Mononuclear Cell Extravasation in an Inflammatory Response Is Abrogated by All-Trans-Retinoic Acid through Inhibiting the Acquisition of an Appropriate Migratory Phenotype

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ABSTRACT

The inflammatory response is tightly regulated by several mediators that promote the adhesive and migratory capacities of different cell types, including peripheral blood mononuclear cells (PBMCs). Our laboratory has previously characterized the inflammatory response developed in the experimental model of mercuric chloride (HgCl₂)-induced nephritis in Brown Norway rats as an acute inflammatory response dependent on very late antigen (VLA)-4. This response can be modulated by all-trans-retinoic acid (at-RA), a vitamin A metabolite that regulates a broad range of biological processes and exhibits anti-inflammatory properties. Based on this in vivo experimental model, we have established a VLA-4-dependent ex vivo system to study the effect of at-RA on PBMC polarization, adhesion, and migration and to elicit new mechanisms triggered by at-RA for abrogating an inflammatory response. We found that at-RA significantly reduces the VLA-4-dependent migration of PBMCs activated in vivo. In addition, we demonstrated by spreading assays that in vivo at-RA treatment abrogates the acquisition of a polarized cell phenotype. In fact, at-RA inhibits the actin polymerization required for cell morphology changes, and it alters the distribution of F-actin and VLA-4 integrin in focal contacts, essential for cell adhesion. Moreover, we describe that at-RA also abrogates the redistribution of Rac1 and RhoA, important proteins implicated in the dynamic process of cell movement. In summary, we demonstrate the capacity of at-RA to block the acquisition of an appropriate migratory phenotype in PBMCs as a new mechanism underlying the anti-inflammatory effects of this compound.

Leukocytes extravasation from the bloodstream into the tissue is of key importance in several physiological and pathological processes (Tanaka, 2001). During infiltration, these cells show a polarized morphology essential for directional movement. This polarization is characterized by a flat lamella extending in the direction of migration, which ends in a lamellipodium (the leading edge) and by a narrow retracting tail at the rear of the cell (for review, see Fais and Malorni, 2003). Although migratory phenotypes and factors that promote migration vary greatly among cell types and infiltration stimuli, a universal mechanism triggered by chemotactic factors that underlies cell migration is actin polymerization (Pollard and Borisy, 2003).

Retinoic acid has been described as a potent modulator of immune responses and inflammatory diseases (Perez de Lema et al., 2004; Adams et al., 2005). Additionally, at-RA exhibited effects on adhesion molecules, abolishing in vitro α4 integrin-dependent rolling in the acute promyelocytic leukemia cell line NB-4 (Brown et al., 1999) and down-regulating the expression and function of β2 integrins in primary human monocytes (Babin and Henz, 2003). These in vitro results support those described in experimental in vivo models and, more in particular, in experimental models of kidney diseases where at-RA

ABBREVIATIONS: at-RA, all-trans-retinoic acid; BN, Brown Norway; PBMC, peripheral blood mononuclear cell; VLA, very late antigen; PAS, periodic acid-Shiff; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; VCAM, vascular cell adhesion molecule; MIP, macrophage inflammatory protein.
treatment significantly reduces the cellular infiltrates in the renal interstitium (Moreno-Manzano et al., 2003; Suzuki et al., 2003; Perez de Lema et al., 2004).

It has been previously reported that Brown Norway (BN) rats receiving sublethal doses of HgCl₂ develop an inflammatory syndrome (Pelletier et al., 1986; Rossert et al., 1988; Pusey et al., 1990). Recently, we have demonstrated that at-RA treatment significantly reduces the inflammatory response observed during HgCl₂-induced nephritis in BN rats by inhibiting the cell infiltration into the renal interstitium as well as the release of proinflammatory mediators (Escribese et al., 2007).

For directional movement, cells must coordinate assembly and disassemble integrin adhesive complexes. Several integrins localize at the leading edge of migrating cells, including αβ1 (Pinc et al., 2002; Goldfinger et al., 2003).

Previous reports from our laboratory have established that PBMC migration from the blood into the renal tissue in HgCl₂-induced nephritis is mainly mediated by the VLA-4 integrin (Molina et al., 1994; Escudero et al., 1998). Rho family members, which include Cdc42, Rac1, and RhoA, cycle between an active GTP-bound state and an inactive GDP-bound state. Members of this family play an important role in regulating cell migration by remodeling the actin cytoskeleton (Allen et al., 1998; del Pozo et al., 1999; Banyard et al., 2000). Cdc42 and Rac1 regulate the assembly of new focal complexes at the leading edge of the cell causing cell polarization (Nobes and Hall, 1995a,b). RhoA is required for the maturation of adhesive contacts into focal adhesions (Ridley and Hall, 1992; Cox et al., 2001). Less is known about the effect of at-RA in the regulation of Rho GTPases and in the formation of focal adhesion complexes, even though some studies identified retinoids as inhibitors of RhoA activity (Shao et al., 2005), thereby altering the cytoskeletal organization and cell motility (Donald et al., 2001).

In this study, we demonstrate that at-RA markedly reduces mononuclear cell migration during an in vivo inflammatory response by abrogating the acquisition of a polarized morphology. at-RA significantly decreases actin polymerization, alters F-actin and α4 integrin distribution in focal complexes, and modifies Rac1 and RhoA location, all essential requirements for PBMC migration. Our data identify new targets and mechanisms through which at-RA modulates inflammatory responses.

Materials and Methods

Animals. BN male rats, weighing 150 to 180 g, were obtained from IFFA-CREDO (Paris, France) and from our own breeding colony, fed with standard laboratory chow ad libitum, and given free access to water. All experimental procedures were performed according to the institutional guidelines that are in compliance with the National Institute of Health Guide for the Care Use of Laboratory Animals.

Experimental in Vivo Design. Animals were separated into four different groups. Group I (n = 8) received five s.c. injections of HgCl₂ (1 mg/kg body weight) over a period of 2 weeks to induce the autoimmune nephritis and served as the control group. The dosage and days were established on the basis of previous optimizing experiments. The following two groups of rats were treated with chow pellet supplemented with 15 mg/kg body weight at-RA (Sigma-Aldrich, St. Louis, MO). at-RA-supplemented chow pellets were prepared daily using a 1.68 mg/ml ethanol at-RA stock solution as previously described (Moreno-Manzano et al., 2003; Perez de Lema et al., 2004) and mixed in a dark cold room with standard chow. After the ethanol evaporated, the food was given to the rats. Group II (n = 8) included rats injected with HgCl₂ and treated with at-RA, and group III (n = 5) included rats only treated with at-RA (15 mg/kg). Finally, group IV (n = 5) included rats injected with H₂O adjusted at the same pH (3.8) as the HgCl₂ solution, following the same protocol of HgCl₂ administration and considering this group as the vehicle group.

Kidney Tissue Processing and Immunohistochemistry. Kidneys were removed at day 13 of the disease, and a piece of tissue was fixed in phosphate-buffered 10% formalin, paraffin-embedded, and then 3-μm sections were stained with periodic acid-Schiff (PAS) and observed using light microscopy (Olympus BX50; Olympus, Tokyo, Japan). In addition, an indirect peroxidase stained method was used to characterize CD3⁺ cells (T lymphocytes) and ED1⁺ cells (monocyte/macrophages), in the renal interstitium, using specific anti-retinoid primary antibodies from Serotec (Oxford, UK). HRP-conjugated secondary antibodies were obtained from Jackson Immunoresearch laboratories Inc. (West Grove, PA). Quantification of interstitial infiltrating cells CD3⁺ and ED1⁺ was performed by counting, in two kidney tissue sections per each rat, the total number of cells examined in 10 randomly chosen areas of interstitial infiltrates.

Isolation of PBMCs. Blood was obtained from the caudal vessel at the animal tail with a heparinized syringe, just after the treatment finished (13 days). Then, the blood was immediately diluted (one fifth) with RPMI 1640 medium supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin and then placed on ice. The blood cell suspension was thereafter layered onto a Ficoll density gradient (Sigma-Aldrich) and centrifuged at 1800 rpm for 30 min following the manufacturer’s protocol. A band lying at the interface corresponding to mononuclear cells was collected, and the cells were washed with RPMI 1640 medium before to be used for further experiments. Previously to any additional assay, cell viability was tested by trypan blue uptake.

Flow Cytometry Analysis of Mononuclear Populations. PBMCs isolated as described above from all the experimental groups were washed in PBS and characterized by specific immunostaining for the mononuclear cell subpopulations using the following labeled antibodies: CD45-FITC, CD45-PE, ED-1-FITC, CD3-FITC, CD4-PE, and CD8-PE (all from Serotec). Then, cells were washed in PBS containing 1% BSA (Sigma-Aldrich) and analyzed using a FACS-Scan Cytometer. In parallel, the viability of the PBMCs was checked by PI uptake.

Migration Assays. Cell migration was evaluated using a 24-well and 5-μm pore size Transwell system (Costar, Cambridge, MA). PBMCs were washed once in chemotaxis medium (RPMI medium containing 0.5% BSA) and then adjusted to 5 × 10⁶ cells/ml in the same medium. A cell suspension containing 5 × 10⁵ cells was placed in the upper compartment of the Transwell. The chemokine MIP-1α (100 ng/ml) was added to the chemotaxis medium (600-μl total volume) at the lower compartment of the Transwell. Cell migration was allowed for 5 h at 37°C in a 5% CO₂ atmosphere, and the cells in the lower chamber were quantified using a FACSscan Cytometer (BD Biosciences, San Jose, CA). To establish the number of cells that migrated nonspecifically, migration assays were performed in the absence of chemotacticant. The results are expressed as percentage of specific migration calculated as follows: [(total number of cells that have migrated in the presence of chemokine – the total number of cells migrating in the absence of chemokine/total number of input cells) × 100].

Cell Spreading Assays. Samples of heparinized blood were obtained from all experimental groups, and mononuclear cells were isolated using Ficoll-Histopaque method, then resuspended in RPMI 1640 medium containing 10% fetal calf serum and 1% BSA and seeded on coverslips coated with 5 μg/ml rhVCAM-1 (R&D Systems, Minneapolis, MN) for 2 h at 37°C. Spreading assays were performed on rh-VCAM-1 matrix, the specific ligand
of VLA-4 integrin, since this adhesion molecule plays an important role in the adhesion and migration of mononuclear cells in the autoimmune nephritis induced by HgCl₂ (Escudero et al., 1998). Cells that exhibited flattened morphology and lamellipodia by phase contrast microscopy observation were scored as positive for spreading.

**Actin Polymerization Assay.** PBMCs from all experimental groups were washed three times in assay medium (RPMI medium containing 0.5% BSA) and resuspended at 1 × 10⁶ cells/ml final concentration. After fixation in 3.7% formaldehyde and permeabilization with 0.5% Triton X-100 in PBS, blocked in 1% BSA in PBS, and stained with 50 U (165 nM) of Phalloidin-Alexa568 (Invitrogen, Carlsbad, CA) during 20 min at 4°C. Then, the cells were centrifuged and resuspended in PBS with 0.25% BSA. Mean fluorescence intensity was measured by FACScan. Total actin in PBMCs from all experimental groups was measured using an anti-total actin antibody (1:100) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by the appropriate anti-goat-Alexa 488 secondary antibody (1:200) (Invitrogen) and flow cytometry analysis.

**Actin Cytoskeleton Staining and Immunofluorescence.** Adhered PBMCs on a matrix of the adhesion molecule VCAM-1, specific ligand for VLA-4 integrin, were fixed in 3.7% formaldehyde, permeabilized with 0.5% Triton X-100 in PBS, blocked in 1% BSA in PBS, and stained with 50 U (165 nM) of Phalloidin-Alexa568 (Invitrogen). For VLA-4, Rac1, and RhoA immunostaining, cells were processed as above and incubated in 1% BSA in PBS containing anti-α4 (BD Biosciences, San Jose, CA), anti-Rac1 (BD Biosciences), and anti-RhoA (Santa Cruz Biotechnology, Inc.) at 1:50, followed by the appropriate secondary antibodies: anti-mouse Alexa488 1:100 (Invitrogen) and anti-rabbit FITC 1:100 (Jackson ImmunoResearch Laboratories). After washing, samples were mounted with mowiol (Calbiochem, San Diego, CA) and observed using a Leica TCS-SP2-AOBS-UV confocal inverted microscope (Leica, Wetzlar, Germany). Images and quantifications shown were obtained and processed with Leica Lite Confocal Software. Immunofluorescence experiments were performed at least three times.

**Statistical Analysis.** The statistical analysis was performed by ANOVA analysis, and p < 0.001 was considered significant.

## Results

**at-RA Reduces VLA-4-Dependent Migration.** We previously reported that at-RA improves the outcome of the inflammatory response in the HgCl₂-induced nephritis in BN rats, in whose development VLA-4 plays an important role (Escribese et al., 2007). Here, we evaluated the effect of at-RA on mononuclear cells migration into the renal interstitium in vivo and ex vivo. Based on these results, which confirmed that lymphocytes and monocytes are the main mononuclear subpopulations involved in the inflammatory response developed during HgCl₂-induced autoimmune response (Molina et al., 1994; Escudero et al., 1998), we have developed a VLA-4-dependent migration assay through a sVCAM-1 matrix using PBMCs.

Quantification of these mononuclear cells subpopulations in all the experimental groups is shown in Fig. 1C.

Based on these results, which confirmed that lymphocytes and monocytes are the main mononuclear subpopulations involved in the inflammatory response developed during HgCl₂-induced autoimmune response (Molina et al., 1994; Escudero et al., 1998), we have developed a VLA-4-dependent migration assay through a sVCAM-1 matrix using PBMCs.
from all the experimental groups. To perform migration assays and taking into account the capacity of at-RA to induce cell differentiation, we first characterized the working population of mononuclear cells isolated from each experimental group as CD45⁺ cells. Then, we analyzed alterations in the mononuclear cell subpopulations ED-1⁺, CD3⁺, CD4⁺, and CD8⁺. The results presented in the Fig. 2, A and B, demonstrate that neither HgCl₂ or at-RA alone nor the combination

![Graphs showing mononuclear cells populations distribution](image)

Fig. 2. Mononuclear cells populations’ distribution was not altered by the experimental treatments. A, PBMCs from the all experimental groups were labeled with specific markers for monocytes (ED1/CD45) and lymphocytes (CD3/CD45, CD8/CD45, and CD4/CD45) and analyzed by flow cytometry. Representative experiment is shown. B, quantification of the number of positive cells for each marker in all the experimental groups. Data are presented as mean ± S.D. of triplicates from three independent experiments; ∗, p < 0.001 versus HgCl₂ group.
of both compounds induced a shift in the different mononuclear cells subpopulations.

Hence, using the PBMCs isolated from all the experimental groups, VLA-4-dependent migration assays toward MIP-1α were performed. As is shown in Fig. 2C, PBMCs from HgCl₂-injected rats exhibit a significant increase in the percentage of migration toward MIP-1α in comparison with those from vehicle-treated rats. PBMCs from HgCl₂-injected rats treated with at-RA show a decrease of around 50% in migration compared with HgCl₂-injected rats. PBMCs from vehicle-treated rats and rats treated only with at-RA show a basal level of migration. These results clearly demonstrate that at-RA significantly reduced VLA-4-dependent migration in vivo and ex vivo, and this reduction is not due to any cell differentiation process triggered by at-RA in the PBMCs.

**PBMC Spreading Is Abrogated by at-RA Treatment.**

Next, spreading assays using PBMCs from all experimental groups were performed on a sVCAM-1 matrix. PBMCs from HgCl₂-injected rats strongly adhered to the substrate and spread over the matrix since these cells lost their round characteristic morphology and showed extended protrusions and a polarized morphology (Fig. 3A, a). On the other hand, PBMCs from HgCl₂-injected rats also treated with at-RA did not exhibit significant morphological changes (Fig. 3A, b) in comparison with the PBMCs from rats treated with vehicle (Fig. 3A, c) or with at-RA only (Fig. 2A, c). In these cases, cells maintained their characteristic round shape. The quantification of these spreading assays is shown in Fig. 3B. PBMCs from HgCl₂-injected rats exhibit the highest percentage of spread cells in comparison with PBMCs from the other experimental groups. at-RA diminished significantly this percentage. These results correlate with the remarkable cellular infiltration observed in the HgCl₂-injected rats described above and support that at-RA abrogates the acquisition of a migratory phenotype in activated PBMCs.

**at-RA Reduces Actin Polymerization in Activated PBMCs.**

Actin polymerization was measured in PBMCs from all experimental groups using Alexa Fluor-468 phalloidin for F-actin staining and flow cytometry. The results presented in Fig. 4 show a significant increase in F-actin levels in PBMCs from HgCl₂-injected rats in comparison with the F-actin levels in PBMCs from vehicle rats. In PBMCs from HgCl₂-injected rats also treated with at-RA, the levels of F-actin decreased to control levels. F-actin levels in PBMCs from rats only treated with at-RA did not show significant differences compared with those in PBMCs from rats treated with vehicle. The amount of total actin estimated by specific anti-actin antibody staining and flow cytometry did not show differences among the experimental groups (data not shown). Thus, we demonstrate that at-RA reduces the F-actin amount to basal levels on PBMCs during an inflammatory response, strongly suggesting that at-RA modulates cytoskeletal dynamics in PBMCs during this process.

**at-RA Abrogates the Polarized Distribution of F-actin in Activated PBMCs.**

In addition to estimate actin polymerization in PBMCs, we have also studied the distribution of F-actin in these cells by Alexa Fluor-488-phalloidin staining and confocal microscopy. As detailed above, PBMCs from all experimental groups were spread on a matrix of sVCAM-1. Phalloidin staining demonstrates that in PBMCs from HgCl₂-injected rats F-actin was distributed in focal spots in the leading edge as well as in the rear of the cell (Fig. 5, left). This focal distribution of F-actin allows PBMCs to acquire the appropriate morphology necessary for adhesion and directional migration. Otherwise, in PBMCs from mercury rats treated with at-RA, F-actin was localized homogeneously, mainly in the periphery of the cells resulting in

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**Fig. 3.** at-RA inhibits the acquisition of a polarized morphology by leukocytes. A, spreading assays on a VCAM-1 matrix have been performed using leukocytes from all experimental groups. B, quantification of spread leukocytes; *, p < 0.001 versus HgCl₂ group.

**Fig. 4.** at-RA reduces actin polymerization in activated leukocytes induced by HgCl₂. Leukocytes from all experimental groups were stained with Phalloidin-Alexa568 and analyzed by flow cytometry. Results are expressed as fold changes of F-actin expression in comparison with vehicle group. The results correspond to five independent experiments. *, p < 0.001 versus HgCl₂ group.
maintenance of their round morphology (Fig. 5, right). The results described in this section demonstrate that at-RA disturbs the normal distribution of F-actin, which is necessary for leukocyte polarization.

at-RA Modulates VLA-4 Distribution in PBMCs. To study the distribution of VLA-4 in the spread leukocytes, PBMCs from all experimental groups were immunostained with anti-α4 and observed by confocal microscopy. The results are presented in Fig. 6 and show that PBMCs from HgCl₂-injected rats formed VLA-4-dependent focal contacts on a VCAM-1 matrix (Fig. 6a). However, PBMCs from HgCl₂-injected rats also treated with at-RA did not exhibit focal location of VLA-4 (Fig. 6b), showing a similar pattern of VLA-4 distribution than PBMCs from rats only treated with at-RA (Fig. 6c) or vehicle (Fig. 6d).

at-RA Inhibits Rac and RhoA Redistribution during Cell Polarization. The dynamics of the actin cytoskeleton is regulated by small GTPases from the Rho family. Here, we have studied the distribution of Rac1 and RhoA in PBMCs from all experimental groups, by immunofluorescence combined with F-actin staining and confocal microscopy. In Fig. 7, left, and Fig. 8, left, spread PBMCs from HgCl₂-injected rats show a focal distribution of F-actin (red), localized at the rear and at the cell edge. Rac1 and RhoA immunofluorescences (green) reveal a marked accumulation of these proteins in the leading edge of the cell, where actin reorganization is taking place. Moreover, in the leading edge of the cell, both F-actin and Rac1 colocalized (yellow) strongly, suggesting the contribution of Rac1 to the lamellipodia formation. PBMCs from rats injected with HgCl₂ and treated with at-RA maintained their characteristic round morphology (Fig. 7, right, and Fig. 8, right), as mentioned above, and Rac1 and RhoA were distributed uniformly around the cell surface. These results indicate that at-RA treatment impairs the proper distribution of Rac1 and RhoA in mononuclear cells, which would contribute to the failure to acquire a migratory phenotype.

Discussion

Mononuclear cells extravasation from the bloodstream during an inflammatory response is tightly regulated by several proinflammatory mediators that promote the adhesive and migratory capacities of these cells. In pathological conditions, an excess of these mediators leads to excessive cell extravasation (Springer, 1994). The acquisition of an appropriate migratory phenotype in PBMCs is an essential requirement for extravasation. To allow firm adhesion and transmigration, these cells must change their round morphology and polarize toward the migratory stimuli. These processes require the redistribution of several proteins within the cells (Vicente-Manzanares et al., 2005). Retinoic acid has been extensively used as an anti-inflammatory agent in many inflammatory diseases including nephropathies, due to its inhibitory effect on the expression of proinflammatory mediators (Moreno-Manzano et al., 2003; Vicente-Manzanares et al., 2003, 2005; Perez de Lema et al., 2004; Adams et al., 2005; Escribese et al., 2007).
In this work, a novel and direct effect of at-RA on PBMCs during an inflammatory response is described. Based on an in vivo model of HgCl₂-induced nephritis, where we have previously demonstrated the beneficial effect of this treatment on the outcome of this nephritis (Escribese et al., 2007), we have performed ex vivo experiments to identify new mechanisms underlying the anti-inflammatory properties of at-RA by affecting mononuclear cells diapedesis.

Herein, we have reproduced PBMCs migration during an inflammatory response toward MIP-1α/H9251, a key chemokine released during renal inflammation (Zhou et al., 2003; Li et al., 2006). This assay can be considered an ex vivo VLA-4-dependent migration system to evaluate in vitro the effect of the in vivo at-RA treatment on lymphocyte and monocyte migration in the model of HgCl₂-induced nephritis since both populations are the major mononuclear subpopulations involved in this inflammatory response, as we have probed here and in previous works (Escudero et al., 1998; Mittelbrunn et al., 2004). The results obtained in this work corroborate what we have previously observed in vivo (Escribese et al., 2007): PBMCs from HgCl₂-injected rats showed a significant increase in their migratory capacity in comparison with PBMCs from vehicle rats. The treatment with at-RA of the HgCl₂-injected rats drastically reduces the percentage of migration. This inhibitory effect of at-RA on PBMCs migration was comparable with that observed by Wu et al. (2006), which demonstrated a clear inhibition of migration and invasion in a hepatocellular carcinoma cell line upon at-RA treatment. Additionally, Johst et al. (2003) demonstrated that 9-cis-retinoic acid treatment inhibited migration and proliferation in human aortic smooth muscle cells.

As we have mentioned above, cell polarization is essential for migration. Thus, we have studied the PBMC’s capacity to acquire an appropriate migratory phenotype performing spreading assays on a VCAM-1 matrix. The results show that HgCl₂ treatment induced a polarized morphology in PBMCs with the formation of significant protrusion in the leading edge of these cells. This correlates with the highest percentage of migration obtained with PBMCs isolated from HgCl₂-injected rats group as well as with the in vivo response of these rats, in terms of a significant inflammatory response development. On the other hand, the administration of at-RA to the rats drastically reduces the acquisition of a migratory phenotype by the PBMCs that kept their round characteristic morphology, which might explain the reduction in the migratory capacity of these cells.

An essential mechanism that drives cell polarization and the consequent cell migration is actin polymerization. Our results show that in vivo-activated cells by HgCl₂ exhibited a significant increase in F-actin in comparison with PBMCs from vehicle-treated rats. On the other hand, at-RA caused a drastic reduction of F-actin, demonstrating that at-RA abro-
gates actin polymerization in PBMCs. This reduction supports our results describing the abrogation of cell polarization and, as a consequence, the reduction of the cell migration and the inflammatory response development in vivo by at-RA treatment.

In addition to studying the levels of F-actin in PBMCs from each experimental group, we also evaluated the distribution of F-actin in the cell, finding that in PBMCs from HgCl₂-injected rats, F-actin localized mainly in small dots or focal contacts along the cell that let it adhere to the substratum. These focal contacts contribute to the dynamic spread of the cell and to the acquisition of an appropriate migratory phenotype as mentioned above. In contrast, PBMCs from rats treated with at-RA did not exhibit F-actin location in focal contacts, failing these cells to adhere and subsequently to spread and migrate. Very little is known about the signaling pathways involved in at-RA-mediated modulation of actin polymerization and distribution, which could even comprise changes in expression of actin binding proteins (Yen et al., 2004). It has been described that retinoic acid regulates the expression levels of proteins associated with early stages of HL-60 leukemia cell line differentiation and, interestingly, F-actin capping proteins (Yen et al., 2004). Additionally, proteomic analysis of all-trans-retinoic acid-treated hepatocarcinoma cell lines showed up-regulation of profilin-1, an ubiquitous important regulator of actin dynamics that contributes to the inhibition of cell proliferation and migration (Wu et al., 2006). Regarding all this and since it was described that at-RA can induce cell differentiation, thus leading to morphological alterations, we assessed here this hypothesis finding that there were no changes in the percentages of monocytes and lymphocytes populations composing the PBMCs among all the experimental groups. Then, our results demonstrate that the morphological alterations described here are direct effects of the treatments, i.e., HgCl₂ and at-RA, not the consequence of any differentiation process triggered by them.

Ongoing works from our laboratory suggest that at-RA might protect epithelial tubular cell cytoskeleton structure during renal ischemia/reperfusion, contributing to the reduction of epithelial cell detachment. These might support the results presented in this work regarding an inhibitor effect of at-RA in the actin cytoskeleton remodeling. Moreover, at-RA also reduces the inflammatory renal infiltrate in other autoimmune diseases such as lupus (Perez de Lema et al., 2004), even though no direct effect of at-RA on leukocyte migratory phenotype has been reported.

Since VLA-4 is an essential molecule in the development of the HgCl₂-induced nephritis (Escudero et al., 1998; Mittelbrunn et al., 2004) and, as an adhesion protein, plays an important role in PBMCs adhesion and extravasation, we studied the distribution of VLA-4 integrin in the PBMCs from all experimental groups, using our ex vivo system. In PBMCs from HgCl₂-injected rats, VLA-4 integrin was mainly localized in the focal contacts, allowing a VLA-4-dependent adhesion of these PBMCs to a VCAM-1 matrix. However, the administration of at-RA to the rats caused a diffuse distribution of the integrin VLA-4 in PBMCs, which correlated with the absence of focal contacts described above. There are previous reports that show a modulation of integrins function by retinoids administration in several cell lines (Babina and Henz, 2003) as well as the effect of at-RA on o4-dependent adhesion of promyelocytic leukemia cell lines (Brown et al., 1999). Our results using the ex vivo VL4-dependent system demonstrated that at-RA also affects the distribution of this adhesion molecule in PBMCs.

We have also studied the role of the RhoGTPases Rac1 and RhoA in the control of mononuclear cells polarity and migration by analyzing the distribution of both proteins in these cells. Activated PBMCs from HgCl₂-injected rats exhibit a distribution of Rac1, mainly at the leading edge of polarized leukocytes. Moreover, Rac1 colocalized with F-actin at the lamellipodia, suggesting that Rac1 was redistributed to these sites to contribute to actin reorganization. In PBMCs from rats injected with HgCl₂ and also treated with at-RA, which retained a round morphology and did not exhibit increased migration capacity, Rac1 redistribution to the leading edge and its colocalization with F-actin was completely abrogated, similar to the nonactivated PBMCs.

In the case of RhoA, we obtained related results; this protein, when localized at the focal contacts (PBMCs from HgCl₂-injected rats), might mediate actin reorganization and contribute to the firm adhesion of PBMCs from HgCl₂-injected rats. In PBMCs from HgCl₂-injected rats also treated with at-RA, RhoA redistribution to focal contacts was abrogated, and RhoA remained located at the cell periphery, correlating with the maintenance of a round morphology. Notice that in nonactivated PBMCs from vehicle-treated rats that neither polarize nor migrate, both Rac1 and RhoA were located continuously all around the cell periphery.

There are not many studies on the effect of at-RA on expression, distribution, and activity of Rho GTPases in relation to the anti-inflammatory properties of this compound. It has been described that Rac1 and RhoA activities are involved in the differentiation induced by at-RA in several cell lines (Alsayed et al., 2001; Pan et al., 2005). Although the system used was different from ours, the effect described in these studies supports our results on the modulation of Rho GTPases by at-RA.

In summary, we have reported here a novel mechanism underlying the anti-inflammatory effect of at-RA treatment in vivo. We demonstrated for the first time that at-RA abrogates the acquisition of an appropriate migratory phenotype in PBMCs, an essential requirement for cell extravasation and for the development of an inflammatory response. By means of an ex vivo system using PBMCs from rats that developed an inflammatory response induced by HgCl₂ during the autoimmune nephritis, we have demonstrated that at-RA reduces actin polymerization, inhibits the formation of VLA-4-dependent adhesion contacts, and blocks the appropriate redistribution of Rac1 and RhoA, which are all required for PBMC polarization and migration toward a migratory stimulus. Therefore, this work reveals a new mechanism triggered by at-RA to modulate an inflammatory response.

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