the spleen in mice is the major site of extramedullary haematopoiesis in pathological conditions. Therefore we preferred to study changes in immunological parameters in LNs as this is the site in which the immune response is initiated in both mice and humans, and also to avoid the contribution of proliferative progenitors that could have interfered with the results. An initial approach was performed counting total lymphocytes using optical microscopy (Figure 2A). The IS group had a decrease in total lymphocyte number that was restored by ATRA treatment, reaching higher levels than those found in the control group. We then characterized the presence of B-cells ($CD19^+$) and T-cells ($CD8^+$ and $CD4^+$) within the LNs using flow cytometry (Figure 2B–2D). Decreases in the absolute numbers of $CD19^+$, $CD4^+$ and $CD8^+$ cells were observed in the IS group, although for CD8 the differences did not reach statistical significance. However, treatment with ATRA increased the number of these populations, reaching values even higher than those observed in the control group. The percentages of the different lymphocyte populations present in the LNs were similar in all groups (see Supplementary Table S1 at http://www.clinsci.org/cs/126/cs1260355add.htm, which shows the percentages of $CD19^+$, $CD4^+$ and $CD8^+$ cells in the different experimental groups).

To investigate further the modulatory effects of ATRA on T-cells, we studied the double $CD4^+CD8^+$ and single $CD4^+$ or $CD8^+$ populations in the thymus (Figure 2E). We observed a decreased number of both double- and single-positive lineages in the IS group compared with the control group. The administration of ATRA to the IS group re-established the number of $CD4^+CD8^+$ cells compared with the IS group, but single $CD4^+$ or $CD8^+$ cells were still decreased in the IS + ATRA group compared with the control group.

ATRA modulates the number of suppressor populations in LNs of LPS-treated mice

T_{reg}-cells and MDSCs are two suppressor populations that could be mediating the decrease in T-cell proliferation in the IS group. When we analysed the T_{reg}-cell population ($CD4^+CD25^+Foxp3^+$; where Foxp3 is forkhead box p3), we found that, in the IS group, their number was decreased (Figure 2F), although this was restored to normal levels by ATRA. The percentage of T_{reg}-cells was similar in all groups (see Table S1, which shows the percentages of $CD4^+CD25^+Foxp3^+$ in the different experimental groups).

In contrast, the number of total MDSCs ($Gr\text{-}1^+CD11b^+$) was considerably increased in the IS group (Figure 3A). These results suggest that it is more likely that MDSCs are involved in the inhibition of T-cell proliferation observed in this mouse model. ATRA treatment did not modify the number of MDSCs compared with those in the IS group. In mice, two main subsets of MDSCs have been identified that are equally able to suppress antigen-specific T-cell proliferation: a granulocytic subset with a $Gr\text{-}1^{high}CD11b^+$ phenotype and a monocytic subset characterized as $Gr\text{-}1^{low}CD11b^+$ [35]. Both subsets were increased in the LNs of the IS group (in both percentage and absolute number) and the concomitant treatment with ATRA did not decrease any of these subsets (see Supplementary Figure S1 at http://www.clinsci.org/cs/126/cs1260355add.htm, which shows the percentage and absolute number of the different subsets of MDSCs, namely $Gr\text{-}1^{high}CD11b^+$ and $Gr\text{-}1^{low}CD11b^+$).

The results described above prompted us to study the relevance of MDSCs in the inhibition of T-cell proliferation in immunocompromised mice. For this purpose, we performed *in vitro* depletion experiments using cells from LNs. Cells were incubated with an anti-Gr-1 antibody followed by magnetic beads. Depletion was successfully achieved, with less than 10% of the initial number of $Gr\text{-}1^+$ cells remaining after depletion. When T-cell proliferation was determined using the immunocompromised-depleted fraction, the values obtained were increased compared with the non-depleted fraction, reaching values similar to those observed for the control group (Figure 3B). This result indicates

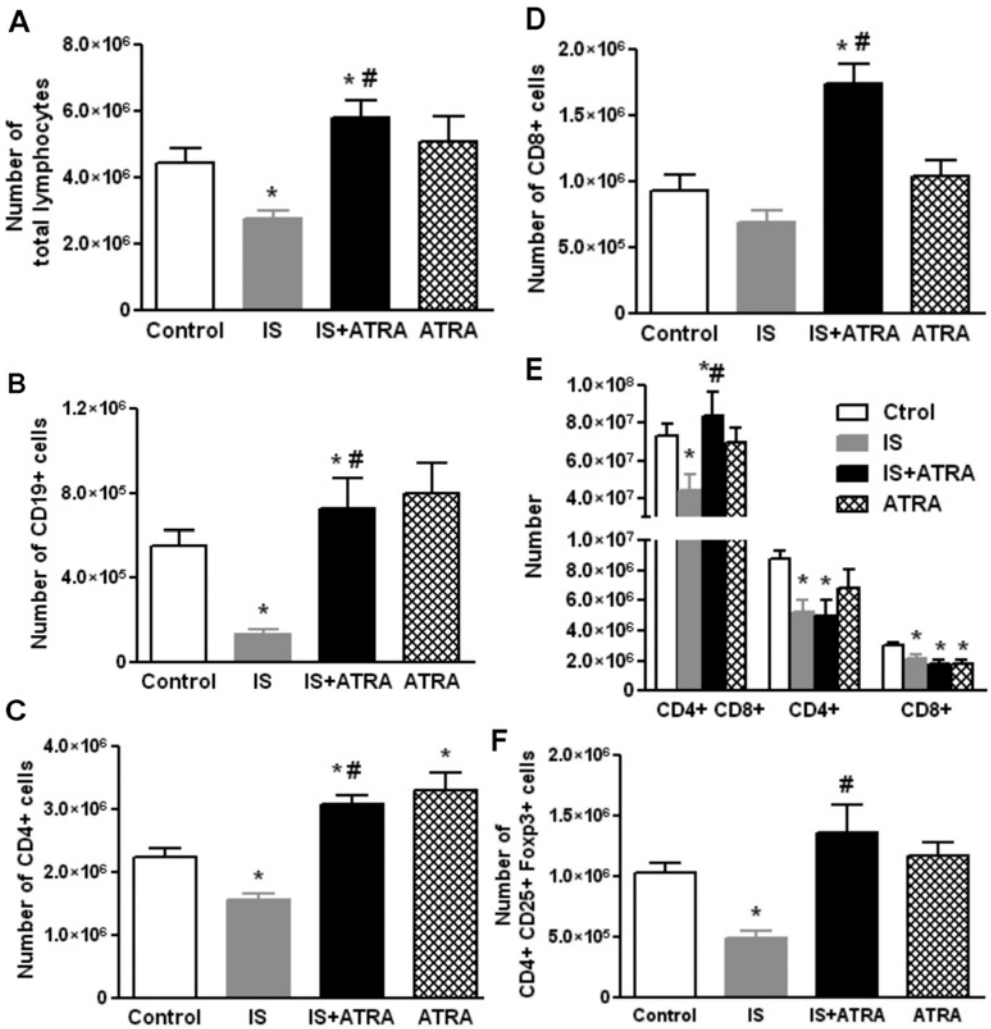

Figure 2 **ATRA increases lymphocytes numbers in LPS-immunocompromised mice**
Absolute number of total lymphocytes (**A**), $CD19^+$ B-cells (**B**), $CD4^+$ T-cells (**C**), $CD8^+$ T-cells (**D**) and $CD4^+CD25^+Foxp3^+$ T_{reg}-cells (**F**) in LNs. (**E**) Double or single $CD4^+/CD8^+$ T-cells in the thymus. Results are expressed as means ± S.E.M., $n = 12–20$ per group, $^*P < 0.05$ compared with the control group; $^\#P < 0.05$ compared with the IS group.

that MDSCs mediate the inhibition of T-cell proliferation in immunocompromised mice.

MDSC suppressor mediators are decreased in T-cell proliferation assays in the IS + ATRA group

Even though the absolute number of total MDSCs was unchanged by ATRA treatment, there was a clear restoration in T-cell proliferation. Therefore we investigated whether the inhibition and restoration of T-cell proliferation levels in the IS and IS + ATRA groups were associated with MDSC activity. MDSC suppressor mediators include NO, ROS and arginase-1 activity. When ROS production was evaluated, the values obtained for all groups were similar (results not shown). In contrast, both the amount of NO (Figure 4A) and arginase-1 activity (Figure 4B) were augmented in the IS group. In agreement with the restoration of T-cell proliferation, ATRA administration decreased both of the suppressor mediators.

ATRA increases apoptosis of MDSCs in LPS-treated mice

As no differences in absolute numbers of total $Gr\text{-}1^+CD11b^+$ cells (considering that these numbers include dead and live cells) were observed between the IS and IS + ATRA groups (Figure 3) and to explain the absence of MDSC activity found in the IS + ATRA group (Figure 4), we studied the effects of ATRA on the modulation of MDSC survival. We used AnV, Gr-1 and CD11b, and AnV and CD3 to determine the percentage of apoptotic MDSCs and T-cells respectively. As shown in Figure 5(A), the percentage of AnV^+ cells within the $Gr\text{-}1^+CD11b^+$ cell population was increased in the IS + ATRA group. ATRA alone also caused an increase in apoptosis compared with the control group. When the absolute number of AnV^+ $Gr\text{-}1^+CD11b^+$ cells was calculated, there was a significant increase only in the IS + ATRA group (Figure 5B). In contrast, when only live $(AnV^-)Gr\text{-}1^+CD11b^+$ cells were analysed, this population was

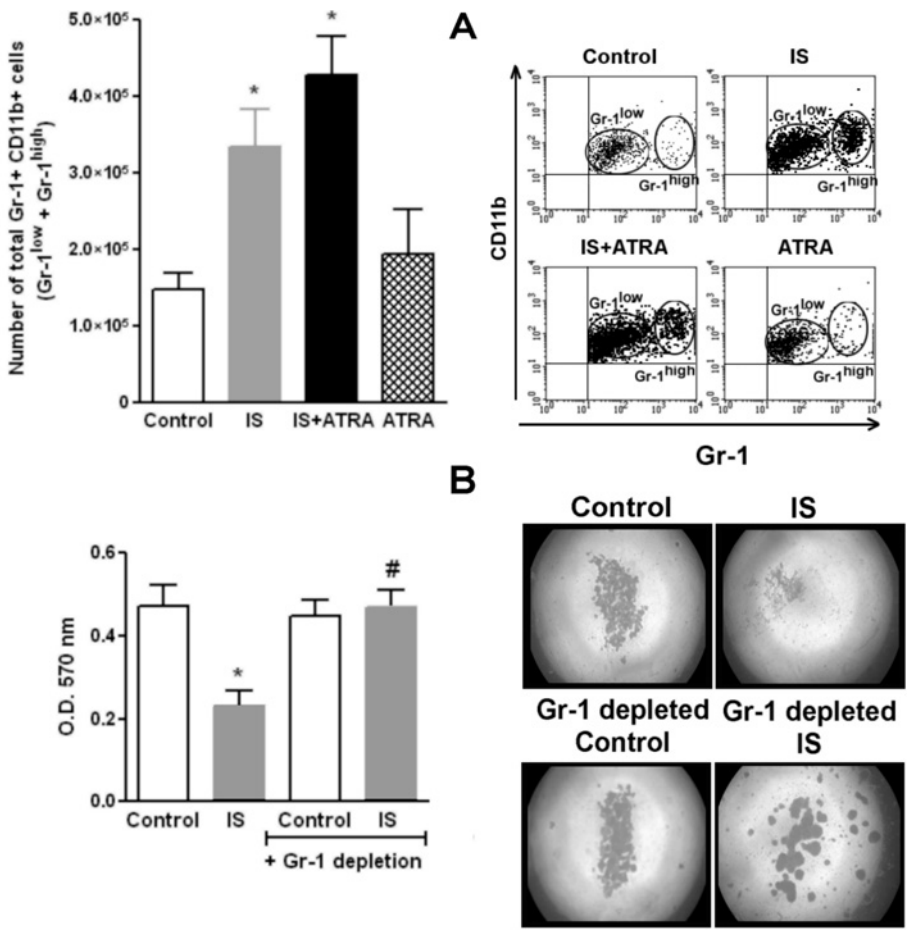

Figure 3 MDSCs mediate the inhibition of T-cell proliferation in LPS-immunocompromised mice

(**A**) Left-hand panel, absolute number of Gr-1$^+$CD11b$^+$ MDSCs. $n = 20-24$ per group. Right-hand panel, representative dot plots showing the gate of cells corresponding to the total Gr-1$^+$CD11b$^+$ MDSC population (Gr-1^{low} plus Gr-1^{high}) for the different experimental groups. (**B**) Left-hand panel, T-cell proliferation in Gr-1$^+$-depleted and non-depleted fractions. Results are expressed as means $\pm$ S.E.M., $n = 6$ per group. O.D., absorbance. Right-hand panel, representative microphotographs of wells showing proliferative foci of Gr-1$^+$-depleted and non-depleted fractions. $^*P < 0.05$ compared with the control group; $^\#P < 0.05$ compared with the IS group.

in fact decreased in the IS + ATRA group, whereas the IS group still had an increased number of live MDSCs (Figure 5C). Values of apoptosis obtained for CD3$^+$ lymphocytes were similar for all of the groups (AnV$^+$CD3$^+$ cells: control group, $27.3 \pm 1.1\%$; IS group, $25.7 \pm 0.5\%$; IS + ATRA group, $32.3 \pm 0.5\%$; ATRA group, $32.6 \pm 2.4\%$; $n = 6$), indicating that ATRA specifically causes an increase in apoptosis in MDSCs. In summary, the reduction in live MDSCs in the IS + ATRA group revealed a mechanism that may explain the restoration in T-cell proliferation and the decrease in the amounts of NO and arginase-1 observed in the IS + ATRA group.

ATRA is able to reverse LPS immunosuppression

In the results described above, ATRA was given concomitantly with LPS from day 1. However, as patients usually arrive in the ICU after sepsis has developed, we performed different LPS/ATRA administration protocols in order to mimic more closely the clinical scenario and corroborate whether ATRA was able to reverse immunosuppression after its establishment. As shown in Figure 6(A), one protocol consisted of the administration of increasing doses of LPS alone for 1 week. After this, animals were left untreated (IS/- group) or given ATRA alone (IS/ATRA group) for an additional 2 weeks. When left untreated, the IS/- group had a partial recovery of their humoral immune response. However, and most importantly, the administration of ATRA to the initially immunosuppressed group increased this response, indicating that ATRA was able to reverse the immunosuppressive state. Additionally, another protocol was performed (Figure 6B), where mice were given LPS alone for the first week (exactly as in the protocol in Figure 6A) and after that we continued with the inoculation of LPS (IS/IS group) or LPS and ATRA (IS/IS + ATRA group) for an additional 2 weeks. Using this protocol, a partial, but statistically significant, improvement in the immune response was observed, similar to what was found when ATRA was given concomitantly with LPS from day 1 (Figure 1A). Thus our results indicate that ATRA is able to rescue mice from a state of previously established immunosuppression whether LPS is still present or not.

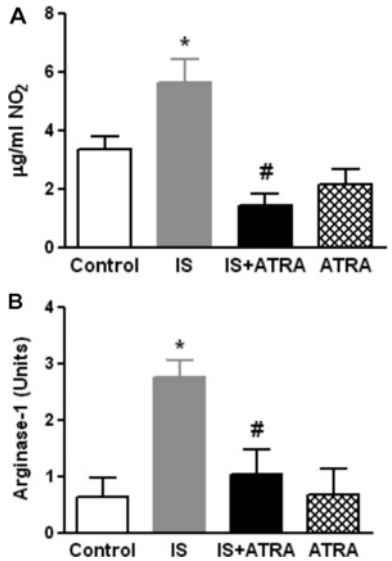

Figure 4 Suppressor mechanisms of MDSCs in T-cell proliferation assays are decreased in LPS-immunocompromised mice treated with ATRA

(**A**) NO levels. (**B**) Arginase-1 activity. Results are expressed as units of arginase-1, where 1 unit is defined as the amount of enzyme that catalyses the formation of 1 μg of urea in 60 min. Values are means $\pm$ S.E.M., $n = 14$ per group. $^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with the IS group.

DISCUSSION

Through improvements in supportive care measures, physicians have been able to help patients survive the initial phase of sepsis, but this has been associated with an increased frequency of late sepsis-associated immunosuppression [36]. In fact, in recent years, this loss of immune competence has been considered to be one of the main problems in late sepsis [37]. For that reason, many investigators consider that efforts to recover or preserve host immune function will be the next major advance in the management of patients with sepsis [37]. In agreement with this assumption, in the present study we have attempted to overcome LPS-induced immunosuppression at the humoral and cellular levels using ATRA. We found that LPS-associated immunosuppression was related to an increased number of MDSCs and a decreased *in vitro* T-cell proliferation, which was associated with higher levels of NO and arginase-1 activity. We also demonstrated, using depletion experiments, that the inhibition of T-cell proliferation was, in fact, mediated by MDSCs. Treatment with ATRA was able to reverse the inhibition of T-cell proliferation, and this was associated with lower amounts of NO and arginase-1 activity and a decrease in live MDSCs. Moreover, ATRA decreased plasma IL-10 levels, counteracted the immunosuppression-induced decrease in lymphocytes and improved the primary humoral immune response that was almost absent in the IS group. The ATRA-induced expansion of $CD19^+$ cells may explain the partial restoration in the primary immune response; however, as this restoration was not complete, other immune-suppressive mechanisms may still be operating. Significantly, and more relevant from a clinical point of view, ATRA was able to reverse the impaired LPS-induced primary humoral immune response in two additional protocols where immunosuppression was established first, highlighting ATRA as a potential therapeutic tool for the treatment of post-sepsis-immunosuppressed patients.

Many animal models of sepsis have been described, each with specific advantages and disadvantages [38]. For the present study, as we were particularly interested in the immunosuppressive phase of the response to sepsis, we chose a model of LPS-induced immunosuppression in mice. This model is simple and reproducible, and is highly controlled and standardized. The disadvantages of our model are that the triggering stimulus is not microbial, as LPS-mediated signalling is strictly TLR4 (Toll-like receptor 4)-dependent. Additionally, it may not reflect all of the complex physiological responses in humans. Nevertheless, as described in our present study and by others, LPS-induced immunosuppression mimics several clinical signs found in patients who succumb to septic shock after 72 h (late sepsis) due to opportunistic infections, such as down-regulation of monocyte HLA-DR expression, lymphocyte depletion, reduced ability to produce TNF-α and other cytokines *in vitro*, and MDSC expansion [39–41]. No model reproduces all aspects of human sepsis; however, each experimental model allows the study of the aspects of human sepsis that the model reproduces. Therefore we believe that the LPS-tolerant model is adequate to investigate and study the immunosuppressive phase that patients may develop after an episode of sepsis.

Numerous studies in animal models and clinical trials have confirmed the ability of vitamin A to prevent infections and strengthen the immune system [28,42]. Although one of the mechanisms by which ATRA improves immunocompetence is by differentiation of immature myeloid progenitors (i.e. MDSCs) into their mature counterparts, in the IS group treated with ATRA the number of MDSCs was apparently not reduced, despite restored T-cell proliferation. However, ATRA did cause an increase in MDSC apoptosis and, when considering only live MDSCs, this population was, in fact, decreased. These results suggest that ATRA may not be interfering with LPS-induced migration of MDSCs to LNs, but counteracts the effects of LPS by increasing MDSC apoptosis. The fact that ATRA alone also caused an increase in the percentage of apoptotic MDSCs and that the percentage of apoptosis observed in the $CD3^+$ population (T-cells) did not change suggests a specific effect of the drug itself in the induction of MDSC apoptosis. Several *in vitro* studies have demonstrated the pro-apoptotic activity of ATRA, and ATRA has been reported to induce apoptosis in the myeloid cell line HL-60 [43], human mesangial cells [44], promyelocytic leukaemia cells [45], canine osteosarcoma cells [46] and a human breast tumour cell line [47]. To our knowledge, the present study is the first to report that ATRA is able to regulate MDSC survival *in vivo*, and therefore our results indicate that the mechanism by which ATRA restored T-cell proliferation is through an increase in MDSC apoptosis. We are now investigating the direct effects of ATRA in the apoptosis of MDSCs and the underlying mechanisms.

Immunosuppression in our model was associated with T-cell depletion, as shown by a decrease in the number of lymphocytes both in the LNs and the thymus. The IS group treated with ATRA had an increase in single $CD4^+$ and $CD8^+$ cells in the LNs,

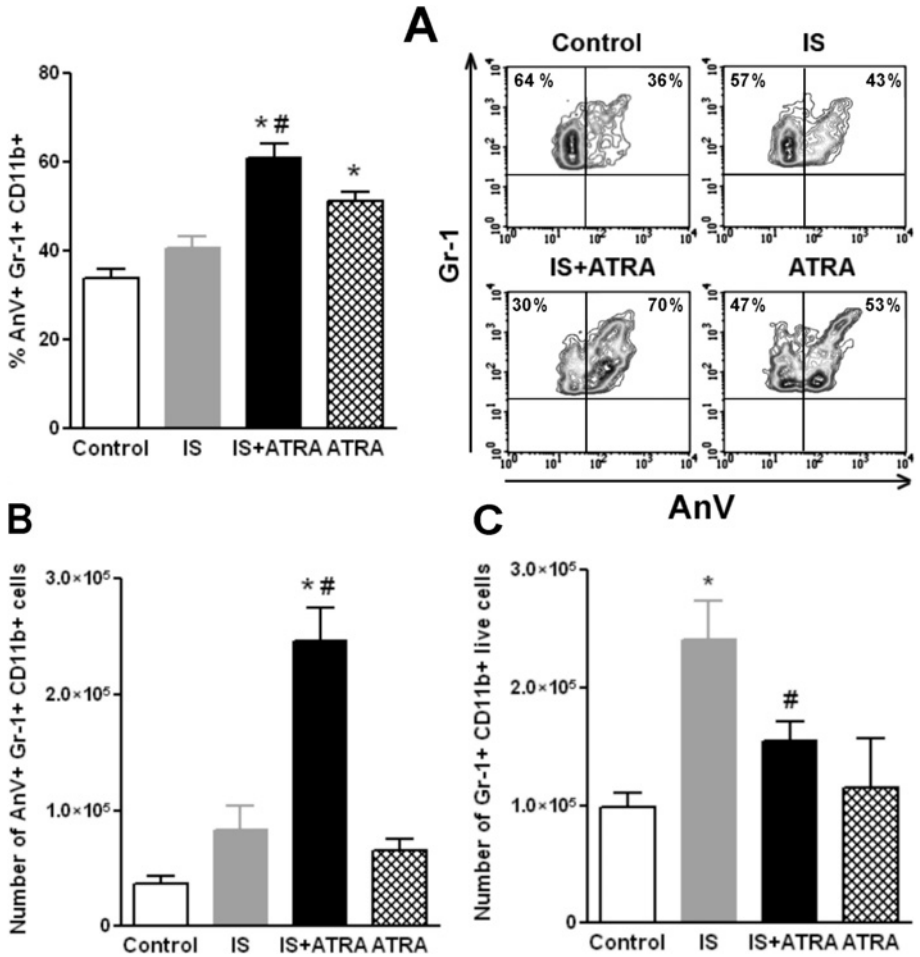

Figure 5 ATRA increases MDSC apoptosis in LPS-immunocompromised mice

(**A**) Left-hand panel, percentage of apoptosis in Gr-1$^+$CD11b$^+$ cells. Right-hand panel, representative dot plots from the different experimental groups showing AnV$^+$ within Gr-1$^+$ cells. (**B**) Absolute number of AnV$^+$Gr-1$^+$CD11b$^+$ cells. (**C**) Absolute number of live (AnV$^-$) Gr-1$^+$CD11b$^+$ cells. Results are expressed as means $\pm$ S.E.M., $n = 12$ per group. $^*P < 0.05$ compared with the control group; $^\#P < 0.05$ compared with the IS group.

whereas only double-positive cell reconstitution was observed in the thymus. This result suggests that, even though ATRA is able to stop/reverse LPS-induced T-cell depletion, it concomitantly induced migration of single CD4$^+$ or CD8$^+$ cells from the thymus to the LNs, similar to what has been reported in mucosal tissues [48]. The increased T-cell numbers observed with ATRA may be crucial in the improvement of immunocompetence found in our LPS-immunocompromised model. This may also have clinical relevance as a direct correlation between lymphocyte counts and patient's survival has been demonstrated [49]. A number of studies have shown that T-cell loss in sepsis is caused by increased apoptosis [50,51]. We could not detect an increase in apoptosis of CD3$^+$ cells obtained from the LNs in the IS group. However, as our model involves a chronic exposure to LPS, we cannot discard that apoptosis was involved in T-cell loss during the acute phases of the establishment of immunosuppression. It has been suggested that the decrease in the number and functional unresponsiveness of T-cells in immunosuppressed patients with sepsis may be caused by cell 'exhaustion' [41]. This mechanism has been widely described for persistent viral infections and involves the progressive loss of cell function due to protracted antigen exposure and cell stimulation [52]. In our model, the chronic exposure to LPS may be mimicking the state of cell 'exhaustion' observed in patients. As this state is phenotypically characterized by persistent expression of multiple inhibitory receptors, we are now investigating the presence of these molecules in T-cells from immunocompromised mice.

An increase in T_{reg}-cells has been reported in splenocytes recovered from patients with sepsis and LPS-treated mice [9,41]. However, the lungs of patients with sepsis showed no variations in T_{reg}-cells, but an increase in cells with a MDSC phenotype was observed [41]. In our model, no expansion of T_{reg}-cells was found in the LNs, but, consistent with what has been observed in other lymphocyte subpopulations, their numbers were decreased in the IS group. Although ATRA administration to the IS group restored T_{reg}-cell numbers, this could be an epiphenomenon as a

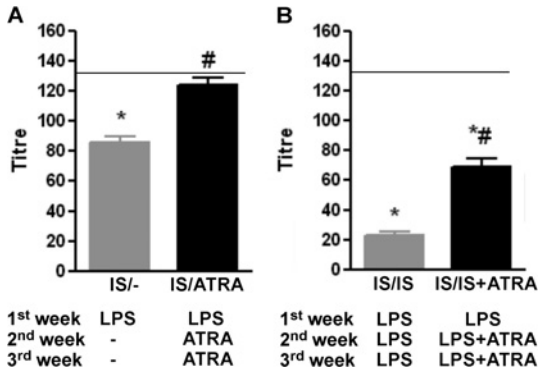

Figure 6 ATRA reverses the immunosuppression induced by LPS

Two different LPS/ATRA administration protocols were assayed, where immunosuppression was first established by the inoculation of increasing doses of LPS alone in the first week. (**A**) Animals were left untreated (IS/- group) or given ATRA alone (IS/ATRA group) for an additional 2 weeks. (**B**) Mice were given LPS (IS/IS group) or LPS + ATRA (IS/IS + ATRA group) for an additional 2 weeks. In both protocols, the primary humoral immune response was evaluated at week 4. The horizontal line represents the titre obtained in the control group. Results are expressed as means ± S.E.M., $n = 12$ per group. $^{*}P < 0.05$ compared with the control group; $^{\#}P < 0.05$ compared with respective the IS/- or IS/IS group.

consequence of the generalized lymphocyte expansion caused by ATRA.

In summary, our results demonstrate that ATRA administration induced MDSC apoptosis *in vivo*, concomitantly with an increase in the number of lymphocytes. This restoration of the normal proportion between effector/suppressor (lymphocyte/MDSC) populations might explain, in part, the effects of ATRA in improving immunocompetence in a model of immunosuppression induced by chronic exposure to LPS. Additionally, even when the immunosuppressive state was already induced, ATRA was still able to improve the immune response, and this improvement was observed whether LPS continued to be present or not.

Although immunosuppression is a complex multifactorial phenomenon and may involve diverse cellular and humoral mechanisms interconnected with each other, we believe that ATRA administration may represent a supplementary potential strategy in the treatment of the immunosuppressive state of sepsis and may be beneficial in improving the adaptive immune response both in the absence or presence of an on-going infection.

CLINICAL PERSPECTIVES

- The loss of immunocompetence in patients with sepsis is one of the current major concerns in ICUs. For that reason, many investigators consider that efforts to recover or preserve host immune functions will be the next major advance in the management of patients with sepsis.
- In the present study, we found that ATRA overcame LPS-induced immunosuppression at the humoral and cellular levels. Moreover, ATRA reversed the LPS-induced inhibition of T-cell proliferation, increasing MDSC apoptosis. ATRA restored the LPS-mediated impairment in the primary humoral immune response and the number of lymphocytes in lymph nodes.
- Thus ATRA may represent a potential strategy in the treatment of the immunosuppressive state in sepsis.

AUTHOR CONTRIBUTION

Daiana Martire-Greco, Veronica Landoni, Martin Isturiz and Gabriela Fernandez conceived and designed the experiments. Daiana Martire-Greco, Veronica Landoni, Paula Chiarella, Nahuel Rodriguez-Rodrigues, Pablo Schierloh and Barbara Rearte performed the experiments. Daiana Martire-Greco, Veronica Landoni, Pablo Schierloh, Martin Isturiz and Gabriela Fernandez analysed the data. Pablo Schierloh and Barbara Rearte contributed with reagents/materials/analysis tools. Gabriela Fernandez wrote the paper. Martin Isturiz revised the paper.

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Original Paper

SUPPLEMENTARY ONLINE DATA

all-*trans*-Retinoic acid improves immunocompetence in a murine model of lipopolysaccharide-induced immunosuppression

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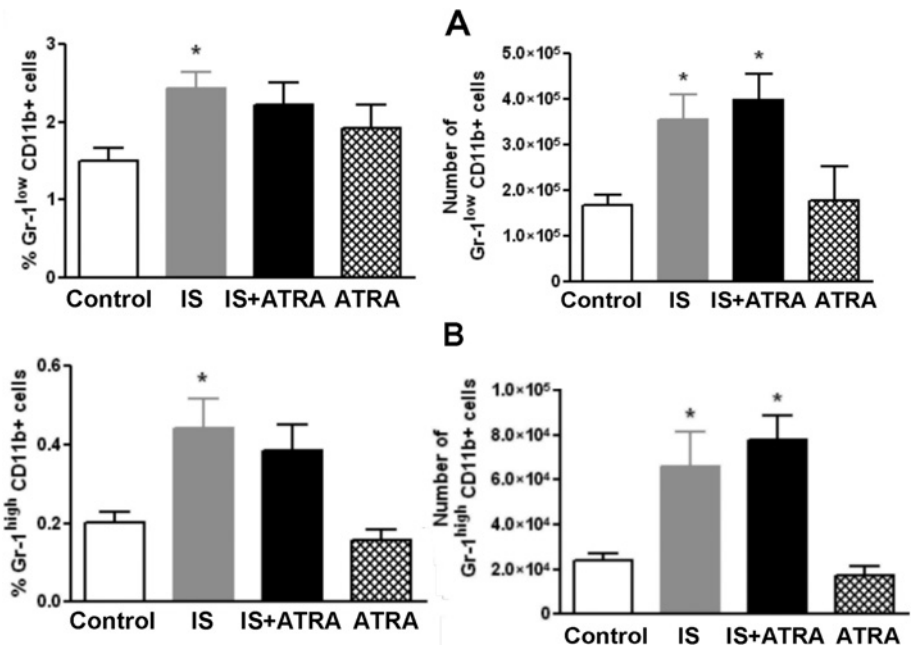

Figure S1 **Proportion and absolute numbers of the two subsets of MDSCs found in the LN in mice from the different experimental groups**
(**A**) Gr-1^{low}CD11b^{+}. (**B**) Gr-1^{high}CD11b^{+}. $n = 20$–24 per group. *$P < 0.05$ comapred with control.

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Table S1 Lymphocyte populations found in the lymph nodes during treatment
Values are means ± S.E.M, $n = 20–24$.

Lymphocyte population	Control	IS	IS + ATRA	ATRA
$CD19^+$	19.1 ± 4.9%	20.0 ± 1.4%	25.1 ± 1.1%	13.8 ± 3.5%
$CD4^+$	54.1 ± 2.0%	48.7 ± 0.8%	41.6 ± 1.0%	56.3 ± 2.9%
$CD8^+$	21.2 ± 0.7%	22.1 ± 0.4%	23.5 ± 1.2%	21.7 ± 1.4%
$CD4^+CD25^+Foxp3^+$	11.1 ± 0.3%	14.9 ± 0.7%	15.0 ± 0.8%	11.8 ± 0.2%

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