0022-3565/10/3343-854–862$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
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JPET 334:854–862, 2010

Vol. 334, No. 3
161760/3612531
*Printed in U.S.A.*



# Impaired Function of Dendritic Cells Deficient in Angiotensin II Type 1 Receptors

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Received September 17, 2009; accepted May 28, 2010

## ABSTRACT

Dendritic cells (DC) are highly specialized antigen-presenting cells with a unique ability to activate resting T lymphocytes and initiate primary immune responses. Angiotensin II (AII) is involved in key events of the inflammatory response. Because our previous work implicated an effect of AII on differentiation and function of murine and human DC, we investigated the impact of AII type 1 receptor ($AT_1$) deficiency on the phenotypical and functional properties of mouse DC in vitro and in vivo. Bone marrow (BM) cells isolated from mice lacking AII subtype 1a receptor ($AT_{1a}$), AII subtype 1b receptor ($AT_{1b}$), or both receptor isoforms and control littermates [wild type (WT)] were cultured for 7 days in the presence of recombinant mouse granulocyte/macrophage colony-stimulating factor to generate myeloid DC in vitro. Generation of $CD11c^+$ cells was less efficient in both $AT_{1a}$- and $AT_{1b}$-deficient BM cells than in WT BM cell cultures. Moreover, DC generated from $AT_1$-deficient progenitors showed lower levels of expression of major histocompatibility complex II (MHC-II) and CD11c ($p < 0.01$) and a marked reduction in their allostimulatory activity ($p < 0.01$ or 0.001). Although $AT_1$-deficient DC released comparable levels of interleukin (IL)-10 and IL-12p70 to WT DC, they produced significantly lower levels of tumor necrosis factor $\alpha$ (TNF-$\alpha$) ($p < 0.05$). Remarkably, $CD11c^+$ cells isolated from the spleen of $AT_1$ knockout mice challenged with lipopolysaccharide in vivo up-regulated MHC-II, CD40, and CD80 as did WT, but released significantly lower levels of TNF-$\alpha$ ($p < 0.01$). These data provide clear evidence that $AT_1$ controls differentiation and functionality of DC and thus may have a crucial impact on inflammatory processes where local angiotensinergic systems are known to be activated.

## Introduction

Angiotensin II (AII), the major bioactive peptide of the renin-angiotensin system (RAS), plays a critical role in controlling cardiovascular and renal homeostasis. AII acts through two main cell surface receptors, $AT_1$ and $AT_2$. Most of the recognized functions of AII involve $AT_1$, which represents a critical pharmacological target in the treatment of cardiovascular disorders, such as hypertension, heart failure, and diabetic nephropathy. Humans possess a single $AT_1$ (de Gasparo et al., 2000), whereas there are two $AT_1$ isoforms described in rodents, $AT_{1a}$ and $AT_{1b}$, which are products of separate genes (*Agtr1a* and *Agtr1b*, respectively) (Ito et al., 1995). These two isoforms share substantial DNA sequence homology, but are pharmacologically indistinguishable and differ in distribution and regulation among tissues and cell types. A growing body of evidence supports the notion that, in addition to its role in homeostasis of cardiovascular and renal systems, AII activates different inflammatory pathways. It increases vascular permeability, enhances the recruitment of leukocytes, and stimulates the production of inflammatory molecules such as reactive oxygen intermediates, adhesion molecules, growth factors, chemokines, and inflammatory cytokines (Fernandez-Castelo et al., 1987; Wolf et al., 1997; Sadoshima, 2000; Phillips and Kagiyama, 2002). In this way,

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.109.161760.

**ABBREVIATIONS:** DC, dendritic cells; AII, angiotensin II; $AT_1$, AII type 1 receptor; $AT_{1a}$, AII subtype 1a receptor; $AT_{1b}$, AII subtype 1b receptor; $AT_2$, AII type 2 receptor; KO, knockout; DKO, double KO; BM, bone marrow; LPS, lipopolysaccharide; RAS, renin-angiotensin system; rmGM-CSF, recombinant mouse granulocyte/macrophage colony-stimulating factor; WT, wild type; TNF-$\alpha$, tumor necrosis factor $\alpha$; IL, interleukin; MHC-II, major histocompatibility complex II; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; DXT, dextran; FCS, fetal calf serum; CFSE, carboxyl fluorescein succinimidyl ester; PE, phycoerythrin.

acting mainly through the $AT_1$, AII seems to contribute to the development of a great number of inflammatory diseases, such as cardiovascular diseases, immune-mediated glomerulonephritis, allograft rejection, and arthritis. Thus, independent of their hemodynamic benefits, the effectiveness of $AT_1$ blockers has been attributed in part to inhibition of the proinflammatory actions of AII (Hisada et al., 1999; Nataraj et al., 1999; Dragun et al., 2005).

Dendritic cells (DC) are highly specialized antigen-presenting cells with a unique ability to activate resting T cells directing the differentiation of T $CD4^+$ cells into different profiles (Banchereau and Steinman, 1998). DC progenitors from the BM enter the bloodstream and sparsely distribute in all tissues, where they differentiate into immature DC characterized by a high ability to capture and process antigens. Inflammatory cytokines and microbial products promote the maturation of DC, characterized by a high ability to present antigens to lymphocytes, triggering the adaptive immune response. Thus, they represent a crucial link between innate and adaptive immunity (Banchereau et al., 2000). We have reported that the blockade of $AT_1$ by specific antagonists such as losartan or candesartan impaired differentiation and function of DC, supporting the existence of an autocrine/paracrine loop through which AII supports the normal development of DC. In the present study we investigated the impact of AII type 1 receptor deficiency on the differentiation and function of DC in vitro and in vivo. We report that the absence of $AT_{1a}$ and/or $AT_{1b}$ subtypes results in the generation of DC with major alterations in their phenotypical and functional properties.

## Materials and Methods

**Materials.** RPMI medium 1640 with L-glutamine and fetal calf serum (FCS) was purchased from Invitrogen (Grand Island, NY). Recombinant mouse GM-CSF (rmGM-CSF) was purchased from R&D Systems (Minneapolis, MN). Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4, β-mercaptoethanol, BSA-FITC, and dextran-FITC (DXT-FITC) were purchased from Sigma-Aldrich (St Louis, MO). Carboxyl fluorescein succinimidyl ester (CFSE) was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK), and [$^3$H]thymidine was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Mouse anti-CD4 microbeads, mouse anti-CD11c microbeads (clone N418), Macs separation columns, and preseparation filters were from Miltenyi Biotec (Bergisch Gladbach, Germany). Collagenase D was from Roche Diagnostics (Mannheim, Germany). The following antibodies were purchased from BD Pharmingen (San Diego, CA): anti-CD 16/32 purified (2.4G2 clone), anti-CD4-FITC (L3T4) (RM4-5 clone), anti-Gr-1-PE (Ly-6G) (1A8 clone), anti-CD40-FITC (clone 3/23), anti-CD80-PE, anti-IA/IE-PE (clone M5/114.15.2), anti-$Ia^b$–FITC (AF6-120.1 C57BL/10J), and anti-CD11c-PE (clone HL-3).

**Animals and Experimental Design.** Mice lacking $AT_{1a}$ or $AT_{1b}$ for AII were generated by using homologous recombination in embryonic stem cells as described previously. $AT_{1a}$ (−/−//+/+), and $AT_{1b}$ (+/+//−/−) single-knockout (KO) mice were used to generate the $AT_{1a}/AT_{1b}$ double-knockout mice (DKO) (−/−//−/−) (Ito el al., 1995; Oliverio et al., 1998). To generate homozygous wild-type (WT) (+/+//+/+) and DKO animals, heterozygous offspring was intercrossed and bred on a 129 × C57BL/6 background at the animal facilities of the Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany, whereby experimental homozygous animals were generated by cross-breeding of heterozygous mice to ensure the most similar genetic background. Sets of males 3 to 6 months old were used. Experiments were performed in accordance with the *American Physiological Society Guiding Principles for the Care and Use of Animals in Research* and the guidelines of German federal law on the use of experimental animals (Animal Welfare Act) and were approved by the local authorities.

For the in vivo challenge with LPS, groups of four mice were injected intraperitoneally with either a sublethal dose of LPS (10 mg/kg) from *Escherichia coli* (serotype 0111:B4) or solubilized in 0.9% NaCl or 0.9% NaCl alone (control group). The dose of LPS was sufficient to elicit a significant response without the risk of endotoxic shock according to the literature (De Smedt et al., 1996; McCartney-Francis et al., 2004). Eighteen hours after injection the animals were killed by cervical dislocation and the spleens were removed.

**Polymerase Chain Reaction for Genotyping.** Homozygosity in the three knockout genotypes used ($AT_{1a}$KO, $AT_{1b}$KO, and DKO) was proved by polymerase chain reaction after weaning and again after experiments were performed using specific primer pairs for each gene locus. Specific pairs of primers to detect $AT_{1a}$ have been described (Ito et al., 1995). For $AT_{1b}$-specific polymerase chain reaction, two pairs of primers that amplified products for the $AT_{1b}$ WT (P1, 5′-CCA GGG CAA GAT TCA GAA GG-3′ and P2, 5′-CCA ACA AAG AGA CAT GAT C-3′) or knockout (P3, 5′-CCT GCG TGC AAT-3′ and P4, 5′-CCA TCT TGT TCA ATG-3′) allele were designed and used as described previously (Gembardt et al., 2008).

**Generation of Mouse DC.** Mouse DC were generated as described previously with minor modifications (Lutz et al., 1999). In brief, after all muscle tissues were removed from the femurs, the bones were washed twice with PBS and transferred into a fresh dish with RPMI medium 1640. Both ends of the bones were cut with scissors in the dish, and then the marrow was flushed out by using 2 ml of RPMI medium 1640 with a syringe and 25-gauge needle. The tissue was suspended and passed through a 100-μm cell strainer (BD Biosciences, Franklin Lakes, NJ) to remove small pieces of bone and debris. Red cells were lysed with 0.45 M ammonium chloride, washed, and suspended at a concentration of $1 \times 10^6$ cells/ml in RPMI medium 1640 containing 10% heat-inactivated FBS, 100 IU/ml penicillin G, 100 g/ml streptomycin, 2 mM glutamine, and $5.5 \times 10^{-5}$ M β-mercaptoethanol (Sigma-Aldrich), supplemented with 200 IU/ml rmGM-CSF (R&D Systems) and cultured for 7 days in 100-mm Petri dishes ($5 \times 10^6$ cells/dish). Approximately 70% of the harvested cells expressed MHC class II and CD11c. DC were identified by flow cytometry as being positive for CD11c. At day 6, immature DC were further stimulated for 24 h with 100 ng/ml LPS to obtain mature DC.

**Western Blot Analysis.** DC lysates were prepared with ice-cold lysis buffer, subjected to SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membranes, which were blocked with 5% (w/v) skimmed milk in PBS, pH 7.4 and 0.05% (v/v) Tween 20 for 1 h at room temperature. Membranes were then incubated with rabbit polyclonal anti-$AT_1$ antibody (N-10) (1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4°C. After incubation with peroxidase-linked secondary antibody (1:500) for 1 h at room temperature, immunoreactive proteins were visualized by ECL reagent and autoradiography film (Amersham Hyperfilm ECL; GE Healthcare). The same blots were stripped and reprobed with anti-β-actin antibody (1:1000) (Cell Signaling Technology, Danvers, MA). Quantification of the relative amount of protein was performed with ImageQuant Software (GE Healthcare) and normalized for the amount of β-actin in each experiment.

**Purification of Splenic CD11c-Positive Cells.** Spleens were removed and digested with collagenase D (2.5 mg/ml; Roche Diagnostics) for 20 min at 37°C, then treated for 5 min with EDTA to disrupt T cell–DC complexes. The spleens were then suspended in RPMI medium 1640 with 10% FCS and softly passed through a 70-μm sterile cell strainer (BD Biosciences) to make a single cell suspension. Cells were washed twice with complete medium and stained directly with antibodies or further purified to isolate the $CD11c^+$ splenocytes. Cells were sorted for positive selection with Mini-MACS by using the mouse CD11c cell isolation kit (clone N418)

(Miltenyi Biotec). In brief, splenocytes were incubated with biotinylated N418 antibody and afterward with streptavidin microbeads. Cells were then positively enriched on a Mini-MACS column. Cells were centrifuged and washed twice with PBS containing 0.5% BSA and then suspended in 1% BSA/PBS. Cells were stained for 20 min at 4°C with the corresponding antibodies. More than 90% of these cells expressed CD11c.

**Flow Cytometry.** Cells were harvested by pipetting with cold PBS. The cells were first blocked with anti-CD16/32 antibodies and then stained in phosphate-buffered saline (Invitrogen) at 4°C for 30 min with FITC- or PE-conjugated monoclonal antibodies [antigen-specific or control immunoglobulin G (IgG)]. Analyses of stained cells were performed on a FACS scan or FACSCalibur by using CellQuest software (BD Biosciences).

**Allogeneic T Cell Proliferation.** Spleens were removed from BALB/c mice, and the tissue was disrupted to release a single cell suspension, which was then passed through a 70-μm sterile cell strainer. $CD4^+$ cells were sorted by positive selection with Mini-MACS using a mouse $CD4^+$ cell isolation kit (Miltenyi Biotec). Cells were centrifuged and washed twice with PBS containing 0.5% BSA, and then resuspended in culture medium. DC were washed with complete medium and irradiated (3000 rad from a 137-Cs source), and $2.5 \times 10^4$ cells were cocultured with $2.5 \times 10^5$ allogeneic splenic $CD4^+$cells (BALB/c) for 96 h in U-bottom 96-well plates (Corning Life Sciences, Lowell, MA). Cells were pulsed with 1 μCi/well of [$^3$H]thymidine (specific activity, 5 Ci/mM; DuPont, Wilmington, DE) for 18 h before harvesting on day 4 to measure proliferation. Plates were harvested with a Filter Mate (Canberra Industries, Meriden, CT), and incorporation of [$^3$H]thymidine was determined by liquid scintillation spectroscopy using a TopCount device (Canberra Packard). Proliferation of allogeneic $CD4^+$ splenocytes in response to the coculture with DC was also measured by CFSE dilution analysis by flow cytometry. Proliferation of splenocytes was calculated as the percentage of dividing cells.

**Endocytosis Assay of BSA-FITC.** DC were incubated at 37°C in complete medium in the presence of BSA-FITC or DXT FITC (100 μg/ml) for 60 min. To determine unspecific binding a control at 4°C was incubated in parallel. Cells were washed three times with ice-cold 1% FCS/PBS and fixed with 1% paraformaldehyde. The BSA-FITC and DXT-FITC uptake was then evaluated by flow cytometry as described previously (Sallusto and Lanzavecchia, 1994).

**Cytokine Assay.** TNF-α, IL-10, and IL-12p70 levels were measured in the culture supernatant by using a commercially available Quantikine ELISA Kit (R&D Systems) according to the manufacturer's instructions.

**Statistical Analysis.** Results are expressed as the mean ± S.E.M. Data were analyzed by using single-factor analysis of variance (Prism 4.01; GraphPad Software Inc., San Diego, CA). If statistically significant effects were found, data were further analyzed by Dunnett's test. Statistical significance was assumed for $p < 0.05$.

## Results

**$AT_1$-Deficient BM Cells Give Rise to a Diminished Percentage of DC.** BM cells were obtained from mice lacking $AT_{1a}$, $AT_{1b}$, or both isoforms ($AT_{1a}$KO, $AT_{1b}$KO, and DKO, respectively) and control littermates (WT). We analyzed the percentage of positive cells for the lineage markers CD11b, CD3e, B220, GR-1, and TER-119 in the BM preparations and did not find any difference among the four genotypes (data not shown). After 7 days of culture in the presence of rmGM-CSF, the total number of cells recovered and the percentage of adherent cells were comparable among the genotypes (24 ± 3.0, 22 ± 4.3, 27 ± 3.7, and 26 ± 4.3% for WT, $AT_{1a}$, $AT_{1b}$, and DKO, respectively). DC were recognized for being positive for CD11c expression. Notably $AT_{1a}$KO, $AT_{1b}$KO, and DKO BM cells generated lower percentages of CD11c-positive cells compared with WT BM cells ($p < 0.01$; Fig. 1, A and B).

**DC from $AT_1$-Deficient BM Cells Express Low Levels of MHC-II and CD11c.** It is noteworthy that not only the percentage of $CD11c^+$ cells was lower in cultures of $AT_1$-deficient BM cells, but these $AT_{1a}$ KO- and DKO-deficient $CD11c^+$ cells were also characterized by a lower mean fluorescence intensity of this marker ($p < 0.01$; Fig. 1C), suggesting that differentiation of DC from $AT_1$-deficient progenitors is compromised under in vitro conditions. Supporting this assumption, the expression of MHC-II (IA/IE) molecules was also found to be lower in DC generated from $AT_{1a}$KO, $AT_{1b}$KO, and DKO BM cells as shown in representative histograms in Fig. 1D; CD40 and CD80 expression was comparable in the four genotypes analyzed.

**Immature DC Generated In Vitro from $AT_1$-Deficient Precursors Are Poor Stimulators of Allogeneic T Cells.** Immature DC are characterized for their high endocytic capacity. To evaluate the functional profile of $AT_1$-deficient DC we first examined their ability to endocyte soluble markers such as DXT-FITC and BSA-FITC. We found no significant differences in the four genotypes analyzed (Fig. 2, A and B). Next, the antigen-presenting function of DC was evaluated by studying their ability to stimulate allogeneic T cell proliferation. We found that $AT_{1a}$-, $AT_{1b}$-, and double-deficient DC showed a markedly reduced allostimulatory capacity compared with wild-type DC ($p < 0.01$ or 0.001; Fig. 2C). Similar results were observed when the allostimulatory activity of DC was analyzed by flow cytometry using allogeneic splenocytes stained with CFSE (Fig. 2, D and E), indicating that the absence of $AT_1$s in DC progenitors results in major defects in the function of immature DC.

**LPS Induces Up-Regulation of $AT_1$ Expression in WT DC.** Upon encountering inflammatory or microbial stimuli in peripheral tissues, DC become activated and undergo a number of physiological changes leading to their maturation. These changes enable DC to activate naive T cells and direct the differentiation of $CD4^+$ T cells into different functional profiles. As expected, LPS induced up-regulation of MHC-II (IA/IE), CD80, and CD40 in WT BM-derived DC (Fig. 3A). It is noteworthy that this maturation of DC was associated with a marked increase in the expression of $AT_1$ ($p < 0.05$; Fig. 3, B and C).

**Mature DC Generated In Vitro from $AT_1$-Deficient Precursors Remain Poor Stimulators of Allogeneic T Cells.** We then examined the role of $AT_1$ in the phenotypical and functional maturation of DC triggered by LPS. Immature DC were stimulated for 24 h with 100 ng/ml LPS, and their phenotype was evaluated by flow cytometry. $AT_{1a}$-, $AT_{1b}$-, and double-deficient DC expressed significantly lower levels of IA/IE than WT immature DC ($p < 0.01$; Figs. 1D and 4A), whereas gene deficiencies had no effect on CD80 and CD40 expression (Fig. 4, B and C). LPS stimulation induced up-regulation of MHC-II (IA/IE), CD40, and CD80 in all genotypes. However, significant differences in the up-regulation were observed only for IA/IE, whereas DC from $AT_{1b}$KO and DKO mice have been less responsive (Fig. 4, A–C).

We then studied the functional profile of mature $AT_1$-deficient DC by analyzing their allostimulatory capacity. The proliferation rate of allogeneic T $CD4^+$ cells was evaluated by using a [$^3$H]thymidine incorporation assay (Fig. 4D). We

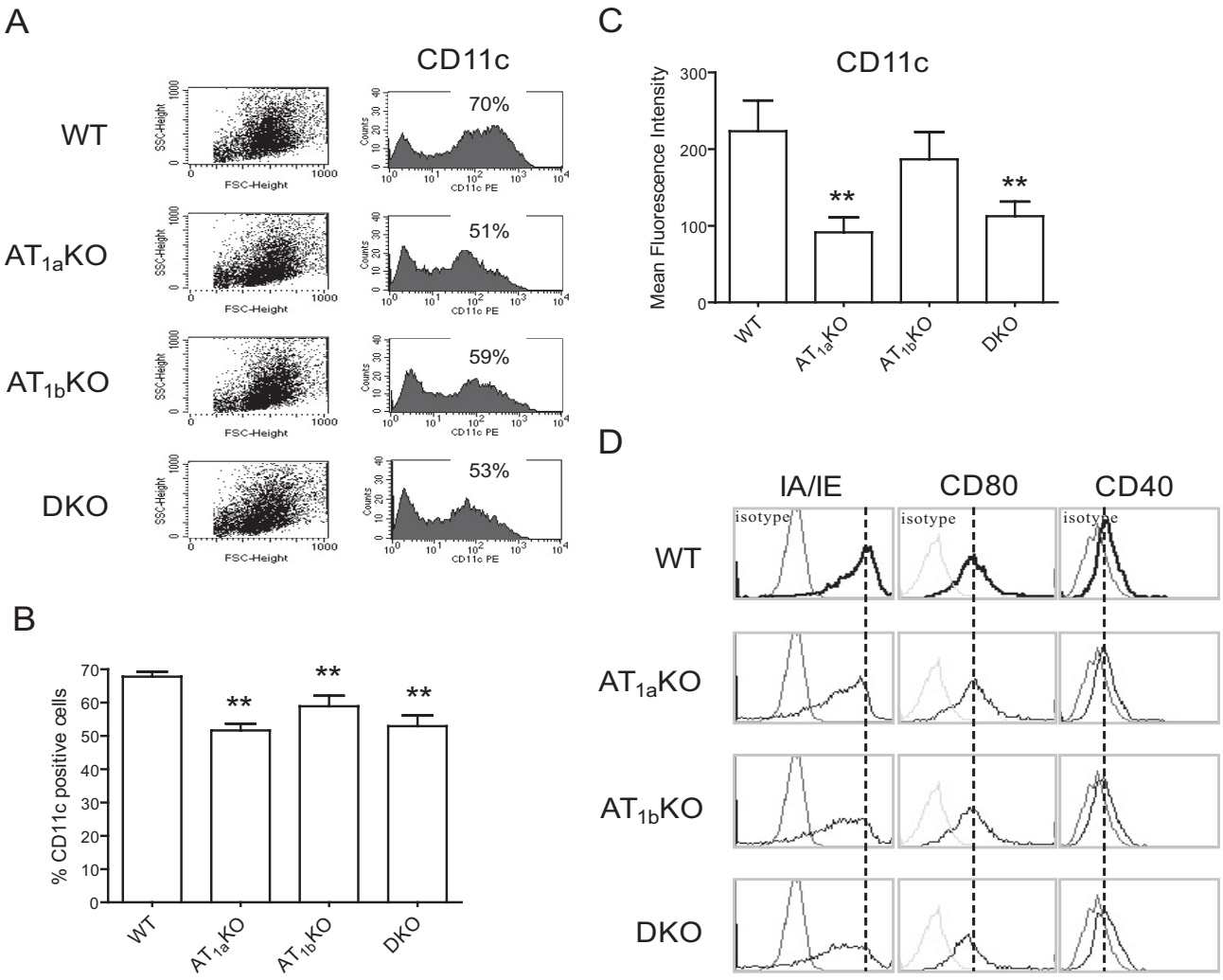


**Fig. 1.** $AT_1$-deficient BM cells give rise to a diminished percentage of $CD11c^+$ cells (DC) that express lower levels of MHC-II and CD11c. BM cells were cultured in the presence of rmGM-CSF for 7 days. Cells were harvested and analyzed by flow cytometry. A, representative dot plots (left) and histograms for CD11c expression of the cells obtained from each of the four genotypes analyzed (WT, $AT_{1a}$KO, $AT_{1b}$KO, and DKO) (right). The percentage of cells expressing CD11c is indicated within each histogram. B, percentages of $CD11c^+$ cells in cultures of BM of the four different genotypes. C, CD11c expression levels are presented as mean fluorescence intensity for the four genotypes. D, CD11c-positive cells were analyzed for the expression of IA/IE (left), CD80 (center), and CD40 (right) as shown in representative histograms. **, $p < 0.01$ versus WT. $n = 6$ mice per group.

observed that LPS-stimulated DC generated from $AT_1$-deficient progenitors show a remarkably lower capacity to stimulate the proliferation of allogeneic T $CD4^+$ lymphocytes compared with WT DC ($p < 0.01$), independent of whether they have been deficient in $AT_{1a}$, $AT_{1b}$, or double-knockout DC.

**Effects of $AT_1$ Deficiency on DC's Ability to Release IL-10, IL-12p70, and TNF-α.** An important property of DC is their capability to produce a great variety of cytokines. While IL-12 p70 is considered the key factor in the polarization of the adaptive immune response toward a T helper 1 profile, the production of IL-10 is usually associated with a tolerogenic profile, and TNF- α is a key inflammatory cytokine. We studied the pattern of secretion of IL-10, IL-12p70, and TNF-α in culture supernatants of immature and mature DC. $AT_1$-deficient DC released IL-10 and IL-12p70 in a similar fashion to wild-type DC (Fig. 5, A and B). In contrast, immature DC generated from $AT_1$-deficient BM cells produced significantly lower levels of the proinflammatory cytokine TNF-α ($p < 0.05$ or 0.01). However, under these in vitro conditions the significant reduction in TNF-α release vanished after LPS stimulation (Fig. 5C).

**Spleen CD11c-Positive Cells Deficient in $AT_1$ Release Lower Levels of TNF-α after In Vivo Challenge with LPS.** We first studied the phenotype of spleen DC isolated from $AT_1$-deficient mice. We found no differences in the percentages of $IA/IE^{high}$ and $CD11c^{high}$ double-positive cells or in the levels of expression of IA/IE and CD11c in the $IA/IE^{high}$ and $CD11c^{high}$ constellation of double-positive spleen cells in the four genotypes analyzed (Fig. 6, A and B). Furthermore, purified $CD11c^+$ spleen cells from $AT_1$-deficient mice showed similar expression of IA/IE (WT: 1370 ± 25; $AT_{1a}$ KO: 1125 ± 109; $AT_{1b}$ KO: 1341 ± 71; DKO: 1349 ± 120), CD40 (WT: 72 ± 6; $AT_{1a}$ KO: 86 ± 11; $AT_{1b}$ KO: 71 ± 5; DKO: 61 ± 4), and CD80 (WT: 79 ± 11; $AT_{1a}$ KO: 84 ± 8; $AT_{1b}$ KO: 70 ± 10; DKO: 79 ± 10) (all numbers are mean fluorescence intensity; $n \geq 5$) compared with the WT mice.

It is well known that LPS administration induces a systemic inflammatory response, which involves the massive production of inflammatory cytokines by splenic macrophages and DC. Our aim was to determine whether $AT_1$ activation is necessary for the complete acquisition of a mature phenotype in spleen DC after administration of systemic

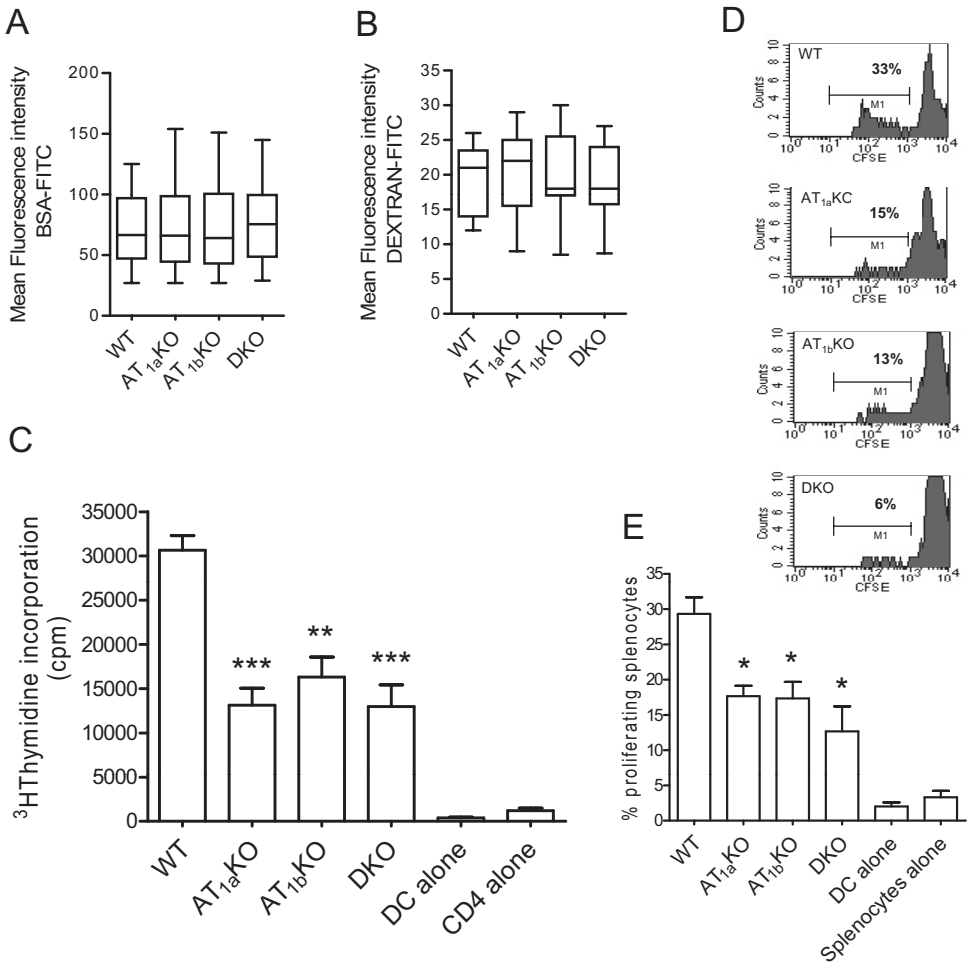


**Fig. 2.** DC generated in vitro from $AT_1$-deficient precursors are poor stimulators of naive allogeneic T cells. A and B, endocytosic activity of DC was analyzed by flow cytometry with BSA-FITC (A) or DXT-FITC (B). Results are expressed as the mean fluorescence intensity ($n = 6$). C, proliferation of $CD4^+$ cells (BALB/c) was measured by [$^3$H]thymidine incorporation in a mixed leukocyte reaction, and results are presented as cpm ($n = 6$ animals per group). D and E, the percentage of proliferating CFSE-labeled splenocytes from BALB/c mice was determined by FACS analysis and is indicated within each representative histogram (D) and in a bar graph (E) ($n = 3$ animals per group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus WT.

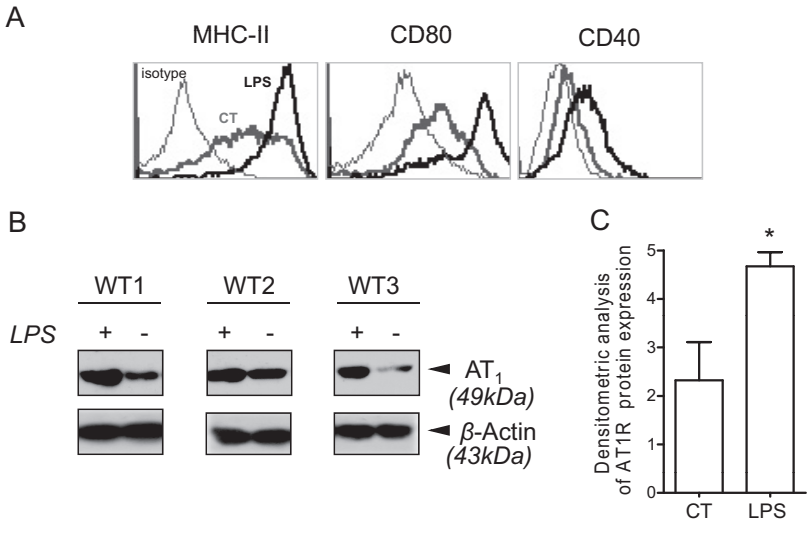


**Fig. 3.** Activation of DC with LPS up-regulates the expression of $AT_1$. A, representative histograms for IA/IE, CD40, and CD80 expression in WT BM-derived DC either stimulated with LPS for 24 h or not. B, protein expression of $AT_1$ in WT BM-derived DC either stimulated with LPS for 24 h or not. Top, $AT_1$ expression was analyzed by Western blot. Bottom, β-actin was used as a housekeeping protein. C, densitometric analysis to quantify $AT_1$ expression in BM-derived DC, expressed in arbitrary units. *, $p < 0.05$. $n = 3$ mice per group.

LPS. WT, $AT_{1a}$KO, $AT_{1b}$KO, and DKO mice were challenged either with LPS or saline solution intraperitoneally. After 18 h, mice were sacrificed and spleens were removed to prepare a single cell suspension. Spleen cells were then analyzed for IA/IE and CD11c expression by FACS scan. $CD11c^{high}$ spleen cells from DKO mice showed up-regulation of IA/IE in response to LPS challenge under these in vivo conditions as did WT. We then analyzed by FACS scan the phenotype of the $CD11c^+$ spleen cells isolated from these mice. We found that WT and DKO $CD11c^+$ spleen cells showed a comparable up-regulation of IA/IE, CD80, and CD40 in response to the systemic administration of LPS.

To further evaluate the $CD11c^+$ spleen cells' response to systemic administration of LPS we studied the secretion of the proinflammatory cytokine TNF-α, a central mediator of

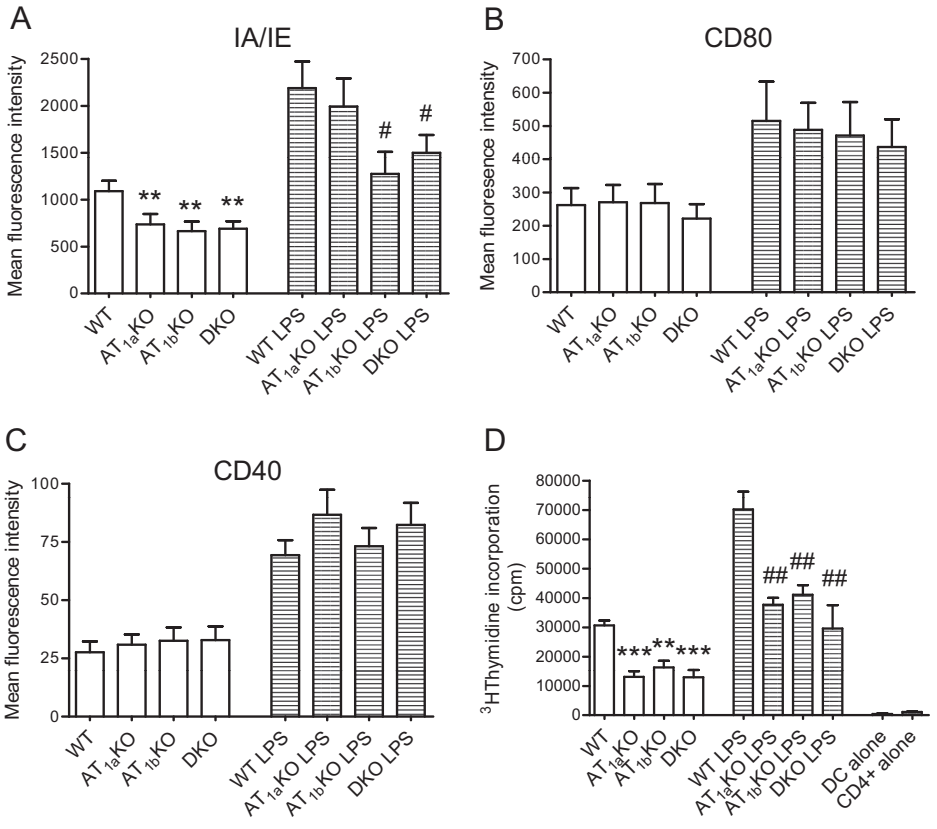


**Fig. 4.** DC generated in vitro from $AT_1$-deficient precursors remain poor stimulators of allogeneic T lymphocyte proliferation after LPS stimulation. A–C, BM-derived DC were cultured in the presence or absence of LPS for 24 h. Their phenotype was analyzed by flow cytometry, gating the CD11c-positive cells. Results are expressed as the mean fluorescence intensity for IA/IE (A), CD80 (B), and CD40 (C). D, DC were either stimulated with LPS for 24 h or not, then harvested, washed, irradiated, and cocultured with allogeneic T $CD4^+$ cells for 96 h. Proliferation of allogeneic T lymphocytes was measured by $[^3H]$thymidine incorporation. Results are expressed in cpm. **, $p < 0.01$; ***, $p < 0.001$ versus WT; ##, $p < 0.01$ versus WT LPS. $n = 6$ mice per group.

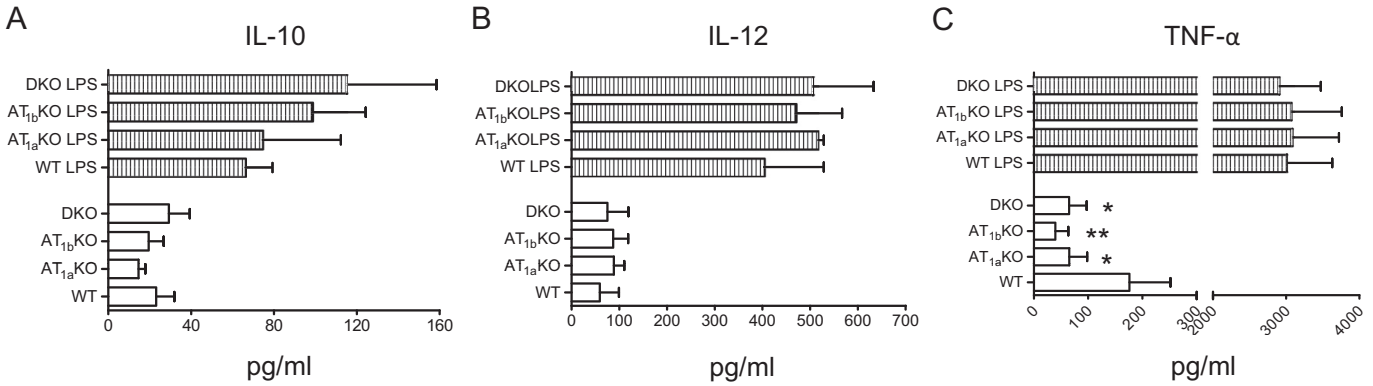


**Fig. 5.** Effects of $AT_1$ deficiency on DC's ability to release cytokines. BM-derived DC were stimulated with LPS for 24 h or not. Concentrations of IL-10 (A), IL-12p70 (B), and TNF-α (C) were measured in culture medium by ELISA. Results are expressed as pg/ml. *, $p < 0.05$; **, $p < 0.01$ versus WT. $n = 5$ mice per group.

the pathophysiological changes associated with endotoxemia. Purified $CD11c^+$ spleen cells from mice challenged with either LPS or saline were cultured for 6 h, and TNF-α production was measured in the culture medium. Remarkably, $AT_{1a}$ and $AT_{1b}$ deficiency nearly abolished the increase in TNF-α release seen in $CD11c^+$ spleen cells from wild-type mice challenged with LPS, suggesting that both isoforms play a critical role in the production of the key inflammatory cytokine TNF-α by $CD11c^+$ spleen cells in response to LPS under in vivo conditions (Fig. 6C).

## Discussion

We investigated the impact of AII type 1 receptor deficiency in the phenotypical and functional properties of mouse DC in vitro and in vivo. Our present results provide clear evidence that AII type 1 receptor is required for the normal development and function of DC. Supporting our findings, other groups suggested that a functional RAS operates in DC. It has been previously demonstrated that human monocyte-derived DC express RAS components (Lapteva et al., 2001; Jurewicz et al., 2007). Lapteva et al. (2002) identified by DNA microarrays a subset of genes that were specifically regulated in human monocyte-derived DC by AII. Nie et al. (2009) observed that AII induces activation of extracellular signal-regulated kinases 1 and 2 in murine BM-derived DC. Furthermore, several lines of evidence suggest a role for the RAS in hematopoietic cell development. It has been demonstrated that there is a local RAS in the BM, present not only in the hematopoietic but also in the stromal compartment, which is thought to affect the growth and differentiation of

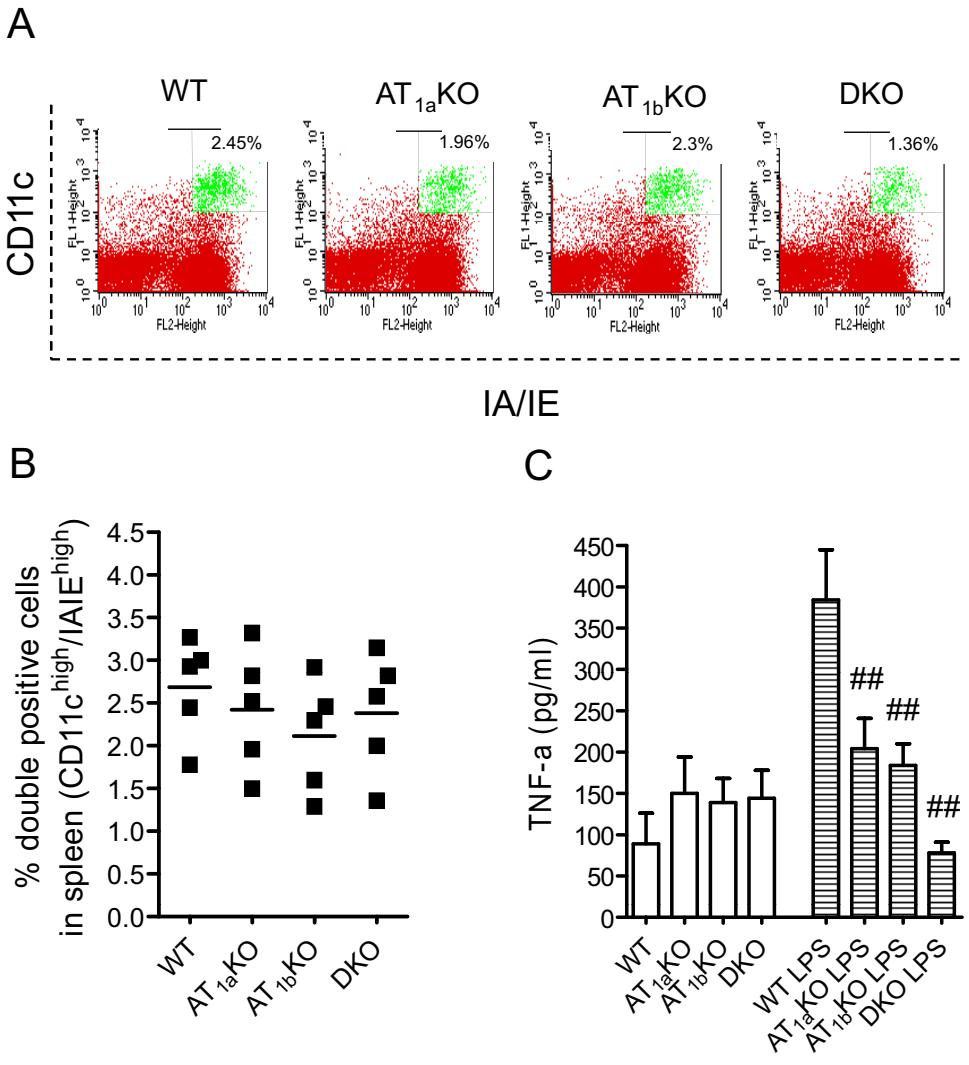


**Fig. 6.** Spleen CD11c$^+$ cells deficient in $AT_1$ release lower levels of TNF-α after in vivo challenge with LPS. The percentages of CD11c$^{high}$ and IA/IE$^{high}$ double-positive cells in the spleen of WT, $AT_{1a}$, $AT_{1b}$, and DKO mice were analyzed. A, representative dot plots for each genotype analyzed are shown. B, scatter plot showing the mean percentage of double-positive cells (CD11c$^{high}$/IAIE$^{high}$) in the spleens ($n = 5$ mice per group). C, WT and $AT_1$-deficient mice were challenged either with LPS or saline solution (sham) intraperitoneally. After 18 h, mice were sacrificed and spleens were removed. CD11c$^+$ spleen cells purified from challenged mice were cultured for 6 h. TNF-α was measured in the culture medium by ELISA. Results are expressed as mean in pg/ml. $n = 5$ mice per group. ##, $p < 0.01$ versus WT LPS.

hematopoietic colonies (Haznedaroğlu and Buyukasik, 1997; Strawn et al., 2004; Hubert et al., 2006).

We have previously examined the role of AII, $AT_1$, and $AT_2$ on the development of human and mice DC in an in vitro model using pharmacological antagonists of both receptors. We demonstrated that AII has a profound regulatory effect on the differentiation of human and mice DC, suggesting that AII supports the normal development of myeloid DC by interacting with $AT_1$, the predominant class of AII receptors (Nahmod et al., 2003). In our previous study, we found that monocytes produce AII and express $AT_1$ and $AT_2$, and blockade of each of these receptors by specific antagonists induced opposite effects during the differentiation of DC. Blockade of $AT_1$ results in the development of DC with very low levels of CD1a, HLA-DR, and CD40 expression and poor endocytic and allogeneic stimulatory activities, whereas blockade of $AT_2$ results in DC with high levels of CD1a, CD40, CD80, and HLA-DR and high endocytic and allostimulatory activities. Results obtained from mice are similar to those in humans (Nahmod et al., 2003).

Those results obtained with $AT_1$ antagonists are consistent with data shown here, because we found that DC differentiated from progenitors deficient in AII subtype 1 receptors express lower CD11c and IA/IE and have a severe defect in their allostimulatory capacity. Nevertheless, we found no differences in endocytic capacity or CD80 and CD40 expression in $AT_1$-deficient DC compared with the WT DC. Differences in the observations made with pharmacological inhibitors versus knockout mice could be explained in different ways. The use of $AT_1$ antagonist does not guarantee a complete inhibition of $AT_1$ along the process of DC differentiation. On the other hand, $AT_1$ blockers are suspected to interact with receptors other than $AT_1$, such as peroxisome proliferator-activated receptor-γ (Schupp et al., 2004; Marshall el al., 2006; Ernsberger and Koletsky, 2007), thromboxane A2/prostaglandin H2 receptors (Liu et al., 1992), and formyl-Met-Leu-Phe receptor (Raiden et al., 1997). Thus, they might not be considered as the most appropriate tool to define the contribution of $AT_1$ in DC physiology.

The model of in vitro DC differentiation with GM-CSF is one of the most common methods used to obtain murine myeloid DC. Here, we demonstrate in this in vitro model that $AT_1$-deficient BM cells give rise to CD11c$^+$ cells less efficiently than WT BM cells, suggesting that AII subtype receptor 1 is necessary for the normal development of DC. It is noteworthy that the absence of $AT_{1a}$ and/or $AT_{1b}$ results in DC expressing low levels of MHC class II molecules, whereas the absence of $AT_{1a}$, but not $AT_{1b}$, results in a marked re-

duction in the levels of expression of the DC marker CD11c. These results suggest that both receptors play a nonredundant function in the development of DC in vitro.

Although it is accepted that DC obtained through culture acquire different phenotypic and functional properties depending on the cytokine milieu used, it is believed that in vivo each tissue provides different microenvironments, which affect DC generation and maturation (Shortman and Liu, 2002). It is noteworthy that we found that $AT_1$-deficient mice showed similar percentages of $IA/IE^{high}$ and $CD11c^{high}$ double-positive cells isolated from spleens compared with the WT. These contrasting results between our in vitro and in vivo data might be related to the fact that during differentiation of DC in vivo the microenvironment is much more complex than the one generated artificially in a culture dish and might contain other factors able to promote DC development.

We also demonstrate here that AII type 1 receptors play a crucial role in DC's functionality, judging by the poor allostimulatory activity of $AT_1$-deficient DC. The absence of $AT_1$ in DC does not affect only the phenotype and function of immature but also mature DC. The main function of mature DC is to activate naive T cells. It is noteworthy that the results observed in allogeneic T cell proliferation assays show that the absence of either $AT_{1a}$ or $AT_{1b}$ isoform results in "mature" DC with a very low ability to act as antigen-presenting cells. These data clearly implicate, similar to our conclusion on CD11c expression pattern, that DC might require signals via both isoforms to be fully functional.

To our knowledge our study is the first to investigate the impact of AII type 1 receptor deficiency on the production of cytokines by DC. We investigated $AT_1$-deficient DC's ability to produce IL-10, IL-12, and TNF-α in vitro. Immature and mature DC deficient in $AT_1$ and WT-DC were able to release comparable amounts of IL-10 and IL-12p70 to the culture supernatant. This suggests that $AT_1$ deficiency is not affecting DC ability to produce cytokines involved in the differentiation of T $CD4^+$ cells into different functional profiles. In contrast, immature $AT_1$-deficient DC produced lower levels of the proinflammatory cytokine TNF-α compared with WT DC.

However, the question arose whether the in vitro finding might have an impact under in vivo conditions, too. We thus examined the role of $AT_1$ in the phenotypical and functional maturation of spleen DC triggered by LPS challenge in an in vivo model. We found that $CD11c^{high}$ splenocytes from $AT_1$-deficient mice produced significantly lower levels of the proinflammatory cytokine TNF-α, a central mediator of the pathophysiological changes associated with LPS challenge. These data clearly demonstrate that $AT_1$ signaling is required for DC full response to LPS. This conclusion is further supported by the observation of other groups using $AT_1$ antagonists or angiotensin-converting enzyme inhibitors, who postulated that AII enhances the LPS-induced production of cytokines in different cell types (Fukuzawa et al., 1997; Constantinescu et al., 1998; Miyoshi et al., 2003, 2008; Shimizu et al., 2004).

The fact that the $AT_1$ is a key player for DC functionality after LPS stimulation is also supported by our molecular findings. We demonstrate for the first time that BM-derived murine DC express $AT_1$ at the protein level and its expression is up-regulated in response to LPS. This novel finding supports the notion that $AT_1$ may play a role in DC functionality during the inflammatory response. It shows that synthesis of RAS components is stimulated by inflammatory signals in distinct cell types or tissues (Brasier et al., 1996; Suzuki et al., 2003; Chan and Leung, 2007; Miyoshi et al., 2008; Sánchez-Lemus et al., 2009).

Our studies extend the importance of AII receptor subtype 1 to mediate physiological functions of the RAS in the immune system. Our results provide direct evidence that endogenously generated AII supports DC differentiation, maturation, and functionality through both $AT_{1a}$ and $AT_{1b}$ isoforms. The findings may also help to understand, at least partially, the beneficial effects of $AT_1$ blockers in inflammatory and autoimmune diseases beyond antagonizing AII classical effects over blood pressure and fluid homeostasis.

In summary, we hypothesize that AII stimulates DC differentiation and function in an autocrine/paracrine manner by signaling via the $AT_1$ to contribute to perpetuating inflammatory responses.

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