Glucocorticoid-mediated regulation of thymic dendritic cell function

Rosa Sacedón, Angeles Vicente¹, Alberto Varas, Eva Jiménez, Juan José Muñoz and Agustín G. Zapata

Department of Cell Biology, Faculty of Biology, and ¹Department of Cell Biology, Faculty of Medicine, Complutense University, 28040 Madrid, Spain

Keywords: dendritic cells, glucocorticoids, thymus

Abstract

The possible effects of glucocorticoids (GC) on the biology of thymic dendritic cells (DC) have been analyzed. Both DC and GC seem to be involved in intrathymic T cell selection but possible relationships, if any, between them remain currently unknown. For the first time, we have proved the expression of GC receptors in thymic DC. Moreover, our data demonstrate that in vitro dexamethasone (Dex) treatment barely affects the viability of mature thymic DC, which are largely resistant to its apoptotic effect. Dex-treated thymic DC also show a slightly reduced surface expression of some adhesion and co-stimulatory molecules in correlation with diminished allostimulatory properties. Furthermore, the production of both IL-1β and tumor necrosis factor (TNF)-α, but not that of IL-6 and IL-10, diminished in the mixed leukocyte reaction established with Dex-treated thymic DC. However, the addition of recombinant rat IL-1β and TNF-α, alone or in combination, did not recover the allostimulatory capacity. Taken together, these results support certain GC-mediated regulation of the activity of thymic DC which could be relevant for the biology of the thymus gland.

Introduction

Glucocorticoids (GC), widely used as anti-inflammatory and immunosuppressive agents, are pleiotropic hormones that constitute a relevant nexus between the neuroendocrine system and the immune system (1,2). GC have also been shown to play a central role in intrathymic T cell selection (3–6) by governing a balance between signals mediated through TCR and GC receptors, both expressed on thymocytes (4,5,7–9). In vivo, T cell selection is a complex process in which thymic epithelial cells and dendritic cells (DC), together with thymocytes under selection, are involved (10,11). Nevertheless, apart from some studies which described the GC-mediated modulation of some components of the thymic extracellular matrix (12), and the expression of cytokeratin (13) and some specific markers (14) in thymic epithelial cells, there is little information on GC and non-lymphoid thymic cells. Therefore, in the present work we analyze the possible relationships between GC and thymic DC, a numerically minor thymic cell population but crucial for intrathymic negative selection (15).

A few recent studies have reported the effects of GC on peripheral DC, a cell population which seems, however, to have a different origin, phenotype and functional characteristics to thymic DC (15–17). In vivo and in vitro treatment with the synthetic GC dexamethasone (Dex) down-regulates the expression of CD80 and CD86, two co-stimulatory molecules involved in T cell activation, on splenic DC and diminishes their T cell stimulatory capabilities (18). Likewise, the effective presentation of an exogenous antigen by airway DC was almost completely inhibited when antigen pulsing was performed in the presence of Dex (19). In this case, Dex failed, however, to regulate the expression of co-stimulatory molecules induced by granulocyte macrophage colony stimulating factor (GM-CSF) (19). Moreover, GC could also govern DC development since Dex prevented the T cell-mediated terminal maturation of an epidermal-derived DC line (20) and our recent results demonstrated an in vivo early maturation of thymic DC developed in the absence of GC (21). On the other hand, all these effects of GC on DC could be mediated through specific GC receptors, the expression of which was recently described in cultured human epidermal Langerhans’ cells (22).

In the present study, we demonstrate the expression of GC receptors on rat thymic DC that are largely resistant to GC-induced apoptosis. In addition, thymic DC responded to
physiologic doses of Dex, slightly down-regulating some costimulatory and adhesion molecules, sharply decreasing their allostimulatory capacities and the production of IL-1β and tumor necrosis factor (TNF)-α, but not in that of IL-6 and IL-10.

**Methods**

**Animals**

Wistar rats were maintained in our animal facilities under conventional conditions. Thymuses were sampled from 5- to 6-week-old male rats.

**GC receptor expression on thymic DC**

Thymic cell suspensions were enriched in DC by anti-CD2 immunomagnetic bead depletion as previously described (23). Cells were cytopsioned onto slides, fixed in acetone for 5 min at –20°C and incubated with a biotin-conjugated mAb specific for rat GC receptor (BuGR2) (ABR, Golden, CO) for 1 h, followed by avidin–Texas red for 45 min (Amersham Iberica, Barcelona, Spain). Cells were finally stained with a FITC-labeled mAb against rat MHC class II molecules (OX6) (PharMingen, San Diego, CA).

**Purification of adult DC and treatment with Dex**

Adult DC isolation was performed according to a modified procedure described by Bañuls et al. (24). Briefly, thymic cell suspensions were layered over NycoPrep 1.068 (Nycomed Pharma, Oslo, Norway) and centrifuged at 600g for 15 min. The low-density fraction was cultured for 1 h in RPMI 1640 medium (2 mM L-glutamine) supplemented with sodium pyruvate (1 mM), streptomycin (100 mg/ml) (all reagents: Gibco/BRL, Eragny, France) and 5% FCS (Biosys, Compiègne, France). Subsequently, the non-adherent cells were removed by gently pipetting, and the remaining cells were incubated for 12 h in complete RPMI 1640 medium with or without 10⁻⁸ M water-soluble Dex (Sigma, St Louis, MO). After this period, non-adherent cells were collected and gently washed with complete RPMI 1640 culture medium. Percentages of DC were determined by morphology and MHC class II expression, and strong acid phosphatase activity.

**Apoptosis assay**

Thymocytes and purified thymic DC were exposed during 4 or 24 h to different doses of water-soluble Dex (0, 10⁻³, 10⁻⁷ and 10⁻⁴ M). The proportion of apoptotic cells was determined by flow cytometry using an apoptosis detecting kit from Boehringer Mannheim (Mannheim, Germany; Annexin V–Fluos), according to the manufacturer’s instructions. Briefly, cells were washed with PBS/2% FCS, and incubated with propidium iodide and FITC-labeled Annexin V for 15 min at 4°C, and immediately analyzed on a FACScan flow cytometer (Becton Dickinson, San José, CA) from the Servicio Común de Investigación (Faculty of Biology, Complutense University of Madrid). Apoptotic cells were considered as those Annexin V-positive and propidium iodide-negative. In most cases 10⁴ cells were analyzed.

**Mixed leukocyte reaction (MLR) assay**

Control and Dex-treated adult thymic DC were used at different numbers (0–15×10⁵) as stimulators for allogeneic T cells (2×10⁵) isolated from rat lymph nodes by non-adherence to nylon wool. The cultures were performed in 96-well flat-bottom culture plates, using 0.1 ml RPMI 1640/5% FCS. After 3 days at 37°C in a 10% CO₂-in-air incubator, the cultures were pulsed for 14 h with 10 µM BrdU. A specific kit from Boehringer Mannheim (BrdU Labeling and Detection Kit III) was used to measure BrdU incorporation into newly synthesized DNA. Briefly, the labeling medium was removed, and cells were dried (2 h at 60°C), fixed in 70% ethanol in HCl (0.5 M) for 30 min at –20°C, treated with nuclease (30 min at 37°C) and then incubated with peroxidase-conjugated Fab fragments of mouse anti-BrdU (30 min at 37°C). The peroxidase reaction was developed with ABTS substrate and the sample absorbance was determined using an ELISA reader at 405 nm with a reference wavelength at 492 nm.

**Flow cytometry analysis**

Thymic DC were purified as previously described and maintained on ice in PBS/2% FCS before use. A total of 1–2×10⁵ cells were incubated with saturating amounts of FITC-conjugated mouse anti-rat MHC class II molecules (OX6), MHC class I molecules (OX18), CD11a (WT.1), CD11b/c (OX42), CD18 (WT.3), CD44 (OX49), CD54 (1A29), CD90 (OX7) obtained from PharMingen, or purified anti-rat integrin α₁β₂ (OX62), CD43 (His 17) (PharMingen), CD49d (HP 2.1) kindly provided by Dr F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain), CD80 (3H5) and CD86 (24F) both gifted by Dr H. Yagita (Jutendo University, Tokyo, Japan). When using unlabeled antibodies, cells were subsequently stained with rat adsorbed FITC-conjugated donkey F(ab’²) anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA). Stained cells were analyzed in a FACSscan flow cytometer. Debris and dead cells were excluded from the analysis by forward and side scatter and propidium iodide gatings. In most cases 10,000 DC were scored.

**Cytokine assays**

Culture supernatants from MLR assays were examined after 2 days of culture with rat-specific ELISA kits for IL-1β, IL-6, TNF-α (Endogen, Woburn, MA) and IL-10 (Cytoscreen; Biosource Europe, Fleurs, Belgium) following suppliers’ indications. Cytokine values are shown in pg/ml as calculated from standard curves carried out with *Escherichia coli*-derived recombinant rat cytokines.

To determine possible correlation between the observed decreased values of IL-1β and TNF-α and the impaired allostimulatory capacities of Dex-treated DC, 100 U/ml of recombinant rat IL-1β (Serotech, Oxford, UK) and/or 2000 U/ml of recombinant rat TNF-α (Endogen) was added on day 0 to MLR cultures established with thymic DC treated or not with Dex. BrdU incorporation into newly synthesized DNA in the MLR assays was evaluated as above described.

**Results and discussion**

**GC receptor expression on thymic DC**

GC are potent immunomodulatory agents, the effects of which are mediated through specific intracytoplasmic receptors.
Glucocorticoids regulate thymic dendritic cell function

Limited information on the effects of steroids on antigen-presenting cells, including DC, indirectly suggests the expression of specific receptors on these cells. However, only Serres et al. (22) have determined their occurrence in human epidermal Langerhans’ cells. To ascertain the capacity of thymic DC to specifically respond to GC we examined by double immunofluorescence the expression of GC receptors on thymic DC, using a specific mAb for rat type II corticosteroid receptor, simultaneously to MHC class II detection. Double staining revealed a clear cytoplasmic but not nuclear expression of GC receptor on thymic DC, identified by their morphology and expression of MHC class II molecules (Fig. 1). Hence, the demonstrated expression of GC receptors on thymic DC suggests that GC could directly modulate thymic DC function as has been reported for other thymic stromal components, such as epithelial cells, also implicated in T cell selection (12–14). Remarkably, our own results on transgenic mice with impaired GC function indicate a GC requirement for establishment of the thymic epithelial network (Sacedón et al., submitted). GC, therefore, seem to be implicated in thymic biology not merely via receptors found in thymocytes.

Resistance of thymic DC to GC-induced apoptosis

GC receptor-derived signals activate a metabolic pathway that leads to apoptosis in different immune cells (25,26), including thymocytes under selection (3,6). The sensitivity of thymic DC to undergo apoptosis in response to Dex was evaluated comparatively to that of adult thymocytes. High percentages of apoptotic cells were detected after Dex treatment of thymocytes for 4 h, becoming evident at a concentration of 10^{-7} M to sharply increase after a 24 h exposure (Fig. 2). Thymic DC were also able to undergo apoptosis in response to GC, as proposed by Kampgen et al. (27) for epidermal Langerhans’ cells. However, the proportion of apoptotic cells found after Dex exposure of purified thymic DC was very low, only detecting 20% of apoptotic cells after 24 h of exposure to the highest concentrations of Dex used (10^{-4} M). In these conditions, ~90% of thymocytes underwent apoptosis (Fig. 2). The marked resistance of thymic DC to undergo apoptosis in response to Dex, also found in thymic macrophages (data not shown), agrees with previous studies which reported normal numbers of DC in 1-day-old thymus after prenatal Dex administration to Wistar rats, despite the profound impairment of thymic cellularity (28). It also agrees with the expression of high levels of bcl-2 molecules described in DC (29,30). By contrast, in vivo and in vitro GC administration results in a reduction of peripheral DC numbers. In vivo, a single injection of Dex provokes a dose-dependent loss of splenic DC (18), and both topical and systemic steroid treatment effectively inhibit the recruitment of DC into the respiratory tract during acute inflammation, reducing their intraepithelial density in the steady status by up to 50% (31). The authors do not demonstrate, however, whether the reduction in DC numbers is a consequence of in situ death or the result of cell migration. In vitro, Moser et al. (18) noted a reduction of splenic DC yield in culture by 10–50% after treatment with low doses of Dex (5 \times 10^{-8} M), compared to the control cultures. These authors suggested, nevertheless, that GC have an indirect effect on DC down-regulating GM-CSF production, since the reduction in DC numbers was prevented by the addition of this growth factor. Thus, the different DC yields obtained could be reflecting differences between thymic and splenic DC, as suggested by others (17), which present, among other features, different GM-CSF requirements (32).

Dex-treatment significantly reduced DC allostimulatory capacity

Intrathymic negative selection, the base of T cell tolerance, is induced by both DC and B lymphocytes, that function as

Fig. 1. GC receptor expression on thymic DC. DC were enriched from adult rat thymuses by anti-CD2 immunomagnetic bead depletion, cytospun onto slides, fixed and incubated with a biotin-conjugated mAb specific for rat GC receptor followed by avidin–Texas red. Cells were finally stained with a FITC-labeled mAb against rat MHC class II molecules. Micrographs demonstrate a clear expression of GC receptor (b) in thymic DC identified by their morphology and MHC class II expression (a). Results shown are representative of three or four experiments (×400).
Glucocorticoids regulate thymic dendritic cell function

Fig. 2. Resistance of thymic DC to undergo apoptosis in response to Dex. Purified thymic DC (▲) were exposed during 4 (a) or 24 (b) h to different doses of water-soluble Dex (0, 10^{-9}, 10^{-7}, and 10^{-4} M) to assess their sensitivity to undergo apoptosis in response to GC, compared to thymocytes (□). Percentages of apoptotic cells were determined by flow cytometry using the Annexin V-binding assay. GC treatment of thymocytes gave rise to high percentages of apoptosis after 4 h of Dex exposure (a, □), being initially detected at a concentration of 10^{-7} M and sharply increasing after 24 h (b, □). On the contrary, the proportion of apoptotic cells found after Dex exposure of purified DC did not exceed 20% after 24 h of exposure to the highest concentrations of Dex used (10^{-4} M) (a and b, ▲), conditions which resulted in ~90% of thymocyte apoptosis (b, □). Data represented are the average values of three or four experiments ± SD. **P < 0.01; ***P < 0.001.

Fig. 3. MLR stimulatory capacity of Dex-treated thymic DC is strongly decreased. Dex-treated (10^{-8} M) (■) or control DC (□) were used at different numbers as stimulators for allogenic T cells (2×10^5) isolated from lymph nodes. After 5 days the co-cultures were pulsed for 14 h with BrdU. A specific kit was used to measure BrdU incorporation into newly synthesized DNA. Full details are given in Methods. Results are the means of the pooled data from three experiments, each with four cultures per point. SD < 10% of the mean values.

antigen-presenting cells establishing similar interactions to those involved in peripheral T cell activation. Accordingly the potency of different antigen-presenting cells for supporting activation appears to correspond with the ability to induce clonal deletion (33). With the aim of investigating the possible effect of Dex on thymic DC function, we compared the allostimulatory capacity of Dex-treated DC with that of control, untreated ones. The MLR assays revealed a strong suppression of allogenic stimulatory function of Dex-treated DC. As shown in Fig. 3, when 10^5 Dex-treated DC were added, proliferation was similar to that observed when only 10^2 control DC were used as stimulatory cells. Thus, overnight exposure of thymic DC to physiological doses of Dex was enough to impair thymic DC allostimulatory function in vitro.

Effects of Dex on the expression of adhesion and co-stimulatory molecules on thymic DC

The surface expression of high levels of MHC antigens, as well as adhesion and co-stimulatory molecules, along with the capacity to secrete different soluble growth factors seem to determine the allostimulatory properties of DC (34). As demonstrated by flow cytometry analysis, the incubation of purified thymic DC with a physiological dose (10^{-8} M) of Dex for 12 h resulted in a small reduction of the expression of MHC class I molecules but not of MHC class II antigens (Fig. 4). Other studies report, however, a GC-mediated decrease of surface expression of MHC class II molecules (31,35). On the other hand, in agreement with our results, impaired allostimulatory capacities of splenic DC without changes in the surface expression of MHC class II antigens have been described after in vitro Dex treatment (18). These authors
explained their result as the down-regulation of the expression of co-stimulatory molecules, known to be involved in the activation of alloreactive T cells (36) as well as in clonal deletion of double-positive thymocytes (33). Therefore, we analyzed the possible changes in the expression of co-stimulatory molecules, CD80 (B7-1) and CD86 (B7-2), occurring in Dex-treated thymic DC. Our results showed that only CD80 expression slightly decreased, whereas CD86 levels remained unchanged (Fig. 4). On the other hand, the expression of several adhesion molecules such as CD11b/c, CD18, CD44, CD49d and the integrin αEβ2 was not affected by Dex exposure, whereas the expression of other molecules like CD11a, CD43, CD54 and CD90 slightly diminished (Fig. 4). The concomitance of all these slight changes observed on the expression of such molecules, crucial for the allostimulatory properties of DC, could account, at least in part, for the impaired allostimulatory capacity exhibited by Dex-treated thymic DC.

**GC regulated cytokine secretion in MLR assays**

Previous works have demonstrated the capacity of DC to produce soluble factors that mediate their allostimulatory capacities (20,34,37). To determine whether Dex-treated DC used for the establishment of MLR assays exhibited altered profiles of cytokine secretion we analyzed IL-1β, IL-6, IL-10 and TNF-α levels in the culture supernatants (Fig. 5). The results demonstrated a significant reduction of both IL-1β and TNF-α levels in the MLR assays established from Dex-treated thymic DC. The values of both IL-6 and IL-10 remained, however, normal as compared to control cultures which used non-treated thymic DC. In agreement with these results, Kitajima et al. (20) demonstrated that Dex inhibited the IL-1β and TNF-α secretion in an epidermal Langerhans’ cell line. However, they also observed a decrease of IL-6 levels.

Decreased values of IL-1β and TNF-α observed in the MLR cultures established with Dex-treated DC could be a direct consequence of the known effects of GC on cytokine production (38–42), although IL-6 and IL-10, the levels of which did not change in our experimental condition, seem to be also targets for the GC action (43,44). Alternatively, the slightly reduced expression of both adhesion molecules and co-stimulatory molecules observed in the Dex-treated DC could be also mediating the variations of cytokine profile. Down-regulated CD80 expression on Dex-treated thymic DC could, therefore, account for the decreased IL-1β secretion, since its production is triggered by coupling of the co-stimulatory

---

**Fig. 4.** Effects of Dex on thymic DC phenotype. The surface expression of different molecules on purified (80–95%) adult thymic DC after in vitro treatment with a physiological dose of water-soluble Dex (10^{-8} M) was analyzed by flow cytometry. Histograms show the expression of MHC antigens as well as adhesion and co-stimulatory molecules in non-treated thymic DC (open histograms) with respect that of Dex-treated ones (dark gray histograms). Isotype controls are shown in light gray histograms. Histograms shown are representative of three or four experiments with homogenous results.

---

**Fig. 5.** IL-1β (a), IL-6 (b), IL-10 (c) and TNF-α (d) production of control (black bars) and Dex-treated (white bars) DC in MLR assays. Culture supernatants from MLR assays were examined after 2 days of culture for cytokine production with rat-specific ELISAs. Cytokine concentrations are shown in pg/ml as calculated from standard curves produced with E. coli-derived recombinant rat cytokines. Data represented are the average values of three or four experiments ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.
molecules on DC with their respective ligands expressed on T cells (45).

On the other hand, the reduced secretion of pro-inflammatory cytokines, such as IL-1β and TNF-α, could be contributing to the impairment of allostimulatory capacities of thymic DC observed after Dex treatment. Remarkably, the addition to the MLR assays of 100 U/ml of recombinant rat IL-1β and/or 2000 U/ml of TNF-α did not recover the lymphocyte proliferation (data not shown). Accordingly, the above-mentioned changes in the expression of adhesion molecules and co-stimulatory molecules on Dex-treated DC and/or another non-tested soluble factors rather than decreased levels of IL-1β and TNF-α may be involved in the process.

Concluding remarks

Taken together, these results demonstrate the ability of thymic DC to respond specifically to GC. This response does not result in apoptosis but in the down-regulation of their allostimulatory function, presumably associated with a slight modulation of their expressed surface molecules, and in decreased production of some pro-inflammatory cytokines, such IL-1β and TNF-α. Accordingly, GC could participate on T cell selection not merely acting on thymocytes but also on the thymic DC directly implicated in the process.

Acknowledgements

This work was supported by grants PR181/96-6824 from the Universidad Complutense de Madrid, PB94-0332 and PB97-0332 from Direcció n General de Investigació n Cientı́fica y Desarrollo Tecnoló gico (DGICYT), 98/0041 from Fondo de Investigaciones Sanitarias, and 08/30014/1997 from Comunidad de Madrid. R. S., J. J. M. and E. J. are recipients of fellowships from the Ministerio de Educació n y Cultura. The technical assistance of Alfonso Cortés and Catalina Escribano is greatly appreciated. We also thank Drs F. Sánchez-Madrid and H. Yagita for the generous gifts of antibodies.

Abbreviations

DC dendritic cells
Dex dexamethasone
GC glucocorticoids
GM-CSF granulocyte macrophage colony stimulating factor
MLR mixed leukocyte reaction
TNF tumor necrosis factor

References