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AT_1 receptor-mediated angiotensin II activation and chemotaxis of T lymphocytes

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ABSTRACT

Angiotensin II (Ang II), a central renin–angiotensin system (RAS) effector molecule, and its receptors, AT_1 and AT_2, have been shown to be involved in the inflammatory aspects of different diseases, however the cellular mechanisms underlying the regulation of immunity are not fully understood. In this work, using spleen-derived $CD4^+$ and $CD8^+$ T lymphocytes activated in vitro, we tested the influence of Ang II on different aspects of the T cell function, such as activation and adhesion/transmigration through endothelial basal membrane proteins. The addition of 10^{-8} M Ang II did not change any of the parameters evaluated. However, 10^{-6} M losartan, an AT_1 receptor antagonist: (i) reduced the percentage of $CD25^+$ and $CD69^+$ cells of both subsets; (ii) inhibited adhesion of these cells to fibronectin or laminin by 53% or 76%, respectively and (iii) significantly reduced transmigration through fibronectin or laminin by 57% or 43%, respectively. In addition, 10^{-6} M captopril, an angiotensin-converting enzyme inhibitor had similar effects to Ang II, however its effects were reverted by exogenous Ang II (10^{-8} M). None of these responses was modified by 10^{-7} M PD123319, an AT_2 antagonist. These data reinforce the notion of endogenous production of Ang II by T cells, which is important for T cell activation, and adhesion/transmigration induced on interaction with basal membrane proteins, possibly involving AT_1 receptor activation. Moreover, AT_1 receptor expression is 10-fold higher in activated T lymphocytes compared with naive cells, but AT_2 receptor expression did not change after T cell receptor triggering.

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1. Introduction

New components and functions of the renin–angiotensin system (RAS) are still being unraveled. Studies aimed at RAS effector molecules, especially angiotensin II (Ang II) and its receptors, AT_1 and AT_2, has shifted the attention toward its nonclassic effects, beyond the regulation of intravascular volume and systemic blood pressure. Ang II has been proposed to be central in the inflammatory aspects of different diseases (Bush et al., 2000; Donadelli et al., 2000), however the cellular mechanisms underlying the regulation of immunity are not fully understood.

The adaptive immune response and T cells in particular have been shown to be important in the pathogenesis of different inflammatory diseases (Platten et al., 2009; Suzuki et al., 2002). In addition to the classic components required for T cell activation, these cells are equipped with all RAS components and are potentially able to produce and deliver Ang II to sites of inflammation (Jurewicz et al., 2007). In these cells, Ang II triggers proliferation and interferon γ (IFN-γ) production, suggesting that it induces differentiation to the Th1 phenotype (Costerousse et al., 1993; Jurewicz et al., 2007). In an experimental autoimmune encephalomyelitis (EAE) model, the use of AT_1 receptor antagonist or angiotensin-converting enzyme (ACE) inhibitor suppressed antigen-specific Th1 and Th17 cells and induced activity of $CD4^+FoxP3^+$ regulatory T cells (Platten et al., 2009). In non-T cells, there is evidence that Ang II has chemoattractant properties which are reduced by the administration of ACE inhibitors (Kato et al., 1999; Morrissey and Klahr, 1998; Weinstock and Kassab, 1986). Furthermore, Ang II induces the chemokine RANTES, a chemotactic molecule for human monocytes, through the Ang II type 2 receptor (AT_2R) (Wolf et al., 1997). Several RAS genes have also been demonstrated to be expressed in imma-

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ture and mature dendritic cells (DCs) identified by human cDNA microarray (Lapteva et al., 2001). Blockade of the Ang II type 1 receptor (AT_1R) with losartan or candesartan caused poor endocytic and allostimulatory activity in DCs differentiated from human monocytes; conversely, treatment of differentiated DCs with AT_2R blockade induced higher endocytic and allostimulatory activity (Nahmod et al., 2003).

In the present work, we investigate the role of Ang II on T cell activation and adhesion/transmigration of these cells through endothelial basal membrane proteins. We studied this point by using AT_1/AT_2 receptor antagonists and the ACE inhibitor, captopril, to provide information about the corresponding effects of endogenously produced Ang II by T cells.

2. Materials and methods

2.1. Animals

BALB/c mice (18–20 g) were provided by Oswaldo Cruz Foundation Breeding Unit (Rio de Janeiro, Brazil) and bred at the Laboratory of Applied Pharmacology Experimental Animal Facility, Farmanguinhos (Fundação Oswaldo Cruz). The mice were caged with free access to food and fresh water in a temperature-controlled room (22–24 °C) with a 12 h light/dark cycle until used. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Ethics Committee of Federal University of Rio de Janeiro (permit number IBCCF098).

2.2. Compounds

N-2-Hydroxyethylpiperazine *N*-2-ethanesulfonic acid (HEPES), (tris350 hydroxymethyl)-aminomethane (Tris), glucose, sodium bicarbonate, sodium azide, bovine serum albumin (BSA), PD123319, Ang II (Asp–Arg–Val–Tyr–Ile–His–Pro–Phe), were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). The AT_1 receptor selective antagonist, losartan, was obtained from Merck S.A. (Rio de Janeiro, Brazil) and captopril, the ACE inhibitor, was obtained from Laboratório Farmacêutico da Marinha (Rio de Janeiro, Brazil). Purified NA/LE hamster IgG1 anti-mouse CD3e (145-2C11), PeCy5.5-conjugated hamster IgG1 anti-murine CD3 (145-2C11), FITC-conjugated rat IgG2b anti-murine CD4 (L3T4), PE-conjugated rat IgG2a anti-murine CD8a (53-6.7), FITC-conjugated rat IgG2a anti-murine CD45R/B220 (RA3-6B2), APC-conjugated rat IgG1 anti-murine CD25 (PC61.5), APC-conjugated hamster IgG anti-murine CD69 (H1.2F3), PE-conjugated rat IgG2b anti-murine VLA4 (R1-2), were purchased from eBioscience (San Diego, CA, USA). PE-conjugated rat IgG2a anti-murine VLA5 (5H10-27), PerCP/PE/FITC-conjugated hamster IgG1 and IgG2, goat IgG2a isotype controls were all purchased from BD PharMingen (San Diego, CA, USA). Dulbecco's minimal essential medium (DMEM) high glucose, RPMI 1640, fetal bovine serum (FBS), ACK lysing buffer, minimal essential medium (MEM) non-essential amino acid solution (MEM AAs), 2-mercaptoethanol, L-glutamine, sodium pyruvate, penicillin/streptomycin and trypan blue were obtained from GIBCO (Grand Island, NY, USA). Human fibronectin was purchased from Chemicon International Inc. (Temecula, CA, USA) and human laminin was purchased from Invitrogen (Eugene, OR, USA). All other reagents were of the highest purity available.

2.3. Splenic T cell isolation and culture

Male intact BALB/c mice, approximately 18–20 g, were used as spleen donors. Mouse spleens were aseptically removed and placed in DMEM supplemented with 2 mM L-glutamine, 10 mM non-essential amino acids, 5×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin and 10 mM HEPES. Single cell suspension from the spleens were treated with ACK solution to induce erythrocytes lysis and washed twice with Hank's solution supplemented with 10% FBS. Cells were re-suspended in complete medium (DMEM with 10% FBS), placed into T cell enrichment nylon wool columns and maintained for 45 min in a humidified atmosphere with 5% CO_2 at 37 °C. The purity of the isolated cells was checked by flow cytometry analysis with PeCy5.5-conjugated anti-CD3 and FITC-conjugated anti-B220 staining (about 80% of T lymphocytes and 20% non-T cells, but mainly $B220^+$ cells) and viability was checked by 0.25% trypan blue dye exclusion method and found to be greater than 95% viable. One-milliliter aliquots of the cell suspension, each containing 1.5×10^6 cells were distributed into 48-well plates (Corning Inc., New York, USA), previously coated with immobilized anti-CD3mAb (2C 11) (5 μg/mL) and maintained for 48 h in a humidified atmosphere with 5% CO_2 at 37 °C.

2.4. T cell activation profile analysis

To analyze expression of the surface markers of T cell activation, naive T lymphocytes were cultured in 48-well plates coated with anti-CD3 as described above. During activation, the cells were pre-treated or not with 10^{-6} M losartan or 10^{-6} M captopril, for 30 min, prior to Ang II incubation for 24 h in a humidified atmosphere with 5% CO_2 at 37 °C. The same treatment with drugs and Ang II was repeated for the following 24 h to complete 48 h of incubation. Activated cells not treated with Ang II but which received 10^{-6} M losartan or 10^{-6} M captopril treatments were used as control. Cells were then harvested and stained with fluorescent antibodies against CD3, CD4, CD8, CD25 and CD69, and analyzed by flow cytometry. Results are expressed as the percentage of $CD4^+$ or $CD8^+$ T lymphocytes expressing CD25 or CD69.

2.5. In vitro adhesion assay

Splenic T lymphocytes were harvested from cultures and re-suspended at 1×10^6 cells in 300 μL of RPMI 1640 supplemented with 1% BSA (assay medium). Cells were placed in individual wells of a 24-well culture plate, previously coated with fibronectin (10 μg/mL), laminin (10 μg/mL) or BSA (10 μg/mL), which was used as a negative control to discount unspecific adhesion. 10^{-8} M Ang II was added or not as stimulus. Plates were incubated for 40 min at 37 °C in 5% CO_2. Adhered cells were collected from the wells and counted in a Neubauer hemocytometer. The results are expressed as the number of adhered cells from endothelial basal membrane proteins minus the number of adhered cells from BSA $\times 10^4$.

2.6. Transwell migration assay

Splenic T lymphocytes were harvested from culture and re-suspended at 5×10^5 cells in 100 μL of RPMI 1640 supplemented with 1% BSA (assay medium). Cells were added to the upper chamber of 5.0-μm pore diameter transwell tissue-culture inserts (Millipore, MA, USA), coated with fibronectin (10 μg/mL), laminin (10 μg/mL) or BSA (10 μg/mL), which was used as a negative control to discount unspecific migration. The inserts were placed in individual wells of a 24-well cell-culture plate containing 600 μL of assay medium in the absence or presence of 10^{-8} M Ang II. To discard a possible chemokinesis effect, 10^{-8} M Ang II was added to both the insert and the corresponding well. Plates were incubated for 3 h at 37 °C in 5% CO_2. Transmigrated cells were collected from the lower chamber, counted, stained with fluorescent antibodies against CD3, CD4 and CD8 and analyzed by flow cytometry. The results are expressed as the number of transmigrated cells through

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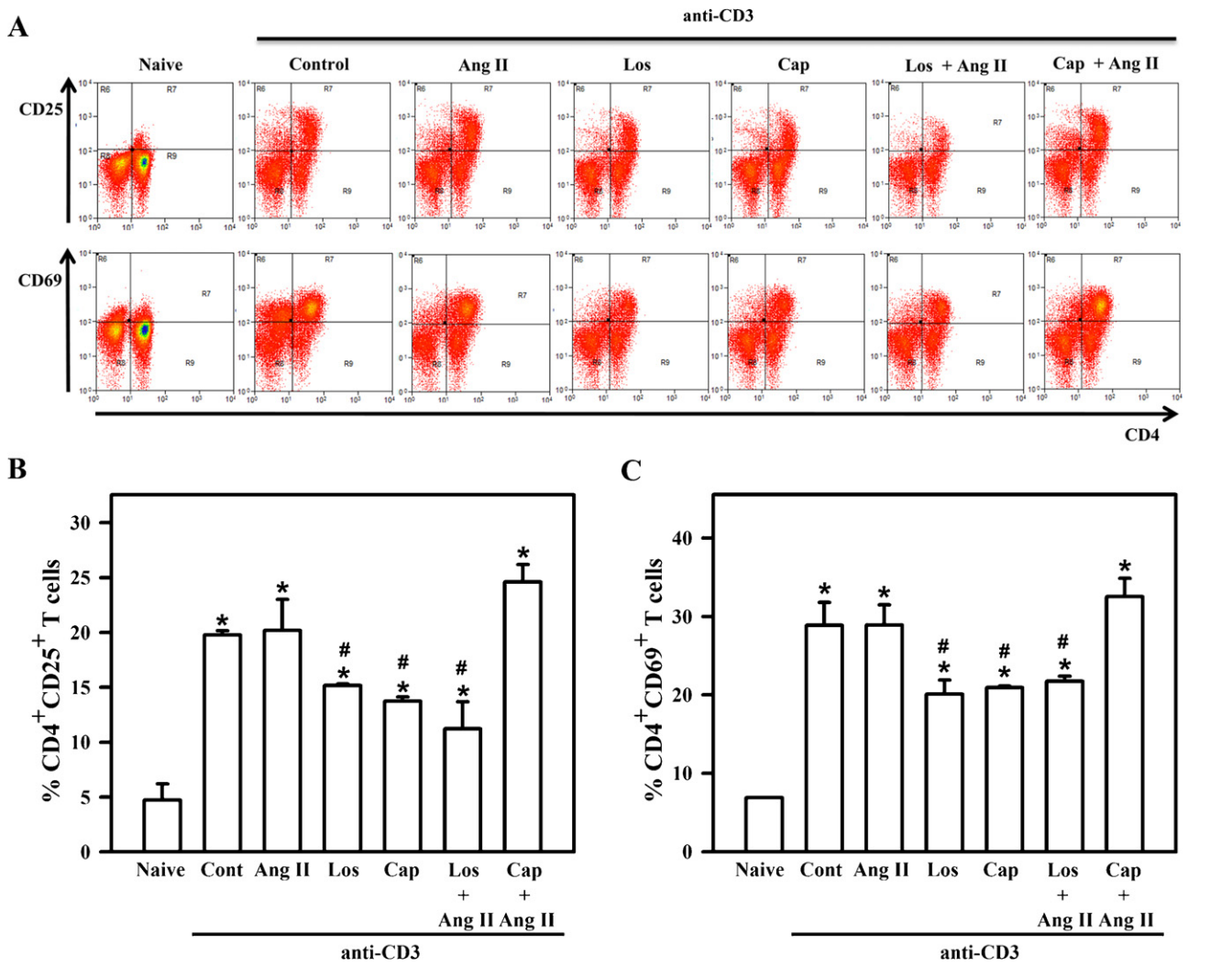

Fig. 1. Role of endogenous Ang II on the activation of $CD4^+$ T cells. Total T lymphocytes obtained from the spleen of naive mice were cultured or not in 48-well plates coated with anti-CD3 for in vitro activation as described in Section 2. During activation, T cells were incubated or not with 10^{-8} M Ang II for 48 h in the presence or absence of 10^{-6} M losartan or 10^{-6} M captopril. After treatments, cells were harvested and stained with fluorescent antibodies and analyzed by flow cytometry. (A) Representative dot plots of $CD4^+CD25^+$ and $CD4^+CD69^+$ cells obtained from gated $CD3^+$ T cells; (B) the percentage of $CD4^+CD25^+$ T cells; (C) the percentage of $CD4^+CD69^+$ T cells. The results are expressed as means ± SE ($n = 5$). Statistically significant compared with *naive ($p < 0.05$) and #anti-CD3 plus Ang II values ($p < 0.05$).

endothelial basal membrane proteins minus the number of transmigrated cells through BSA × 10^4. The number of $CD4^+$ or $CD8^+$ T lymphocytes is reported as the numbers of cells after multiplying the percentage of $CD4^+$ or $CD8^+$ T lymphocytes by the total number of leukocytes.

2.7. Flow cytometry analysis

Cells harvested from culture or adhesion/transmigration assays were incubated with the appropriate concentration of anti-CD3 mAb, anti-CD4 mAb, anti-CD8 mAb, anti-CD25 mAb, anti-CD69 mAb, anti-VLA4 mAb, anti-VLA5 mAb or IgG isotype controls for 30 min at 4 °C, after incubation with rat serum to block nonspecific binding sites. Surface markers analysis was performed using the Summitt software after acquisition in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). At least 10^4 lymphocytes were acquired per sample. All data were collected and displayed on a log scale of increasing fluorescence intensity and presented as dot plots. Percentages of T lymphocytes were determined in a specific $CD3^+$ T lymphocytes gate.

2.8. Immunodetection of angiotensin receptors

Naive or anti-CD3 activated T lymphocytes were re-suspended and lysed on ice for 40 min in Ripa buffer (25 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate and 0.1% sodium dodecyl sulfate) freshly supplemented with phosphatase and protease inhibitors (10 mM NaF, 5 mM Na_3VO_4, 5 mM $Na_4P_2O_7$ and 1× protease inhibitor cocktail, Roche, IN, USA). The final protein concentration in each condition was determined by the Bradford method (Bradford, 1976) using BSA as a standard. Aliquots containing 30 μg of protein were re-suspended in SDS-PAGE loading buffer, resolved on SDS 9% acrylamide gels and transferred onto Immobilon-P membranes (Millipore, MA, USA). After blocking with 5% non-fat dry milk/Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature, membranes were probed overnight at 4 °C with primary specific antibodies, followed by horseradish peroxidase-labeled secondary antibodies (Amersham Biosciences) and visualized with ECL®-plus reagent (Enhanced Chemiluminescence, Amersham Biosciences). Mouse monoclonal anti-human AT_1 and rabbit polyclonal anti-human AT_2 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz,

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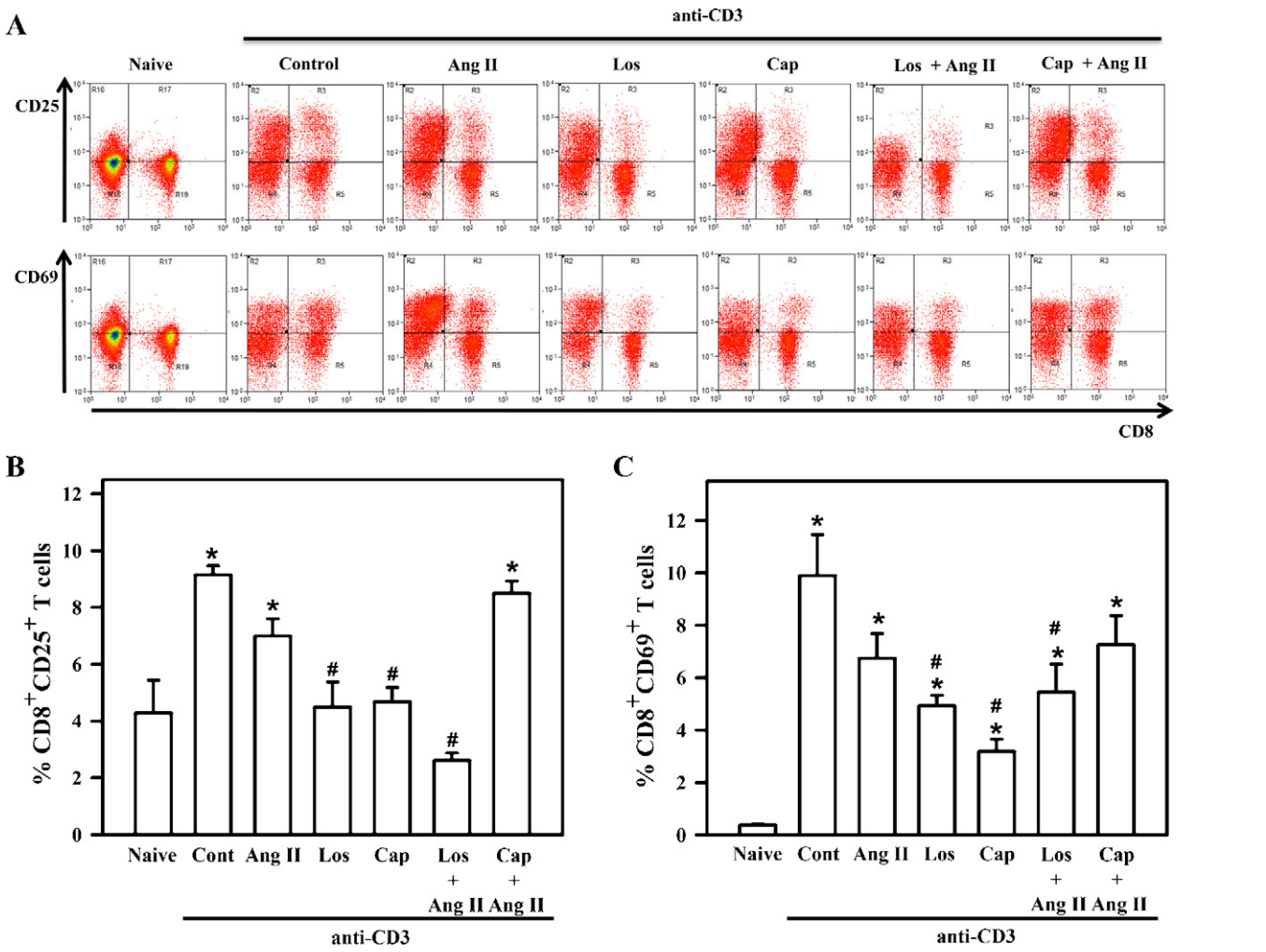

Fig. 2. Role of endogenous Ang II on the activation of $CD8^+$ T cells. Total T lymphocytes obtained from the spleen of naive mice were cultured or not in 48-well plates coated with anti-CD3 for in vitro activation as described in Section 2. During activation, T cells were incubated or not with 10^{-8} M Ang II for 48 h in the presence or absence of 10^{-6} M losartan or 10^{-6} M captopril. After treatments, cells were harvested and stained with fluorescent antibodies and analyzed by flow cytometry. (A) Representative dot plots of $CD8^+CD25^+$ and $CD8^+CD69^+$ cells obtained from gated $CD3^+$ T cells; (B) the percentage of $CD8^+CD25^+$ T cells; (C) the percentage of $CD8^+CD69^+$ T cells ($n = 5$). Statistically significant compared with *naive ($p < 0.05$) and #anti-CD3 plus Ang II values ($p < 0.05$).

CA, USA). The probed membranes were stripped with Re-Blot Plus Western blot stripping solution (Millipore) for 30 min at room temperature and reprobed with rabbit polyclonal β-actin to detect total levels of protein.

2.9. Statistical analysis

Each experiment was performed in independent cell suspensions. The data were analyzed by two-way analysis of variance (ANOVA), considering the treatments as factors. The significance of the differences was verified by the Bonferroni post-test. Statistical analysis was performed using absolute values.

3. Results

3.1. Ang II enhances the activation profile of $CD4^+$ and $CD8^+$ T lymphocytes

To examine the effect of Ang II on the activation profile of T cells, the expression of the activation markers CD25 and CD69 was evaluated in both $CD4^+$ and $CD8^+$ T lymphocytes isolated from the spleen of naive BALB/c mice. The $CD4^+$ and $CD8^+$ T lymphocytes were purified and cultured in 48-well plates containing anti-CD3, in the presence or in the absence of 10^{-8} M Ang II. After 48-h exposure to exogenous Ang II, no changes in the acute activation markers CD25 and CD69 were observed in both lymphocyte subsets in relation to cells treated with anti-CD3 alone (Fig. 1, $n = 5$ and Fig. 2, $n = 5$). However, the addition of 10^{-6} M losartan in the presence of anti-CD3, a well-known AT_1 angiotensin receptor antagonist (Touyz and Berry, 2002), significantly reduced the $CD25^+$ expression by 25% and 56% in $CD4^+$ (Fig. 1) and $CD8^+$ T cells (Fig. 2), respectively. Losartan reduced the percentage of $CD69^+$ by 33% and 50% in $CD4^+$ and $CD8^+$ T cells, respectively. These data indicate that endogenous production of Ang II could mediate anti-CD3 activation of both $CD4^+$ and $CD8^+$ T lymphocytes. Then, in the next experimental group we used captopril, an ACE inhibitor (Touyz and Berry, 2002). The addition of 10^{-6} M captopril significantly reduced $CD25^+$ and $CD69^+$ expression in both $CD4^+$ and $CD8^+$ T cells. Captopril had a similar effect to losartan. Moreover, the effect of captopril on $CD25^+$ and $CD69^+$ expression was completely reversed by exogenous addition of Ang II; the effect of losartan was not changed (Figs. 1 and 2).

Based on these results, we suggest the presence of a functional RAS during anti-CD3-induced activation of T cells. In some way, anti-CD3 leads to the production of Ang II endogenously, which mediates, at least in part, the increase in CD25 and CD69 expression. This effect possibly involves AT_1 receptor activation.

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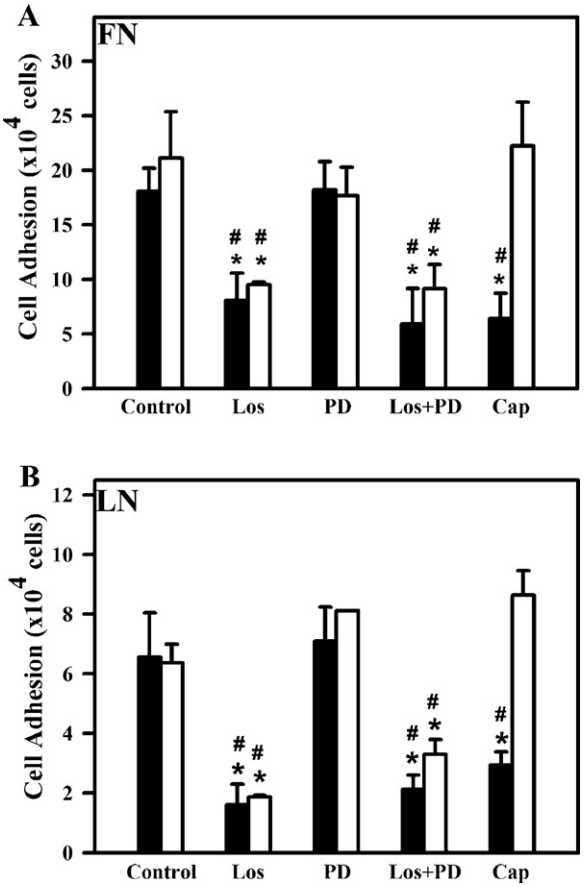

Fig. 3. Endogenous Ang II induces adhesion of T lymphocytes to endothelial basal membrane proteins. 1×10^6 splenic T lymphocytes were placed in individual wells of a 24-well culture plate, previously coated with fibronectin (10 μg/mL), laminin (10 μg/mL) or BSA (10 μg/mL) as negative control, in the absence (black bars) or presence (open bars) of 10^{-8} M Ang II, and allowed to adhere for 40 min at 37 °C. After incubation, adhered cells were collected and counted using a Neubauer hemocytometer. (A) The number of T cells that adhered to the fibronectin matrix; (B) the number of T cells that adhered to the laminin matrix. The results are expressed as the number of migrated T cells ($n = 6$). Statistically significant compared with *medium alone (black bars; $p < 0.05$) and #Ang II values (open bars; $p < 0.05$).

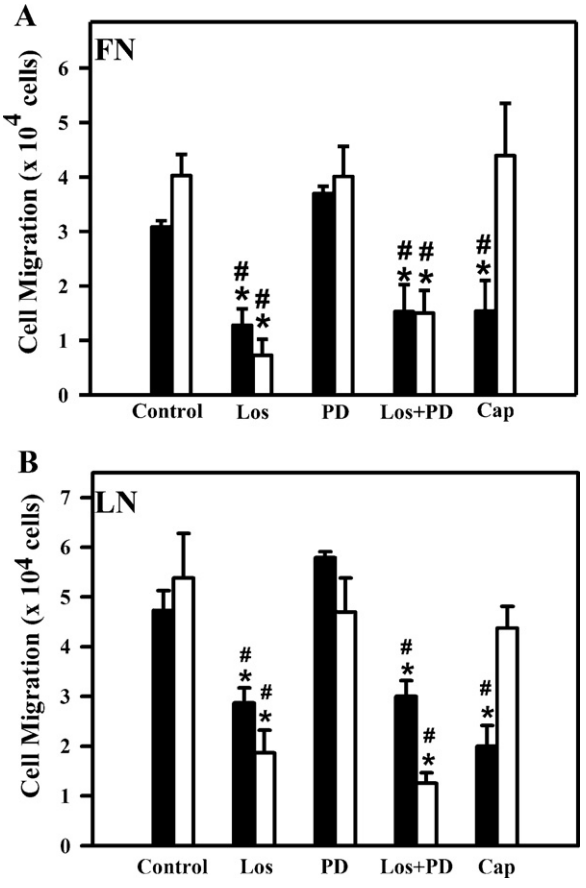

Fig. 4. Endogenous Ang II induces transmigration of T lymphocytes on contact with endothelial basal membrane proteins. 5×10^5 splenic T lymphocytes were added to the upper chamber of transwell tissue-culture inserts (5.0 μm pore diameter) previously coated with fibronectin (10 μg/mL), laminin (10 μg/mL) or BSA (10 μg/mL) as negative control. The inserts were placed in individual wells of a 24-well cell-culture plate containing 600 μL of assay medium in the absence (black bars) or presence (open bars) of 10^{-8} M Ang II and incubated for 3 h at 37 °C in 5% CO_2 with or without the addition of 10^{-6} M losartan, 10^{-7} M PD123319 or 10^{-6} M captopril, alone or in combination, as depicted in each panel. Transmigrated cells were collected from the lower chamber and counted in a Neubauer hemocytometer. (A) The number of T cells that migrated through the fibronectin matrix minus the number of cells that migrated from BSA $\times 10^4$; (B) the number of T cells that migrated through the laminin matrix minus the number of cells that migrated from BSA $\times 10^4$. The results are expressed as means ± SE ($n = 6$). Statistically significant compared with *medium alone (black bars; $p < 0.05$) and #Ang II values (open bars; $p < 0.05$).

3.2. Ang II induces adhesion of anti-CD3-activated T lymphocytes to proteins of the endothelial basal membrane

Cell adhesion is a mechanism that plays a pivotal role in the function of the immune system by tethering leukocytes to the endothelium enabling them to emigrate for tissue specific homing and recruitment at sites of inflammation (Weninger and von Andrian, 2003). Here, we tested the effect of 10^{-8} M Ang II on the adhesion properties of anti-CD3-activated splenic T cells to different proteins of the endothelial basal membrane, such as fibronectin or laminin. In Fig. 3 ($n = 6$) we show, in both matrixes, that the addition of Ang II did not change the basal adhesion capacity of these cells (fibronectin matrix: control, 18.07 ± 2.13; Ang II, (21.11 ± 4.27) × 10^4 cells; laminin matrix: control, 6.56 ± 1.48; Ang II, (6.37 ± 0.62) × 10^4 cells). When cells were pre-treated with 10^{-6} M losartan, the basal T cell adhesion to fibronectin or laminin was inhibited by 53% or 76%, respectively. On the other hand, 10^{-7} M PD123319, an AT_2 antagonist, did not change cell adhesion. The concomitant pre-treatment with losartan and PD123319 also blocked the cell adhesion similarly to losartan alone. This data reinforce the notion of an endogenous production of Ang II by T cells which is important for T cells-endothelial basal membrane proteins interaction and adhesion, involving AT_1 receptor activation.

To support this hypothesis, cells were pre-treated or not with 10^{-6} M captopril, the ACE inhibitor. In this case, captopril inhibited the adhesion of T cells by 65% or 55% to fibronectin or laminin, respectively. The addition of exogenous Ang II reverted captopril effect, restoring cell adhesion capacity.

3.3. Ang II induces transmigration of anti-CD3-activated T lymphocytes through proteins of the endothelial basal membrane

Chemokines are known to control cell traffic to the site of inflammation (Weninger and von Andrian, 2003). It has been reported that Ang II is important for the recruitment of some leukocyte subsets such as monocytes, neutrophils, natural killer cells, DCs and T lymphocytes (Geara et al., 2009; Jurewicz et al., 2007). However, the biological properties involved in Ang II-induced T cell transmigration have not been elucidated. We have shown the involvement of Ang II as an inducer of T cell adhesion properties; therefore we tested the effect of Ang II in T cell transmigration through the interaction with fibronectin or laminin. Independently of the matrix applied, 10^{-8} M Ang II did

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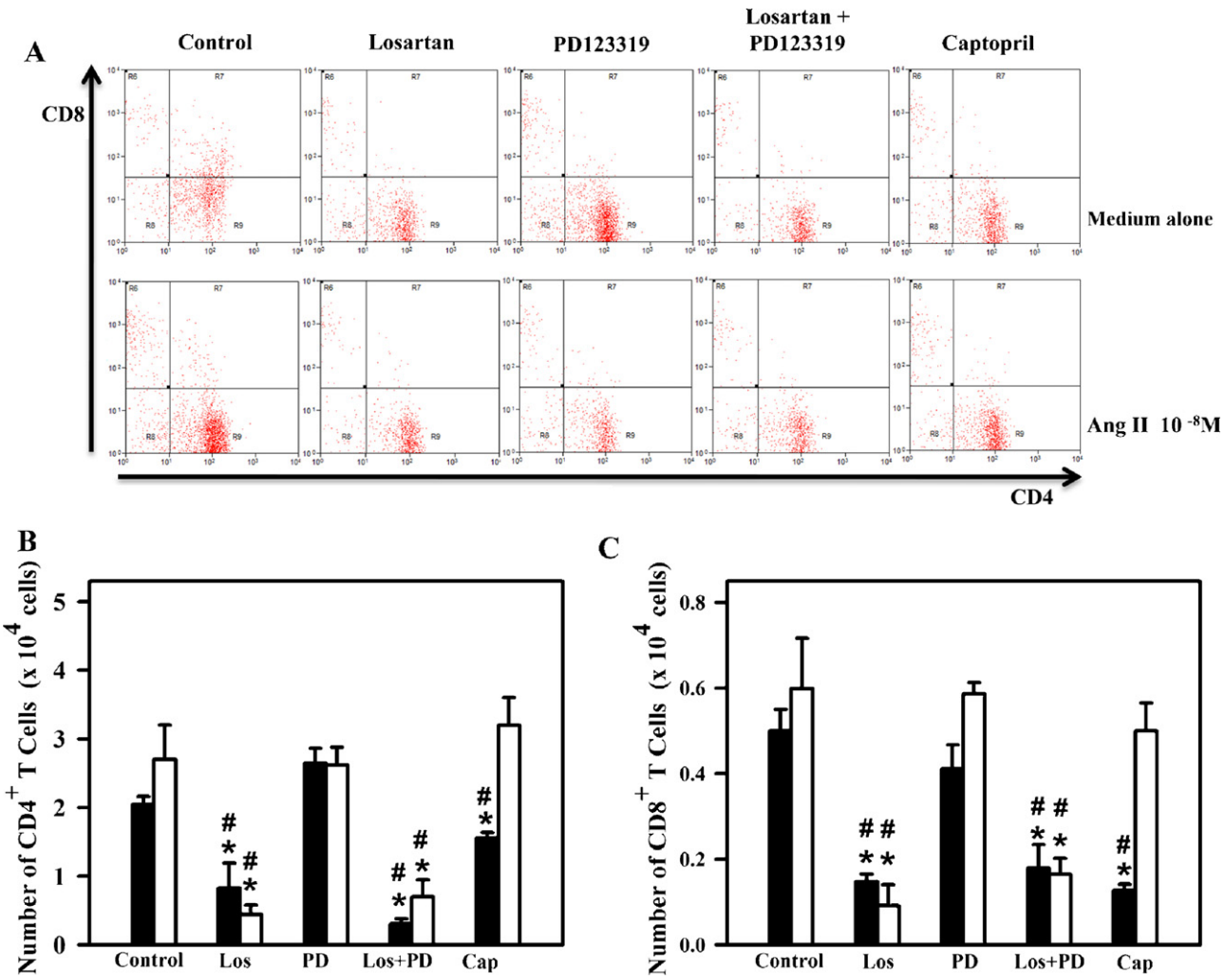

Fig. 5. Differential responsiveness to Ang II by T cell subsets during chemotaxis. The transmigration assay of splenic T cells was performed as described in the legend of Fig. 4. Transmigrated cells were collected from the lower chamber, stained with fluorescent antibodies and analyzed by flow cytometer. (A) Representative dot plots of $CD4^+$ and $CD8^+$ cells gated on $CD3^+$ T cells that migrated through the fibronectin matrix in the absence or presence of 10^{-8} M Ang II; (B) the number of $CD4^+$ T cells that migrated through the fibronectin matrix in the absence (black bars) or presence (open bars) of 10^{-8} M Ang II; (C) the number of $CD8^+$ T cells that migrated through the fibronectin matrix in the absence (black bars) or presence (open bars) of 10^{-8} M Ang II. The results are expressed as means ± SE ($n = 6$). Statistically significant compared with *medium alone (black bars; $p < 0.05$) and #Ang II values (open bars; $p < 0.05$).

not change the chemotaxis profile of anti-CD3-activated T cells, in relation to controls (fibronectin matrix: control, 3.08 ± 0.06; Ang II, (4.03 ± 0.20) × 10^4 cells; laminin matrix: control, 4.73 ± 0.40; Ang II, (5.38 ± 0.90) × 10^4 cells) (Fig. 4, $n = 6$). When the cells were pre-treated for 30 min with 10^{-6} M losartan, before starting the chemotaxis assay, the transmigration capacity through laminin and fibronectin was significantly reduced by 43% and 57%, respectively. The effect of losartan effect was not reversed even when 10^{-8} M Ang II was added in the lower chamber. On the other hand, 10^{-7} M PD123319 did not alter the basal chemotaxis (Fig. 4). When 10^{-7} M PD123319 was used concomitantly with losartan, no modification was observed in the migration profile in relation to losartan alone. The concomitant addition of 10^{-8} M Ang II in the insert and in the lower chamber did not alter the number of cells able to transmigrate through both endothelial basal membrane proteins in relation to the control, excluding any chemokinesis effect. These results likely suggest that the endogenously produced Ang II by T cells is sufficient to induce a matrix-dependent transmigration of cells in a mechanism dependent on AT_1 receptor signaling, which was not improved by the exposure to exogenous Ang II. The pre-treatment with 10^{-6} M captopril was able to reduce T cell transmigration by approximately 50% in relation to the controls in both matrixes. As observed in the adhesion experiments, this phenomenon was restored to the same level as the control when Ang II was added to the lower chamber (captopril + Ang II: fibronectin matrix, 4.39 ± 0.68, laminin matrix, (4.37 ± 0.44) × 10^4 cells) (Fig. 4).

3.4. Differential responsiveness to Ang II by T cell subsets during chemotaxis

So far, we have shown that Ang II is involved in the chemotaxis of anti-CD3-activated total T cells. In the next experimental set, we verified whether Ang II induces chemotaxis of a specific T cell population, in the context of $CD4^+$ or $CD8^+$ subsets. To determine the specific T cell chemotaxis we quantified the specific number of $CD4^+$ or $CD8^+$ T cells that migrate through the fibronectin matrix. The absolute number of $CD4^+$ T cells that migrated followed the same profile as total T cells (compare Fig. 5B, $n = 6$, with Fig. 4A). Exogenous Ang II also did not have a significant effect on $CD8^+$ T

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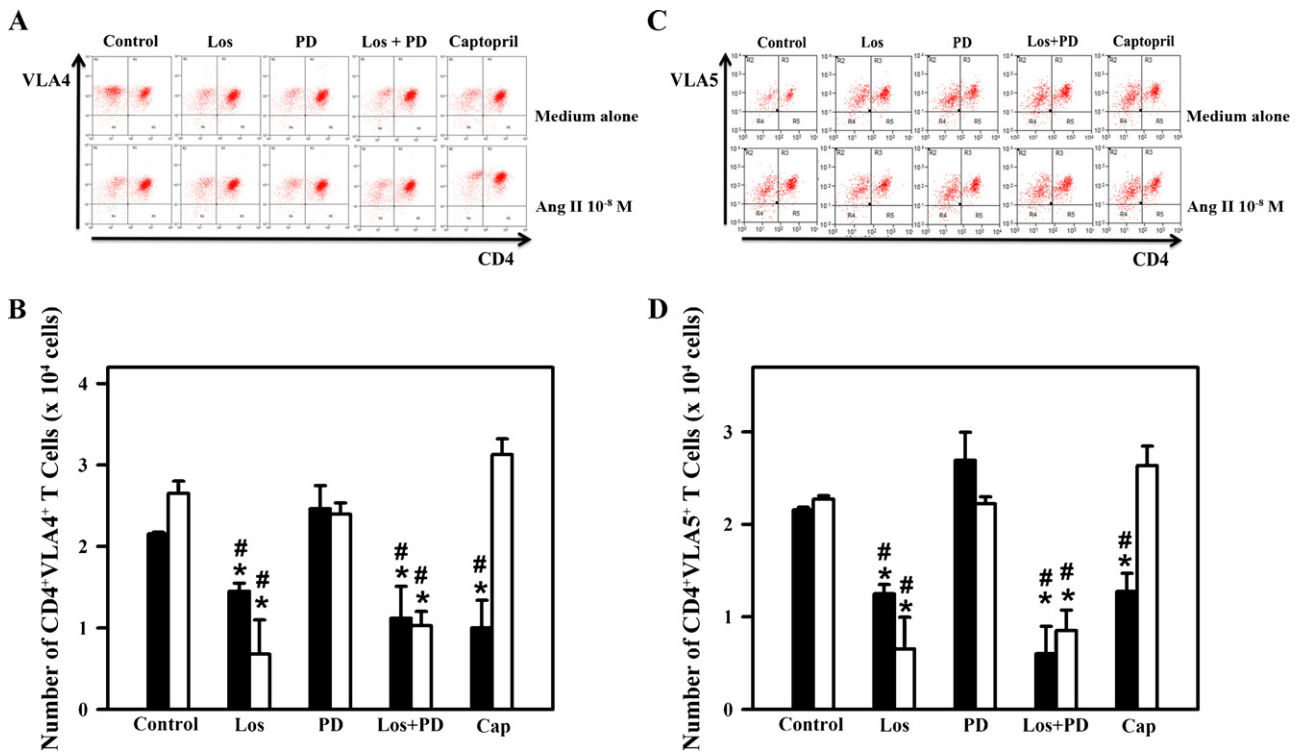

Fig. 6. The expression of adhesion molecules in splenic $CD4^+$ T cells is not modulated by Ang II. The transmigration assay of splenic T cells was performed as described in the legend of Fig. 4. Transmigrated cells were collected from the lower chamber, stained with fluorescent antibodies and analyzed by flow cytometer. (A) Representative dot plots of $CD4^+VLA4^+$ cells gated on $CD3^+$ T cells that migrated through the fibronectin matrix in the absence or presence of 10^{-8} M Ang II; (B) the number of $CD4^+VLA4^+$ T cells that migrated through the fibronectin matrix in the absence (black bars) or presence (open bars) of 10^{-8} M Ang II; (C) representative dot plots of $CD4^+VLA5^+$ cells gated on $CD3^+$ T cells that migrated through the fibronectin matrix in the absence or presence of 10^{-8} M Ang II; (D) the number of $CD4^+VLA5^+$ T cells that migrated through the fibronectin matrix in the absence (black bars) or presence (open bars) of 10^{-8} M Ang II. The results are expressed as means ± SE ($n = 6$). Statistically significant compared with *medium alone (black bars; $p < 0.05$) and #Ang II values (open bars; $p < 0.05$).

cell chemotaxis (Fig. 5 C, $n = 6$). Again we observed that 10^{-6} M captopril inhibited the specific $CD8^+$ T cell chemotaxis, which was also reversed by Ang II.

3.5. Ang II did not modulate adhesion molecules expression in splenic $CD4^+$ T cells

Since the major population able to transmigrate through different extracellular protein matrixes was the $CD4^+$ T subset, in the next experimental group, we evaluated whether these cells also show Ang II-induced modulation of VLA-4 and VLA-5 expression. VLA-4 and VLA-5 are two different integrins that bind with high affinity to fibronectin and laminin (Crisa et al., 1996). Using flow cytometry analysis, we observed that although the expression level of both molecules was not modulated by Ang II, the absolute number of $CD4^+VLA4^+$ and $CD4^+VLA5^+$ T cells followed the same profile observed for total T cells that migrated, in all conditions tested during the chemotaxis assay (Fig. 6, $n = 6$).

3.6. T cell receptor engagement selectively induces AT_1 receptor expression

The scenario indicates that Ang II participates, at least in part, in the anti-CD3 effect on $CD4^+$ and $CD8^+$ T cells. Thus, we tested the hypothesis whether T cell receptor engagement and consequently, T cell activation, could modulate the expression of Ang II receptors, AT_1 and AT_2, making the cells more responsive to Ang II stimuli. Fig. 7A ($n = 6$) demonstrates that AT_1 receptor expression is 10-fold higher in anti-$CD3^+$ activated T cells compared with naive cells, and AT_2 receptor expression did not change after T cell receptor triggering (Fig. 7B, $n = 6$).

4. Discussion

Initially, it was thought that the main physiological role of RAS components was to control blood pressure through the regulation of vascular tonus and electrolytic balance (Basso and Terragno, 2001). However, the observation that RAS components are expressed and regulated in specific tissues and cells, in particular, in different components of the immune system, demonstrates how complex and challenging it is to understand the physiology of RAS components. Different studies show the pivotal role of Ang II in augmenting ongoing immune responses (Basso and Terragno, 2001; Platten et al., 2009; Sánchez-Lemus et al., 2009; Toko et al., 2004; Weninger and von Andrian, 2003; Yanagitani et al., 1999). However, the mechanisms by which Ang II mediates the regulation of T cell functions have not been fully elucidated. In the present work, we found that endogenous Ang II produced by anti-CD3-activated $CD4^+$ and $CD8^+$ T cells stimulated important functions during proinflammatory events, such as activation, adhesion and transmigration of these cells through endothelial basal membrane proteins. The effects produced by Ang II are specific and involve losartan-sensitive AT_1 receptor activation. These results open new avenues to the elucidation of the molecular mechanisms involved in the pathogenesis of diseases and infections that involve exacerbated T cells responses.

Mechanisms of local tissue generation of Ang II have been proposed (Miyazaki and Takai, 2001; Nataraj et al., 1999; Paul et al., 2006). The existence of a functional RAS in lymphoid tissue able to synthesize Ang II de novo, which triggers physiological functions through the activation of specific receptors AT_1 or AT_2 is well established (Geara et al., 2009; Jurewicz et al., 2007; Paul et al., 2006). Hoch et al. (2009) observed that splenic T cells characteris-

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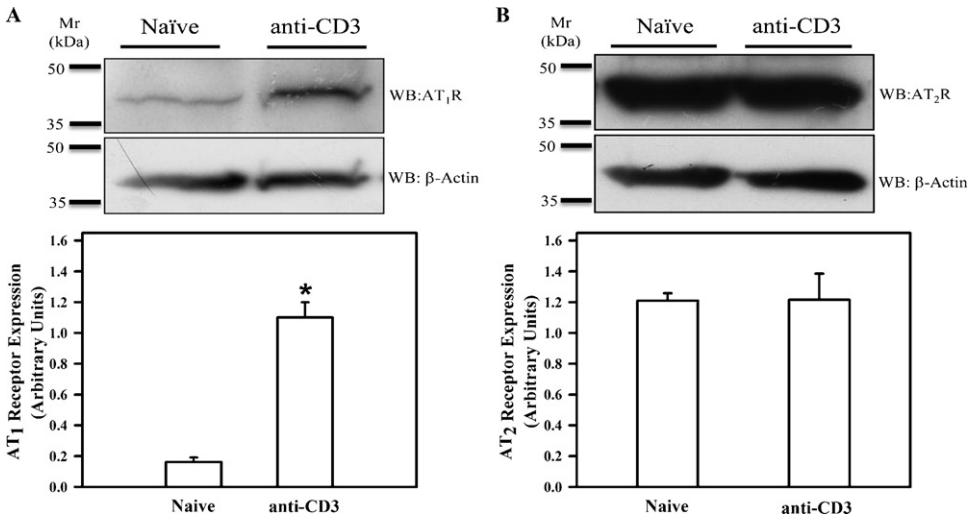

Fig. 7. T cell activation induces upregulation of AT_1 but not AT_2 angiotensin receptor expression. Membrane preparations of naive (lane 1) or anti-CD3-activated T cells (lane 2) underwent immunoblotting for detection of (A) AT_1 or (B) AT_2 angiotensin receptors. AT_1R or AT_2R expression was normalized by β-actin expression. The results are expressed as means ± SE ($n = 6$). *Statistically significant compared with naive T cells ($p < 0.05$).

tically express lower levels of renin, angiotensinogen, ACE and Ang II, compared with circulating T cells and that the addition of Ang II in the concentration range of 10–200 nM did not change splenic $CD4^+$ T cell activation. Similarly, in the present work, it was observed that exogenous Ang II did not change splenic-derived $CD4^+$ and $CD8^+$ T cell activation. However, blockers of RAS such as losartan or captopril significantly inhibited it. Thus, our results indicate that endogenous Ang II of splenic-derived T cells could have a significant effect. This hypothesis is strengthened by the observation that in the presence of captopril, when endogenous production is dropped, the addition of exogenous Ang II increases splenic-derived $CD4^+$ and $CD8^+$ T cell activation.

A previous report showed that Ang II is able to upregulate different components of the RAS and increases mRNA expression levels of AT_1 receptor in lipopolysaccharide-stimulated human T lymphocytes (Coppo et al., 2008). One question remains to be answered: How can Ang II trigger some effect if the levels of RAS components are reduced? We observed that the AT_1 receptor level increased significantly when the splenic-derived T cells were activated but the AT_2 receptor level was not changed. These results indicate that in vitro activated T cells likely express more AT_1 receptor as a result of TCR signaling or as a result of an autocrine loop of AT_1 activation by endogenous Ang II produced by activated T lymphocytes. In addition, a direct correlation between AT_1 receptor and splenic-derived $CD4^+$ and $CD8^+$ T cell activation was established by the inhibition of T cell activation by losartan. Using the same pharmacological approach, other groups have described the influence of Ang II in the proliferation of mouse spleen lymphocytes in vitro, however, it seems that both angiotensin receptors are involved in this response, not only AT_1 as we described in our model of T cell activation (Kunert-Radek et al., 1994; Jurewicz et al., 2007).

Ang II is shown to be involved in the onset and progression of inflammation, by inducing cell recruitment and adhesion (Ruiz-Ortega et al., 2001; Suzuki et al., 2000). In patients with coronary artery disease the upregulation of L-selectin expression is inhibited by AT_1 antagonists (Prasad et al., 2001). In addition, studies with intravital microscopy of the rat mesenteric postcapillary venules showed that infusion of Ang II promotes the adhesion of monocytes and neutrophils to endothelial cells, without any vasoconstrictor activity (Piqueras et al., 2000). This response in inducing L-selectin expression was mediated by both AT_1 and AT_2 angiotensin receptors. Although the role of Ang II in adhesion properties of different leukocytes subsets has been characterized, the mechanism of Ang II-induced T cell adhesion is not fully understood. In this work, we have shown that Ang II enhanced the adhesion properties of spleen-derived $CD4^+$ and $CD8^+$ T cells activated in vitro into fibronectin or laminin, two different endothelial basal membrane proteins. In this case, the adhesion induced by Ang II does not seem to involve upregulation of VLA-4 or VLA-5 expression. One possible hypothesis is that Ang II could activate these integrins, making the cells more able to adhere. Crowley et al. (2008) have shown that AT_1 receptor activation by Ang II on splenic T lymphocytes promotes cytoskeletal rearrangements through a pathway that requires Rho kinase activation. Although the mechanisms described by Crowley's group were shown in the context of T cell activation and proliferation, a similar mechanism could be involved in T cell adhesion and transmigration induced by Ang II, because cytoskeletal rearrangement is downstream integrin signaling. However, additional experiments are necessary to test this hypothesis.

T cells were also able to transmigrate through laminin and fibronectin monolayers, in response to Ang II stimuli. The role of Ang II as a T cell chemoattractant molecule was previously suggested (Jurewicz et al., 2007; Weinstock and Kassab, 1986). In addition to the direct effect of Ang II, there are reports indicating a key role of second mediators, possibly chemokines, induced by Ang II through the activation of both AT_1 and AT_2 receptors (Jurewicz et al., 2007; Kato et al., 1999; Marchesi et al., 2008; Morrissey and Klahr, 1998; Wolf et al., 1997). In the experimental conditions used, we showed a direct effect of endogenous Ang II in T cells that is independent of any other cell type producing specific T cell chemokine. However, we cannot infer whether this direct effect of Ang II involves the modulation of the expression of other cytokines, chemokines and the respective receptors in the T cells, making them more potent to migrate in response to firm contact with the endothelial basal membrane proteins.

There are conflicting data concerning the receptors involved in the transduction of Ang II signaling to induce adhesion/transmigration of T cells (Jurewicz et al., 2007; Morrissey and Klahr, 1998; Wolf et al., 1997). In addition to the notion that both AT_1 and AT_2 receptors could mediate both phenomena, a recent report demonstrating the role of Ang II in the chemotaxis of T cells, using T lymphocytes isolated from peripheral blood of health individuals, showed that Ang II-induced chemotaxis was not abolished by AT_1 or AT_2 antagonists, alone or in combination, suggesting the involvement of a different angiotensin receptor (Jurewicz et al., 2007). Our observation that the AT_2 receptor antagonist, PD123319,

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was not able to interfere in any property evaluated here together with the sensitivity to losartan treatment allows us to suggest that the effects induced by Ang II observed in the present work were mainly mediated by AT_1 receptor. The differences observed in both studies could due to the fact that they used blood-derived T cells isolated from healthy individuals and we used splenic T cells obtained from mice.

5. Conclusions

In conclusion, we have demonstrated that endogenous Ang II could be a key element in the immunomodulation of T cell responses, such as activation and posterior adhesion/transmigration activity. The elucidation of such mechanisms could help to understand the Ang II-induced inflammation process. Therefore, our results provide a rational basis for the use of AngII/AT_1 signaling antagonists in therapeutic strategies to improve treatment outcomes of immune-based diseases.

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