Control of functional T helper cell polarization by dendritic cells
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Citation for published version (APA):

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CHAPTER 8

Glucocorticoids Transiently Inhibit the Maturation of Dendritic Cells but Permanently Downregulate the IL-12 Production

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Glucocorticoids (GC) are potent immunosuppressive agents that are widely used to treat inflammatory diseases. Amongst their various effects, GC affect the function of dendritic cells (DC). GC are applied topically on tissues where DC reside in an immature form (iDC), capable to take up antigen. Upon activation, iDC migrate to the draining lymph nodes while undergoing maturation into mature effector CD83⁺ DC (mDC), which are able to efficiently activate naive Th cells but no longer sample antigen. Recently, it has been demonstrated that GC inhibit cytokine production and the maturation of DC. However, it is unclear whether these effects are permanent or transient and can be overcome by subsequent reactivation. To this aim, monocyte-derived immature DC were induced to mature by LPS in the presence or absence of GC. After removal of the GC by extensive washing, the cells were restimulated with the proinflammatory cytokines IL-1β/TNF-α and their maturation status and cytokine production upon CD40 ligation was analyzed. Our data show that inhibition of maturation by GC could be overcome by a secondary maturation stimulus. However, the downregulation of the production of bioactive IL-12p70 could not be reversed. This study may indicate that topical application of GC on tissues containing iDC may allow for the migration of anti-inflammatory IL-12-deficient effector DC.
Chapter 8

INTRODUCTION
Glucocorticoids (GC) are potent anti-inflammatory and immunosuppressive agents that are widely used in the treatment of inflammatory disorders, such as autoimmune and allergic diseases (1). The therapeutic effects of GC have been generally ascribed to an inhibitory effect on Th cell proliferation due to the downregulation of IL-2 production and signal transduction via the IL-2 receptor (2, 3). Recently, it has been demonstrated that GC also affect T cell activation via interference with early phosphorylation events induced after TCR triggering (4). Moreover, GC inhibit the secretion of both type 1 Th cells cytokine IFN-γ as well as the type 2 cytokines, IL-4 and IL-5 (1, 5, 6). Previously, also the downregulatory effect of GC on cytokine production by monocytes, macrophages and dendritic cells (DC) was demonstrated (7-9).

DC are regarded as professional antigen presenting cells as they have the unique capacity to stimulate naive T cells (10). DC, in their immature form (iDC), reside in epithelial and connective tissues, especially in the skin, lung and gut, and are very efficient in taking up antigen. Upon activation, iDC undergo maturation and most of them will migrate to the draining lymph nodes (11-13). This maturation can be triggered by various signals including inflammatory cytokines, e.g. IL-1β/TNF-α, exposure to microbial products, such as LPS, DNA or dsRNA, and contact with T cells through CD40 ligation. Maturing DC rapidly lose their antigen uptake capacity and, at the same time, gain expression of the costimulatory molecules CD80 and CD86. Consequently, they not only acquire the capacity to stimulate naive T cells antigen-specifically (signal 1), but also to efficiently costimulate them (signal 2) (13-15). Doing so, these immunogenic mature DC (mDC) may polarize (signal 3) Th cells into Th1 or Th2 cells (16). Depending on the conditions during their initial activation as sentinel DC, DC may develop into Th1-promoting DC1 or Th2-promoting, IL-12 deficient DC2 (16, 17). In this respect, the production of bioactive IL-12p70 is an important factor as it is very potent in inducing Th1 responses (18).

When GC are used as topical drugs for the treatment of inflammatory diseases, they normally are applied on tissues containing iDC as well as mDC as a result from the inflammation. At these sites, GC will exert direct effects on the iDC cytokine production, especially IL-12p70, as we and others described before (7, 8, 19). On the other hand, it has been demonstrated that GC inhibit the maturation of iDC into mDC, which may inhibit the recruitment of naive Th cells (20-22). However, it is unknown whether this
GC inhibit DC maturation transiently but IL-12 production permanently

inhibition of maturation and subsequent initiation of the immune response is permanent or that, after withdrawal of the GC, the remaining DC regain the capacity to develop into genuine mature effector DC that will migrate to the draining lymph nodes and prime naive Th cells. Therefore, we investigated to what extent the inhibition of maturation and IL-12 production by GC is transient or can be overcome by the reactivation of the DC. To this aim, human monocyte-derived iDC were matured in the absence or presence of clobetasol-17-propionate (CP). After removal of the GC by extensive washing, the DC received a second boost of maturation factors and after 24 h were analyzed for their expression of cell surface molecules, and their capacity to produce bioactive IL-12p70.

The present data show that GC inhibit the maturation of DC but this can be reversed after a secondary maturation signal. Nevertheless, the obtained GC-treated mature, effector DC do not produce bioactive IL-12p70 and are therefore, DC2-type cells that induce the development of Th2 cells from naive precursors.

MATERIALS AND METHODS

Cytokines, antigens and reagents

Human rGM-CSF (sp. act. 1.11x10^7 U/mg) was a gift from Schering-Plough, Uden, The Netherlands. Human rIFN-γ (sp. act. 8x10^7 U/mg) was a gift from Dr P.H. van der Meide (Utrecht University, Utrecht, The Netherlands). Human rIL-4 (sp. act. 1x10^8 U/mg) and human rTNFα (sp. act 10^8 U/mg) were obtained from PBH (Hannover, Germany). Human rIL-1β (sp. act 5x10^7 U/mg) was obtained from Boehringer Mannheim (Mannheim, Germany). LPS (Difco, Detroit, MI) was used at a final concentration of 100 ng/ml. The GC clobetasol-17-propionate (CP; Sigma) and hydrocortisone (HC; Sigma) were kept as stock solutions of 10^-2 M in 95% ethanol, stored at -20°C, and diluted into complete culture medium just prior to use. In all experiments, a 1:10^4 final dilution of 95% ethanol was included as a vehicle control.

In vitro generation of iDC from peripheral blood monocytes

Venous blood from healthy donors was collected by venipuncture in sodium-heparin containing tubes (VT100H; Venoject, Terumo Europe, Leuven, Belgium). PBMC were isolated by density centrifugation on Lymphoprep (Nycomed, Torshov, Norway). Subsequently, PBMC were centrifuged on a Percoll (Pharmacia, Uppsala, Sweden) gradient, consisting of three density layers (1.076, 1.059 and 1.045 g/ml). The light
density fraction floating on the middle layer, containing predominantly monocytes, was collected, washed and seeded in 24-well culture plates (Costar, Cambridge, MA) at a density of 5x10^5 cells/well. After 60 min of incubation at 37°C, non-adherent cells were removed and adherent cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Life Technologies Ltd., Paisley, UK) containing gentamycin (86 µg/ml; Duchefa, Haarlem, The Netherlands) and 1% FCS (HyClone, Logan, UT), supplemented with GM-CSF (500 U/ml) and IL-4 (250 U/ml) to obtain iDC as described elsewhere (35). At day 3, the culture medium including the supplements, was refreshed. At day 6, CD1a^+CD14^- iDC were ready for use.

**Induction of maturation of iDC in the absence or presence of GC**

At day 6 the maturation of iDC was induced by the addition of either rIL-1β/rTNF-α, or by LPS. After 48 h, fully mature CD1a^+ CD83^+ mDC were obtained. To study the effect of GC on the maturation, CP was added together with the maturation factors in a concentration range from 10^{-6} to 10^{-10} M, whereas HC was used at concentrations of 10^{-6} to 10^{-8} M. To study the reversibility of the inhibition of the maturation of DC by GC, iDC were matured with LPS in the absence or presence of GC. After 48 h cells were thoroughly washed to remove LPS and GC and cultured in IMDM/1% FCS containing IL-4 and GM-CSF alone for 24 h. Subsequently, the maturation was induced by the addition for 24 h of IL-1β/TNF-α after which, at day 10, the expression of CD83 and the production of IL-12p70 was determined.

**Analysis of expression of cell surface molecules by flow cytometry**

Mouse anti-human mAbs against the following molecules were used: CD1a (OKT6; Ortho Diagnostic System, Beerse, Belgium), CD80 and CD86 (B7-24 and 1G10; kindly provided by Innogenetics N.V., Ghent, Belgium), MR (15-2) a gift from Dr F. Noorman, Gaubius Laboratory, TNO Prevention and Health, Leiden, The Netherlands), CD83 (Immunotech, Marseille, France). Bound mAb were detected by FITC-conjugated goat anti-mouse mAb (Jackson Immunoresearch Laboratories Inc., West Grove, PA).

**IL-12p70 production by DC**

CD1a^+ DC (4x10^4 cells/well) were stimulated with mouse CD40L-expressing mouse plasmacytoma cells (J558-CD40L; a kind gift from Dr P. Lane, University of
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Birmingham, Birmingham, UK), 4x10⁴ cells/well in the presence of rhuFN-γ (1000 U/ml), in 96-well flat-bottom culture plates (Costar) in IMDM containing 10% FCS in a final volume of 200 μl. Supernatants were harvested after 24 h and stored at -20°C until the levels of IL-12p70 were measured by ELISA.

Cytokine measurements
Measurements of IL-12p70 levels in the culture supernatants were performed by specific solid-phase sandwich ELISA as described previously. The limit of detection of these ELISA is 3 pg/ml.

Statistical analysis
Data were analyzed for statistical significance (GraphPad, InStat TM, Version 2.02) using ANOVA. Individual comparisons were performed using the Student’s t-test. A P value <0.05 was taken as the level of significance.

RESULTS
GC inhibit the maturation and capacity to produce IL-12p70 of human monocyte-derived iDC
To study the effects of CP and hydrocortisone (HC) on the maturation and capacity to produce IL-12p70 of sentinel iDC, monocyte-derived iDC were cultured for 48 h with the proinflammatory cytokines IL-1β/TNF-α in the absence or presence of several concentrations of CP or HC. In the absence of GC the expression of mDC marker, CD83, as well as the costimulatory molecules CD80 and CD86 were upregulated, whereas the expression of the mannose receptor (MR) was lost. The addition of CP or, to a lesser extent, HC inhibited the full maturation of iDC in part of the cells, as shown by the impaired upregulation of the expression of CD83, CD80 and CD86, as well as the inhibition of the downregulation of the MR (Fig. 1A). The inhibition of the maturation by GC is clearly dose-dependent, CP being active at much lower concentrations as compared to HC (Fig. 1B).

Previously, we have reported that GC strongly inhibit the production of bioactive IL-12p70 by iDC both in a direct fashion and after pre-incubation (19). Here, we studied the effect of the presence of GC during the maturation of DC on the ability to produce IL-12p70 upon subsequent restimulation with CD40L as a mimic for the activation in the
lymph nodes by naive Th cells. In control conditions, mDC stimulated with CD40L produce considerably less IL-12p70 as compared to iDC stimulated similarly with CD40L. The presence of CP during maturation, however, further inhibited the IL-12p70 production up to approximately 70% at $10^{-8}$ M (Fig. 1C), whereas the presence of HC affected the IL-12p70 only slightly at the highest concentration tested ($10^{-6}$ M). These effects are probably the net result of two opposite actions of GC. On the one hand GC strongly inhibit the IL-12p70 production during pretreatment (19). On the other hand GC partially inhibit the maturation of DC, which will result in a less pronounced decrease of the IL-12 p70 levels in the mDC, as iDC produce considerably more IL-12p70.

Figure 1. GC inhibit the maturation and the production of IL-12p70 of DC in a dose-dependent fashion. (A) Maturation of iDC was induced with IL-1β (10 ng/ml)/TNF-α (50 ng/ml) in the absence or presence of CP ($10^{-8}$ M) or HC ($10^{-6}$ M). After 48 h, the cells were analyzed by FACS for the expression of CD83, the costimulatory molecules CD80 and CD86, and the MR. (B) The presence of CP ($10^{-10}$-$10^{-7}$ M) and HC ($10^{-8}$-$10^{-6}$ M) inhibited the maturation dose-dependently as determined by CD83 expression. (C) After maturation in the absence or presence of GC, DC were washed and stimulated with CD40L-transfected J558 cell line in the presence of IFN-γ (1000 U/ml). After 24 h, supernatants were harvested and the concentration of IL-12p70 in the supernatant was determined by ELISA. Data were analyzed for statistical significance by Student's t-test. *P<0.05, **P<0.01. Data are from one representative experiment of five.
GC inhibit DC maturation transiently but IL-12 production permanently.

The capacity to produce TNF-α and IL-6 upon restimulation with CD40L was also reduced in DC matured in the presence of GC as compared to control mDC, although to a lesser extent, by approximately 50-40% (data not shown).

The inhibition of maturation by GC is transient and can be reverted by reactivation with maturation factors (IL-1β/TNF-α)

DC reside at sites that are constantly exposed to incoming pathogens. As it is known that the presence of GC inhibit the maturation of sentinel iDC, we wondered whether this inhibition, and therefore the initiation of the immune response, was permanent or could be overcome by subsequent activation of the DC. To this aim, iDC were activated with LPS in the absence or presence of CP, and thoroughly washed after 48 h, rested for another 24 h and subsequently activated with IL-1β/TNF-α. As is clearly shown in Fig. 2 the CP-induced inhibition of maturation as measured by CD83 expression could be overcome by subsequent activation in the absence of CP. When LPS was used to reactivate the DC that were previously activated with IL-1β/TNF-α in the presence of CP no restoration of the maturation was observed (data not shown).

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**Figure 2.** Inhibition of maturation by GC is reversible. Upon resuming the maturation, GC pretreated DC become mature effector DC. Maturation was induced with LPS (100 ng/ml) in the absence or presence of CP (10⁻⁸ M). After 48 h, the cells were thoroughly washed to remove the GC. After 24 h of resting in IMDM/1% FCS/GM-CSF, maturation factors (IL-1β/TNF-α; 10 ng/ml and 50 ng/ml) were added to resume the maturation. After another 24 h cells were harvested, washed and analyzed for their expression of CD83.
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Primary stimulation / Secondary stimulation
none / none
none / MF
none / MF+IFN-γ
LPS / none
LPS / MF
LPS / MF+IFN-γ
LPS+CP / none
LPS+CP / MF
LPS+CP / MF+IFN-γ

Figure 3. The inhibition of the production of IL-12p70 by GC is not restored upon resumed maturation. Maturation was induced in the absence or presence of CP and resumed as described above in the absence or presence of IFN-γ (1000 U/ml). The cells were harvested, washed and stimulated with the CD40L-transfected J558 cell line. After 24 h, supernatants were harvested and the concentration of IL-12p70 in the supernatant was determined by ELISA. Data were analyzed for statistical significance by Student's t-test. *P<0.05, **P<0.01. Data represent one out of five experiments with similar results.

Figure 4. CD40L-induced IL-12p70 production by mature DC is less susceptible to modulation by GC than that of immature DC. DC were matured with IL-1β/TNF-α (open symbols) or kept immature (closed symbols). After washing both cell population were stimulated with the CD40L-transfected J558 cell line in the absence and presence of various concentrations of CP (A) or HC (B). After 24 h supernatants were harvested and the IL-12p70 production was measured by ELISA. Data were analyzed for statistical significance by Student's t-test. *P<0.05, **P<0.01. Data represent one out of five experiments with similar results.
GC inhibit DC maturation transiently but IL-12 production permanently

The inhibition of the capacity to produce IL-12p70 is not restored upon resumed maturation
IL-12p70 is a major inducer of Th1 responses and we and others have shown that GC are potent suppressors of the capacity to produce IL-12, both in iDC (19) as well as during maturation (Fig 1C). Therefore, we questioned whether the capacity to produce bioactive IL-12p70 was also restored by reactivation of GC-arrested DC. The presence of CP strongly downregulated the production of IL-12p70 upon CD40 ligation (Fig 3.), and this capacity was not restored upon resumed maturation. GC-treated DC produced minimal amounts (around the detection level for the ELISA) of IL-12p70. Even the presence of IFN-γ, a potent enhancer of IL-12p70 production, could not further upregulate these levels of IL-12p70 (Fig 3).

Mature DC have a reduced responsiveness to GC
When an epithelial tissue is inflamed, as is the case in for instance atopic dermatitis or psoriasis, this epithelium will not only contain immature DC but also mature effector DC. Therefore we studied the effect of GC on mature DC. As is reported before, immature DC are very susceptible to the inhibitory effects of GC on the IL-12 production upon CD40 ligation (Fig 4). In the presence of CP a dose-dependent inhibition of maximally 90% was observed, whereas in the presence of HC approximately 60% inhibition was observed. However, the ability of DC to produce IL-12 was only mildly affected by GC after the maturation of DC induced by both LPS and IL-1β/TNF-α. Although the potent GC CP dose-dependently downregulated the IL-12 production to maximally 35% (65% inhibition) of the amount observed in control-treated DC, only a minor reduction in IL-12p70 production of approximately 20% was observed in the presence of HC.

DISCUSSION
We and others have previously demonstrated that GC affect DC in various ways in both man and mouse. Not only sentinel DC that reside in peripheral tissues as antigen-trapping DC are inhibited in their cytokine production but also their maturation into T cell stimulatory effector DC is inhibited. The present study, however, shows that the GC-induced inhibition of maturation into effector DC can be overcome with a second activation after withdrawal of the GC. This indicates that the effect of GC on DC-maturation is transient and GC-treated DC can become effector DC. However, once the
capacity to produce IL-12 is downregulated by GC, this cannot be restored. Even the presence of the potent IL-12 enhancer IFN-γ at the time of stimulation with CD40L (to mimic the interaction with T cells) did not enhance the IL-12 production by GC-treated effector DC.

The total loss of the capacity to produce bioactive IL-12p70 suggest that these effector DC have become DC2 that promote the development of Th2 cells from naive precursors. This is in line with previous observations that memory CD4+ Th cells stimulated with GC-treated human monocytes (7) or murine macrophages (8), show a decreased production of IFN-γ, due to a decreased production of IL-12 by these antigen presenting cells.

Based on in vitro experiments we have recently proposed the concept that the type of activation and the type of tissue in which the immature, sentinel DC resides, determines the development in Th1-promoting type 1 DC (DC1) or a Th2-promoting DC2 (16, 17). In this respect, PGE2 not only enhances the maturation of DC (23, 24) but it also induces the development of highly immunogenic mDC that produce very low levels of IL-12 and promote the development of Th2 cells from naive Th cells (DC2) (24). On the other hand, IFN-γ promotes the development of highly stimulatory DC that produce high levels of IL-12 and promote the development of Th1 cells (25). These subtypes of myeloid mDC are fully committed, since their IL-12 production profiles are stable and resistant to further modulation (24, 25). Moreover, also different types of pathogens induce the development of polarized DC1 or DC2 effector cells from sentinel, immature DC. Pathogens associated with type 1 responses in vivo such as intracellular bacteria or viruses induce the development of DC1 whereas toxin from extracellular bacteria and helminth extracts induce the development of DC2 in vitro (17).

The present study demonstrates that also pharmacological compounds may contribute to the development of effector DC1 or DC2 cells. Several studies have indicated potent effects of pharmacological compounds on DC function, not only GC (19-22, 26) but also vitamin D (27), β2-agonists (28) and many more. To our knowledge it was, however, never determined what happens after withdrawal of the compound and whether all functions of the DC are permanently inhibited. In case of GC, part of the inhibitory action could be reversed.

The observation that only the inhibition of maturation can be restored but not the capacity to produce IL-12 could be explained by differences in signal transduction
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routes. It has been demonstrated by Rescignio et al. (29) that survival and maturation of DC are regulated by different pathways, e.g. ERK regulates survival whereas NF-κB is responsible for maturation. NF-κB is a family of transcription factors consisting of 5 members, RelA (or p65), RelB, cRel, p50 and p52. The IL-12 promoter region contains a NF-κB half site (30). It is tempting to speculate that GC transiently inhibit NF-κB and therefore inhibit the maturation whereas a different pathway involved in regulation of cytokine production is irreversibly shot down. The latter could also be due to a effect on AP-1, which has been demonstrated to be affected by GC in T cells, and has recently been demonstrated to be present and active in the IL-12p40 promoter (31).

Previously, we and others suggested that due to the inhibited induction of maturation and upregulation of costimulatory molecules GC-treated DC may induce IL-10-secreting T cells and therefore be tolerogenic DC3. In addition, it has been demonstrated that repetitive activation of Th cells with immature DC induces high levels of IL-10 upon restimulation. As GC inhibit the maturation only transiently, GC-treated DC do induce proper Th cells responses without the induction of IL-10 (data not shown) and cannot, therefore, be designated "tolerogenic".

GC-treated effector DC are anti-inflammatory, due to their reduced production of IL-12, TNF-α and IL-6. This suggests that the anti-inflammatory effects of GC are not only due to a direct inhibition of T cell functions via inhibition of the expression of IL-2 and the IL-2R, but also via inhibition of the DC function. The induced Th2 profile may be responsible for the enhanced levels of IgE antibodies as has been reported in both allergic and non-allergic individuals using GC in long-term therapeutic strategies (32-34). Moreover, the GC-treated DC may hamper the initiation of the appropriate immune response as observed in patients using GC for a longer period of time. These patients have frequent bacterial infections and difficulty in clearing these infections. Although, this may originate in part from a direct downregulatory effect of GC on T cells, the present study suggests that this may also follow from the effects of GC on DC, altering their role in the mounting the proper immune response e.g. a Th1 response.

Acknowledgments
This work was financially supported by the Fundação para a Ciência e a Tecnologia, Lisbon, Portugal, grant PRAXIS XXI/BD/9195/96 (to P.L.V.).
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