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# Steroids inhibit uptake and/or processing but not presentation of antigen by airway dendritic cells

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## SUMMARY

Recent studies from our laboratory indicate that local and (particularly) systemic steroids can modulate the traffic of dendritic cells (DC) through resting and inflamed airway epithelial tissues. The present report focuses upon the T-cell activating properties of DC, which are controlled by granulocyte–macrophage colony-stimulating factor (GM-CSF) signals, and in particular the question of whether the DC-stimulating effects of GM-CSF are susceptible to regulation by steroids. We present evidence that while dexamethasone inhibited GM-CSF-dependent uptake and/or processing of exogenous antigen by DC, it was ineffective in blocking the presentation of preprocessed self antigen to alloreactive T cells in a one-way mixed lymphocyte reaction (MLR). Associated GM-CSF-induced up-regulation of major histocompatability complex (MHC) class II and CTLA4 ligand expression by DC were also unaffected by dexamethasone phosphate (DX), reinforcing the view that the inhibitory effects of steroids on the T-cell activating functions of DC are restricted to steps upstream from presentation of processed antigen to the T-cell receptor (TCR). These findings have potentially important implications in relation to the use of topical steroids in the treatment of atopic asthma, a disease in which local T-cell activation in airway tissue is a key pathogenic factor, and which furthermore is characterized by intense production of GM-CSF within the airway epithelium.


## INTRODUCTION

Dendritic cells (DC) expressing high levels of surface major histocompatability complex (MHC) class II glycoproteins are found in large numbers as a contiguous network throughout the respiratory tract epithelium,[1–5] and the available evidence indicates that they are the sole professional antigen-presenting cell (APC) population resident in these tissues.[6]

Recent studies from our laboratory have drawn attention to the dynamic nature of these DC networks, particularly in response to local tissue stimulation. Thus, in chronic inflammation, the airway DC population expands in number and displays increased class II MHC expression.[7] Moreover, acute inflammation induced by inhalation of microbial agents, stimulates extremely rapid influx of large numbers of DC into the airway epithelium, with kinetics identical to the accompanying neutrophil response.[7,8] Evidence is also accumulating that the airway DC networks up-regulate in human in response to repeated local antigen challenge.[9,10]

We have also recently demonstrated that both topical (inhaled) and systemic steroids effectively inhibit recruitment of DC into the respiratory tract epithelium during acute inflammation, and moreover can reduce the intraepithelial density of these cells in the steady state by up to 50%.[11] However, no information is yet available on the efficacy of steroids in relation to regulation of DC function(s) within these tissues.

Analogous to their counterparts in the epidermis, DC in normal airway tissue are specialized for uptake and processing of antigen, but do not express their full potential for antigen presentation to T cells until they receive obligatory cytokine differentiation signals.[12] The latter differentiation process is believed to normally occur only after the migration of DC from their peripheral host tissues (in this case the airway epithelium) into regional lymph nodes (RLN), where they encounter high levels of T-cell derived granulocyte–macrophage colony-stimulating factor (GM-CSF), which represents the rate-limiting activation signal in this process.[13] This partitioning of DC functions provides a mechanism for protection of the delicate airway epithelium from the potential tissue-damaging consequences of continuous local T-cell activation in response to inhaled environmental antigens, while preserving the capacity for effective translation of relevant information concerning incoming antigens into immunological memory within the central immune system.

We have suggested[12] that premature differentiation of airway DC into fully functional APC during the intraepithelial stage of their cycle, leading in turn to marked up-regulation of the 'tonus' of local T-cell responses in the airway mucosa,

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may be a contributing factor in the pathogenesis of certain chronic inflammatory diseases of the respiratory system.[12] A likely example may be atopic asthma, a disease characterized by high levels of GM-CSF production within the airway epithelium.[14]

It is accordingly of interest to determine whether steroids, which have been shown to modulate the traffic of respiratory tract DC, can exert similar effects on the responsiveness of these cells to GM-CSF signals.

## MATERIALS AND METHODS

*Animals*

Specified pathogen-free adult rats of the BN and WAG strains, supplied by the Animal Resources Centre, Murdoch University, Western Australia, were used in all experiments.

*DC preparation and purification*

The preparation of enriched DC from collagenase-digested respiratory tract tissues was as described previously,[6,12] yielding a cell suspension in which DC constituted the sole source of MHC class II immunostaining. Final purification of DC to 92–95% homogeneity was achieved via immunomagnetic sorting, as follows. The cell suspension was initially labelled at 4° with anti-MHC class II monoclonal antibody (mAb $0 \times 6$;[15]), washed twice with phosphate-buffered saline (PBS)/0·2% bovine serum albumin, and then incubated with gentle rolling for 30 min at 4° with Dynabeads (Dynal A.S., Oslo, Norway) M450 conjugated with goat anti-mouse immunoglobulin, at 5–10 beads per target cell.

Bead-coated cells were separated using an MPC-1 magnetic separator (Dynal A.S.). Purity was assessed on the basis of the percentage of cells in the final preparation carrying surface bound beads; preliminary experiments (R. Steptoe and P. G. Holt, unpublished) employing parallel immunohistochemical staining established that all cells carrying beads indeed stained positively with mAb $0 \times 6$, and further that overall DC yields with this method were $\geq 80\%$ of that predicted on the basis of the frequency of $0 \times 6^+$ cells in the (starting) unseparated cell suspension. A follow-up series of experiments were also performed in which immunomagnetic purification of DC was performed via the MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). Comparable levels of purity were obtained, in this case monitored by flow cytometry as in our earlier studies (e.g.[8,12]).

*Preculture of DC*

Purified DC were incubated for 48 hr in RPMI-1640 plus 5% fetal calf serum (FCS) containing 10 ng/ml murine recombinant GM-CSF (Biosource International; Camarillo, TX), in the presence or absence of $10^{-6}$–$10^{-8}$ M dexamethasone phosphate (DX; DBL Labs, Melbourne, Australia). At the end of the incubation, cells were washed three times and viability assessed by trypan blue exclusion. Viable cell recoveries varied between 40 and 60% of the starting population; overall recoveries were reduced by variable amounts by the presence of the steroid; consistent with reports from other laboratories,[16] at the highest DX dosages employed here overall DC survival in culture was reduced to 20–30% of that in cultures containing GM-CSF alone. In one series of experiments, ovalbumin antigen was included in the cultures at a final concentration of 500 μg/ml. It should be noted that the survival of purified DC in this culture system was absolutely dependent upon the inclusion of exogenous GM-CSF, as cell recoveries in cultures not supplemented with GM-CSF were $<1\%$ of starting numbers.

*Expression of function-associated surface molecules on DC*

To detect the presence of ligands of CTLA4, DC were incubated with the human fusion protein CTLA4–immunoglobulin,[17] and bound CTLA4 was revealed by fluorescent anti-human immunoglobulin G (IgG: Biosource International, Camarillo, TX). Expression of MHC class II was examined via mAb $0 \times 6$, as detailed previously.[8,12] In one series of experiments expression of the invariant chain of MHC class II was analysed employing the mAb RG11[18] kindly provided by Dr K. Reske (Johannes Gutenberg University, Mainz, Germany).

*Antigen presentation*

The capacity of DC to present alloantigens to T cells in a one-way primary mixed lymphocyte reaction (MLR) was assessed employing a micro-modification of the system detailed by Holt *et al.* (1993).[12] Briefly, triplicate cultures of responder (WAG strain) lymph node cells (LNC) were established in round-bottom microplate wells in 40 μl RPMI plus 5% normal rat serum and $2 \times 10^{-5}$ M 2-mercaptoethanol, to which were added 10 μl aliquots of varying dilutions of DC in the same medium. T-cell activation was assessed as [$^3$H]DNA synthesis at 72 or 96 hr.[12]

In parallel, DC presentation of ovalbumin (OVA) to syngeneic OVA-immune LNC was assessed, employing the assay system detailed by Holt *et al.* (1993).[12] OVA-pulsing of DC was performed in GM-CSF supplemented medium in the presence versus absence of $10^{-7}$ M DX, prior to washing and titration into LNC microcultures, as above.

The *in vitro* bioactivity of the DX used here was validated in parallel experiments, involving inhibition of concanavalin A (Con A)-induced T-cell proliferation. These experiments involved stimulating T cells in the presence of 1 μg/ml or 5 μg/ml Con A for 48 hr, in the presence or absence of the steroid, prior to determination of [$^3$H]DNA synthesis.

## RESULTS

### DX fails to prevent GM-CSF-mediated up-regulation of the allostimulatory activity of DC

Figure 1 demonstrates the marked up-regulation of MLR-stimulating activity of respiratory tract DC achieved by pre-culture with GM-CSF, relative to DC tested 'fresh', (i.e. immediately) on completion of immunomagnetic sorting. This finding is consistent with earlier findings.[12,19] DX at levels as high as $10^{-6}$ M, did not modulate the stimulatory effects of GM-CSF.

The possibility that the failure of DX to inhibit the functional up-regulation of DC in this system was related to lack of bioavailable steroid in the cultures was examined in the control experiments in which LNC were stimulated with the polyclonal T-cell mitogen Con A in the presence of DX at the levels employed in Fig. 1. DX at these levels mediated potent inhibition of Con A-induced T-cell activation (not

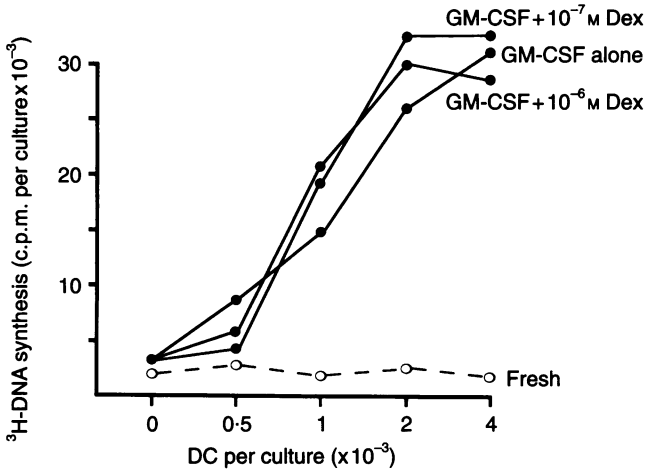


**Figure 1.** GM-CSF-mediated functional maturation of respiratory tract DC: effect of DX. DC were purified to 92·8% homogeneity by immunomagnetic sorting, and employed as stimulator cells in primary MLRs, either immediately after preparation ('fresh'), or after 48 hr pre-treatment with GM-CSF, in the presence or absence of DX. Data shown are median Δ disintegration per minute (DPM) per culture at 96 hr, from a representative experiment; interculture variation was <10% means. The experiment shown was successfully replicated four times.

shown) indicating that steroid bioavailability was not a covert variable in these experiments.

### GM-CSF-induced up-regulation of MHC class II and CTLA4 ligand expression on DC are unaffected by DX

As shown in Fig. 2a, freshly isolated airway DC express high levels of MHC class II, which is further increased upon culture with GM-CSF; a comparable increase in MHC class II expression also occurred in the presence of DX at levels up to $10^{-6}$ M (Fig. 2b).

In contrast, freshly isolated airway DC express only low levels of CTLA4 ligand, and expression of the latter is markedly stimulated by the presence of GM-CSF (Fig. 3a); analogous to the findings with MHC class II, GM-CSF-induced expression of CTLA4 ligand was unaffected by DX (Fig. 3b). In one series of experiments involving the use of the mAb RG11 for detection MHC class II invariant chain staining, no differences in expression attributable to the presence of DX in the cultures was observed (data not shown).

### Inhibition of GM-CSF-induced presentation of OVA

In the experiments of Fig. 4, freshly isolated airway DC were pulsed with OVA in the presence of GM-CSF, with or without concomitant exposure to DX, and then titrated into cultures of OVA-immune LNC. In contrast to the results obtained with presentation of self antigen in a one way MLR, DC pulsed with the exogenous antigen in the presence of DX were unable to deliver an antigen-specific activation signal to T memory cells.

## DISCUSSION

DC populations in peripheral tissues turn over continuously, being simultaneously depleted by the emigration of mature cells to the paracortical regions of RLN, and replenished by an equivalent number of incoming bone marrow derived precursors from the peripheral blood.[13] Recent studies indicate that this process is particularly rapid in the respiratory tract, in which DC populations within the airway epithelium and lung parenchyma are renewed every 2–3 and 7 days, respectively.[20]

Treatment of animals with either high dose (systemic) DX at 10 mg/kg body weight, or lower doses of (topical) nebulised budesonide (0·014 mg/kg) or fluticasone proprionate (0·012 mg/kg), exert unequivocal effects on the density of these DC populations. The most drastic reduction in airway DC numbers was achieved with systemic DX, which produced a 75–80% reduction in this population within a few days of commencement of high-dose systemic treatment.[11] A less marked decline in DC density was seen with topical steroid which reduced steady-state DC levels by 40–50%, but both types of steroid preparations were very effective in preventing the recruitment of DC precursors into the airway epithelium during acute inflammation.[11]

One of the most important inflammatory airway diseases in humans is atopic asthma, in which topical steroids are currently the most widely used anti-inflammatory drugs. While the efficacy of steroids in this context is widely acknowledged, it is also accepted that they are by no means 100% effective, particularly in chronic asthma, and there is currently no cogent explanation for their failure in some cases versus their success in others.

In this context, it is generally accepted that excessive local activation of T helper cells within the airway mucosa in response to inhaled antigen(s) is a key factor in the pathogenesis of this disease.[21] As the primary resident APC population within this tissue,[1,2] there is considerable interest in the potential role of DC in the disease process,[22,23] particularly in view of recent data suggesting up-regulation of local DC networks in the airway mucosa of atopics.[9,10] The issue of the effectiveness of steroids in regulation of airway DC function(s) may accordingly be important in relation to the overall effectiveness of steroids in controlling immunoinflammatory responses in this tissue microenvironment.

In addition to our own experiments on regulation of local DC traffic,[11] there are significant precedents in the existing literature for the expectation that steroids should be effective in damping DC function(s) in the airways. In particular, topical steroids have been demonstrated in several studies to exert potent down-regulatory effects upon epidermal Langerhans' cells (LC), which include downmodulation of MHC class II expression and reduction in population density at application sites (e.g.[24]), and similar effects have been observed on splenic DC following parenteral steroid treatment.[16] Also, high levels of steroids have been reported to induce apoptosis of murine LC *in vitro*.[25]

However, there is only limited information available on the effects of steroids on APC-related function(s) of DC. The most comprehensive report available pertains to effects upon the capacity of splenic DC to present superantigen SEB or self antigen in a primary MLR; both of these functions, together with associated expression of MHC class II and CTLA-4 ligand expression on the DC, was markedly inhibited by steroids.[16] In addition, the inclusion of GM-CSF in the cultures during steroid exposure preserved the DC functions.[16]

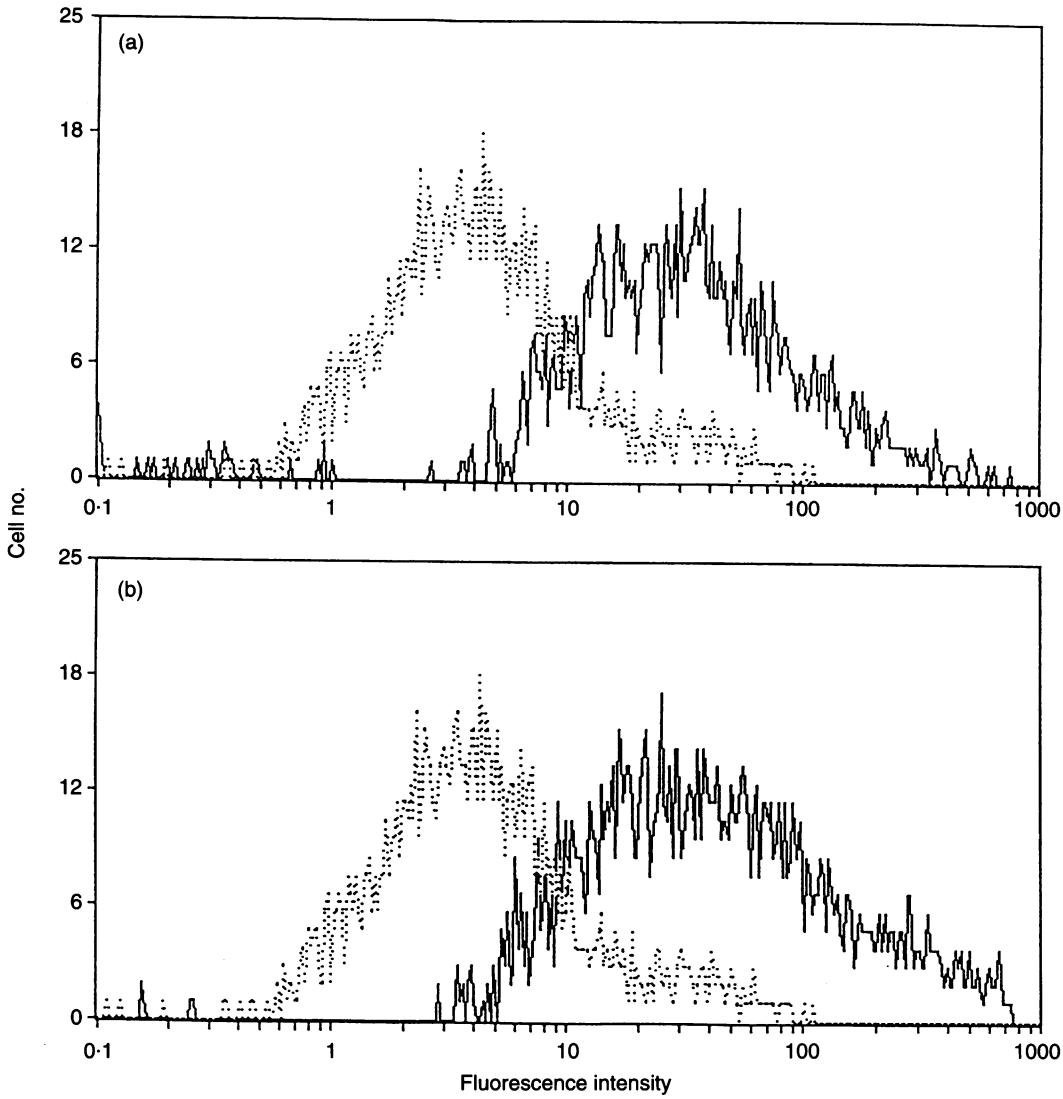


**Figure 2.** GM-CSF up-regulation of MHC class II expression. Purified DC were immunostained with mAb 0 × 6 for analysis of MHC class II expression as detailed in Materials and Methods. (a) Freshly isolated DC (broken line) versus DC cultured 48 hr in GM-CSF supplemented medium. (b) Freshly isolated DC (broken line) versus DC cultured 48 hr in GM-CSF supplemented medium containing $10^{-6}$ M DX.

These important observations, while highly suggestive of a potentially effective role for steroids in modulating of airway DC function(s), do not in themselves resolve this issue. Firstly, while considerable information on APC functions can be gained employing assays involving allostimulation and T-cell triggering with superantigens, these systems do not probe all of the steps involved in uptake, processing and presentation of exogenous soluble protein antigens of the type involved in triggering T-cell responses in atopic asthma. Secondly, it is evident from a variety of studies that DC populations in peripheral tissues and central lymphoid organs are at the opposite poles of a differentiation spectrum, a significant proportion of the latter being the mature end-stage of the former, which migrate to draining lymph nodes or spleen to complete their life cycle.[13] The APC function(s) of DC at the two poles are markedly different, the general rule being that peripheral tissue DC are relatively functionally inert and do not express significant APC activity until they receive inductive cytokine signals (especially GM-CSF), usually after migration to lymphoid organs.[13] Studies on the steroid sensitivity of splenic DC functions may thus not predict all the results obtained with peripheral tissue DC such as those in the airway mucosa, and accordingly the key experiments performed on interactions between splenic DC and DX interaction[16] were repeated and extended with airway DC.

The salient findings from our study are as follows. unlike the situation with splenic DC which are effective APC when freshly isolated from their host tissue,[16] freshly prepared airway DC function poorly as APC in a primary MLR, unless they are precultured with GM-CSF (Fig. 1). DX was completely ineffective in preventing GM-CSF-mediated up-regulation of MLR reactivity (Fig. 1); this latter observation is consistent with findings on splenic DC, where GM-CSF-mediated increases in their already potent APC functions were also resistant to DX.[16]

Similar disparities were observed with respect to MHC

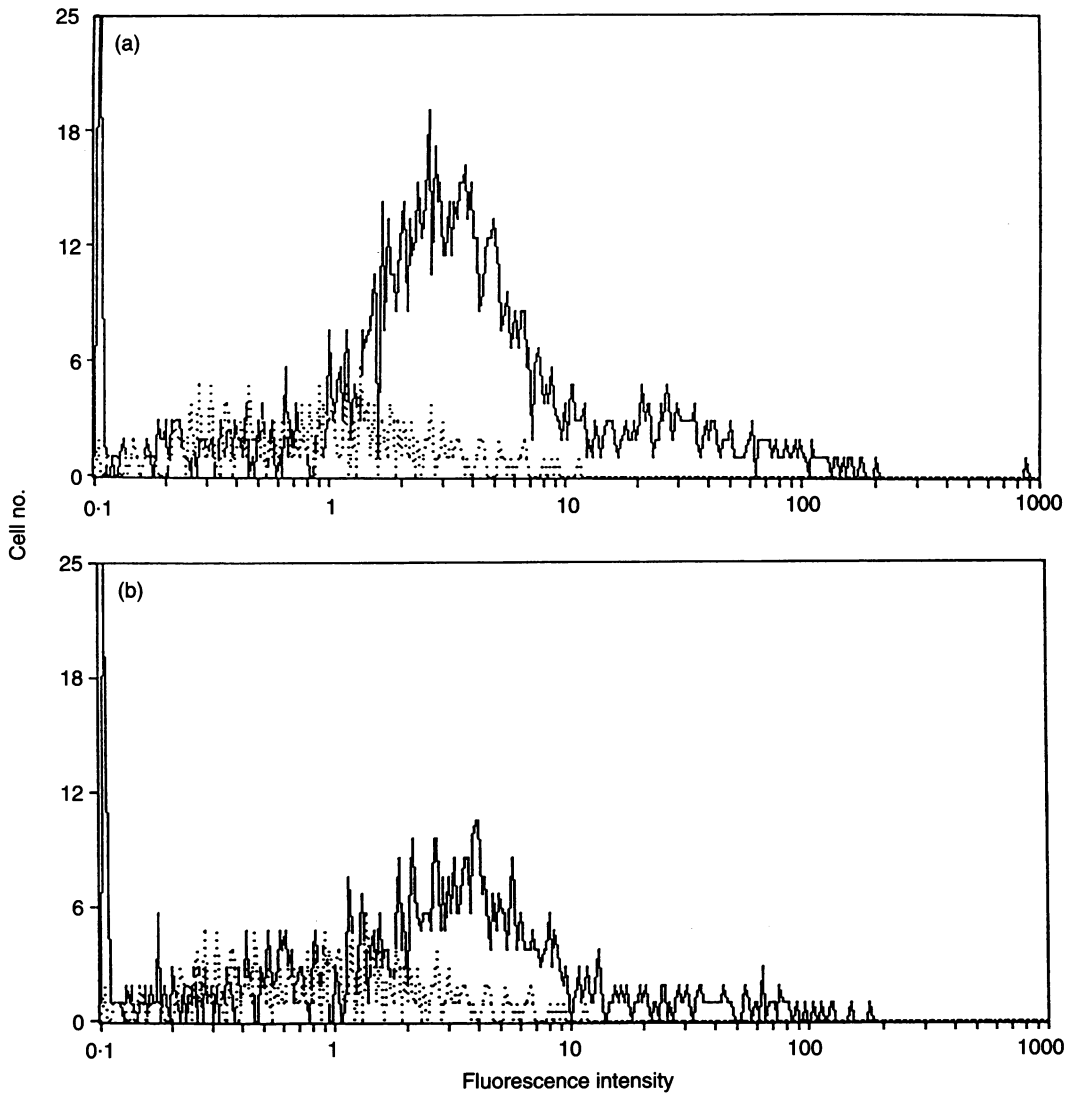


**Figure 3.** GM-CSF up-regulation of CTLA4 ligand expression. Purified DC were incubated sequentially with CTLA4-Ig followed by FITC- anti-Ig, as detailed in Materials and Methods. (a) Freshly isolated DC (broken line) versus DC cultured 48 hr in GM-CSF supplemented medium. (b) Freshly isolated DC (broken line) versus DC cultured 48 hr in GM-CSF supplemented medium containing $10^{-6}$ M DX.

class II and costimulator expression. In the case of CTLA-4 ligand, splenic DC express relatively high levels when freshly isolated and this is down-regulated by DX, whereas airway DC are CTLA-4-ligand low to negative on isolation and do not express the latter unless pre-cultured with GM-CSF (Fig. 3a); the latter process again was DX resistant (Fig. 3b). Thus, in summary, freshly isolated airway DC functioned poorly as APC in a one way MLR until stimulated with GM-CSF, and this change in functional phenotype was attributable to cytokine-induced up-regulation of CTLA-4 ligand expression, a process which was steroid resistant.

An important difference in relation to steroid sensitivity was observed when presentation of a soluble protein antigen OVA to OVA-immune T memory cells was assessed. In these experiments, OVA pulsing of airway DC was performed in the presence/absence of DX prior to their titration into T-cell cultures. In marked contrast to the results obtained with MLR, effective presentation of the exogenous antigen was almost completely inhibited if antigen pulsing was performed in the presence of the steroid.

We speculate that the disparate results obtained with the two assays measuring T-cell activation by DC reflects subtle differences in TCR ligand generation in the two systems. Thus, it is likely that in the case of MLR, a significant proportion of MHC/endogenous peptide complexes on the surface of the DC may be derived from pre-processed peptide, and additionally a significant component of the MLR response may involve recognition of MHC *per se* on the DC surface.[26] In contrast, the generation of TCR ligands comprising OVA peptides complexed to MHC class II involves the full gamut of uptake, processing and assembly steps required for the generation of immunogenic signals from exogenous antigens. On this basis, one interpretation of our results is that the suppressive effects of DX on the APC functions of DC are restricted to steps upstream from presentation; as a consequence the drug is ineffective in preventing presentation of antigens taken up and

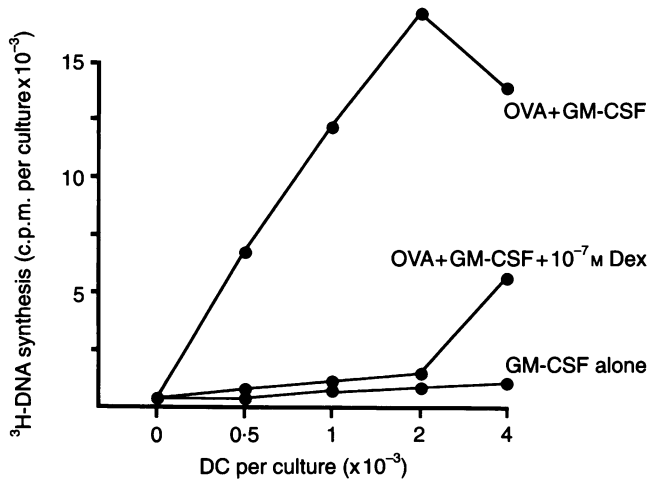


**Figure 4.** Processing and presentation of OVA by GM-CSF-stimulated respiratory tract DC: effect of DX. Purified DC were precultured in GM-CSF and OVA, in the presence and absence of $10^{-7}$ M DX, prior to titration into cultures of OVA-immune LNC which had been depleted of endogenous APC as detailed in.[6] Data shown are median Δ DPM for culture at 96 hr, from a representative experiment; interculture variation was <10% means. The experiment shown was replicated three times.

processed by DC prior to steroid treatment, particularly if (as is the case in atopic asthma[14]) GM-CSF is present in the local tissue microenvironment.

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