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

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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 (AT1) deficiency on the phenotypic and functional properties of mouse DC in vitro and in vivo. Bone marrow (BM) cells isolated from mice lacking AII subtype 1a receptor (AT1a), All subtype 1b receptor (AT1b), 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 AT1a- and AT1b-deficient BM cell cultures. Moreover, DC generated from AT1-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 AT1-deficient DC released comparable levels of interleukin (IL)-10 and IL-12p70 as WT DC, they produced significantly lower levels of tumor necrosis factor α (TNF-α) (p < 0.05). Remarkably, CD11c+ cells isolated from the spleen of AT1 knockout mice challenged with lipopolysaccharide in vivo up-regulated MHC-II, CD40, and CD80 as did WT, but released significantly lower levels of TNF-α (p < 0.01). These data provide clear evidence that AT1 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, AT1 and AT2. Most of the recognized functions of AII involve AT1, which represents a critical pharmacological target in the treatment of cardiovascular disorders, such as hypertension, heart failure, and diabetic nephropathy. Humans possess a single AT1 (de Gasparo et al., 2000), whereas there are two AT1 isoforms described in rodents, AT1a and AT1b, 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,
Impaired Function of DC Deficient in All Type 1 Receptors

Mice lacking AT1a or AT1b for all were generated by using homologous recombination in embryonic stem cells as described previously. AT1α (−/−/+/+), and AT1β (−+/−/+−) single-knockout (KO) mice were used to generate the AT1α/AT1β double-knockout mice (DKO) (−−/−−/−−) (Ito et al., 1995; Olivierio 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 (AT1αKO, AT1βKO, and DKO) was proved by polymerase chain reaction after reaming and again after experiments were performed using specific primer pairs for each gene locus. Specific pairs of primers to detect AT1α have been described (Ito et al., 1995). For AT1α-specific polymerase chain reaction, two pairs of primers that amplified products for the AT1α WT (P1, 5′-CCG CCA GAA GAT TCA GAA GG-3′ and P2, 5′-CCA ACA AAG 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 × 10^6 cells/ml in RPMI medium 1640 containing 10% heat-inactivated FBS, 100 IU/ml penicillin G, 100 μg/ml streptomycin, 2 mM glutamine, and 5.5 × 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 × 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-AT1 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).
Cells were pulsed with 1 Ci/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 co-culture 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 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\(_{1a}\)-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 recovered cells and the percentage of adherent cells were comparable among the genotypes (24 ± 0.0, 22 ± 4.3, 27 ± 3.7, and 26 ± 4.3% for WT, AT\(_{1a}\)-KO, AT\(_{1b}\)-KO, 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\(_{1a}\)-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\(_{1a}\)-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\(_{1a}\)-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\(_{1a}\)-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\(_{1a}\)-deficient DC we first examined their ability to endocytose 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\(_{1a}\)-KO, AT\(_{1b}\)-KO, 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\(_{1s}\) in DC progenitors results in major defects in the function of immature DC.

**LPS Induces Up-Regulation of AT, 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\(_{1a}\)-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\(_{1a}\)-KO, AT\(_{1b}\)-KO, 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\(_{1a}\)-KO and DKO mice have been less responsive (Fig. 4, A–C).

We then studied the functional profile of mature AT\(_{1a}\)-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...
observed that LPS-stimulated DC generated from AT1-deficient progenitors show a remarkably lower capacity to stimulate the proliferation of allogeneic T cell populations compared with WT DC (p < 0.01), independent of whether they have been deficient in AT1a, AT1b, or double-knockout DC.

**Effects of AT1 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 variety of cytokines. While IL-12 p70 is considered the key factor in the polarization of allogeneic T cells to a differentiation 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. AT1-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 AT1-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 AT1 Release Lower Levels of TNF-α after In Vivo Challenge with LPS**

We first studied the phenotype of spleen DC isolated from AT1-deficient mice. We found no differences in the percentages of IA/IEhigh and CD11chigh double-positive cells or in the levels of expression of IA/IE and CD11c in the IA/IEhigh and CD11chigh constellation of double-positive spleen cells in the four genotypes analyzed (Fig. 6, A and B). Furthermore, purified CD11c+ spleen cells from AT1-deficient mice showed similar expression of IA/IE (WT: 1370 ± 25, AT1aKO: 1125 ± 109, AT1bKO: 1341 ± 71, DKO: 1349 ± 120), CD40 (WT: 72 ± 6, AT1aKO: 86 ± 11, AT1bKO: 71 ± 5, DKO: 61 ± 4), and CD80 (WT: 79 ± 11, AT1aKO: 84 ± 8, AT1bKO: 70 ± 10, DKO: 79 ± 10) (all numbers are mean fluorescence intensity; n = 6 mice per group.)

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 AT1 activation is necessary for the complete acquisition of a mature phenotype in spleen DC after administration of systemic...
LPS, WT, AT1aKO, AT1bKO, 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<sup>high</sup> 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<sup>+</sup> spleen cells isolated from these mice. We found that WT and DKO CD11c<sup>+</sup> 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<sup>+</sup> spleen cells’ response to systemic administration of LPS we studied the secretion of the proinflammatory cytokine TNF-α, a central mediator of
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, AT₁a and AT₁b 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 AT₁ 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 AT₁ 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.
hematopoietic colonies (Haznedaroğlu and Buyukasik, 1997; Strawn et al., 2004; Hubert et al., 2006).

We have previously examined the role of AII, AT1, and AT2 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 AT1, the predominant class of AII receptors (Nahmod et al., 2003). In our previous study, we found that monocytes produce AII and express AT1 and AT2, and blockade of each of these receptors by specific antagonists induced opposite effects during the differentiation of DC. Blockade of AT1 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 AT2 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 AT1 antagonists are consistent with data shown here, because we found that DC differentiated from progenitors deficient in AT1 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 AT1-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 AT1 antagonist does not guarantee a complete inhibition of AT1 along the process of DC differentiation. On the other hand, AT1 blockers are suspected to interact with receptors other than AT1, such as peroxisome proliferator-activated receptor-γ (Schupp et al., 2004; Marshall et 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 AT1 in DC physiology.

Fig. 6. Spleen CD11c⁺ cells deficient in AT1 release lower levels of TNF-α after in vivo challenge with LPS. The percentages of CD11chigh and IA/IEhigh double-positive cells in the spleen of WT, AT1a, AT1b, 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 (CD11chigh/IA/IEhigh) in the spleens (n = 5 mice per group). C, WT and AT1-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.
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 ATI 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$_{1b}$ isoforms 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 ATI 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 cytokines by DC. We investigated AT$_1$-deficient DC’s ability to acquire signals via both isoforms to be fully functional.


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