

Glucocorticoids drive human CD8⁺ T cell differentiation towards a phenotype with high IL-10 and reduced IL-4, IL-5 and IL-13 production

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Glucocorticoids are highly effective in the treatment of allergy and asthma and inhibit the synthesis of IL-4, IL-5 and IL-13 by disease-promoting CD4⁺ Th2 cells. CD8⁺ T cells also synthesize these cytokines, and the aim of this study was to investigate how glucocorticoids effect cytokine production by these cells. When CD8⁺ T cells are stimulated with anti-CD3 and IL-2 plus IL-4 or dexamethasone, production of the anti-inflammatory cytokine IL-10 is low in both primary and secondary cultures restimulated with anti-CD3 and IL-2 alone. However, when both are present, a synergistic effect on IL-10 synthesis is observed. The additional presence of antigen-presenting cells (APC) in the priming culture maintains IL-10 levels, but inhibits IL-4 and IL-5 production. CD4⁺ T cells develop a similar glucocorticoid-induced phenotype. These cells demonstrate regulatory activity and inhibit CD4⁺ T cell activation in an IL-10-dependent manner. Earlier reports show glucocorticoids promote a Th2 phenotype by effects on purified naive T cells or pretreatment of APC. This study demonstrates, more critically, that when APC are present, glucocorticoids induce CD4 and CD8 T cell populations synthesizing high levels of IL-10, but greatly reduced amounts of disease-promoting IL-4 and IL-5.

Key words: CD8 / IL-10 / Glucocorticoid

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1 Introduction

CD4⁺ T cells play a central role in allergy through the release of Th2 cytokines such as IL-4, IL-5 and IL-13, which regulate the disease process [1]. These cytokines have well-defined effects on the synthesis of IgE, mast cell and eosinophil growth and activation as well as on bronchial hyperresponsiveness. Human and mouse CD8⁺ T cells can also be divided into distinct cytokine-secreting subsets: type 1 or Tc1 characterized by high levels of IFN- γ secretion and type 2 or Tc2 characterized by the secretion of IL-4 and IL-5 [2–6]. Secretion of type 2 cytokines by CD8⁺ T cells has been proposed to explain the observed association between virus infection and the acute exacerbation of allergic asthma [7]. These types of studies suggest that CD8⁺ T cells may also contribute to the pathogenesis of allergy [6–8]. However, a large body of work has focussed on the immunoregulatory potential of CD8⁺ T cells in allergy based on

their capacity to synthesize large amounts cytokines such as IFN- γ and inhibit IgE synthesis by B cells (reviewed in [8]).

Glucocorticoid hormones represent a common and highly effective treatment in atopic asthma and other inflammatory conditions, and are reported to inhibit cytokines synthesized by Th2 and other cells in patients [9]. However, concern has been raised over reports that chronic steroid therapy may result in the generation of a Th2 response. This is based, in part, on *in vitro* studies that firstly describe how secondary restimulation of naive, purified human CD4⁺ T lymphocytes in the presence of steroid can enhance IL-4 and IL-10 production [10]. Secondly, it has been shown that pretreatment of APC with glucocorticoids inhibits their production of IL-12 and enhances IL-10 production, leading to the generation of Th2 cytokine production [11, 12]. The purpose of the present study was to investigate whether glucocorticoids act directly on human CD8⁺ T lymphocytes to influence cytokine profiles, and the effects of APC on this process. This is of interest since human CD8⁺ T cells are reportedly less sensitive than CD4⁺ T cells to glucocorticoid-mediated inhibition of mitogen-induced proliferation [13, 14]. When glucocorticoid hormones

[19859]

were added to CD8⁺ T cells activated in the presence of APC, and thus more analogous to an *in vivo* scenario, this resulted in development of CD8⁺ T cells producing high levels of IL-10, but low to undetectable levels of IL-4, IL-5 and IL-13.

2 Results

2.1 Dexamethasone and IL-4 act in synergy to induce high levels of IL-10 production by human CD8⁺ T cells

To assess the effects of glucocorticoids on cytokine production, purified CD8⁺ T cells were cultured with anti CD3 and IL-2 in the presence or absence of IL-4 (50 U/ml) and 10⁻⁷ M dexamethasone. CD8⁺ T cell populations (CD8⁺) enriched from peripheral blood mononuclear cells were >99% CD8⁺ and contained less than 1% CD4⁺ (Fig. 1), CD14⁺ or HLA-DR⁺ cells (data not shown) as assessed by flow cytometry. These cells remained >99% CD8⁺ following 7 days in culture with no outgrowth of CD4⁺ T cells (Fig. 1e), or NK cells (<0.5% CD56⁺; <0.5% CD8⁺, CD3⁻) in all treatment groups. Culture supernatants were harvested at 24 or 72 h and cytokine content analyzed by ELISA. Stimulation with anti-CD3 and IL-2 alone induced low levels of IL-4 and readily detectable amounts of IL-5, IL-13, GM-CSF and IFN- γ (Fig. 2). As previously shown, IL-4 enhanced IL-5, IL-13 and GM-CSF production. Dexamethasone, in the presence or absence of IL-4, inhibited IL-5, IL-13, GM-CSF and IFN- γ synthesis. IL-10, however, showed a very different pattern of regulation. IL-10 was low or undetectable in cultures stimulated with anti-CD3 and IL-2 alone or plus dexamethasone. Addition of IL-4 induced detectable levels of IL-10. However, when both IL-4 and dexamethasone were added a synergistic induction of IL-10 production was observed.

2.2 Priming of CD8⁺ T cells with dexamethasone and IL-4 enhances IL-10 production in secondary restimulation cultures lacking these mediators

Purified CD8⁺ T cells were cultured with anti-CD3 and IL-2 and combinations of IL-4 and dexamethasone as described above. After 7 days the cells were washed extensively and recultured at 1 $\times$ 10⁶ cells/ml with anti-CD3 and IL-2 alone for analysis of cytokine production in the secondary culture. Mean cytokine values from eight individuals (except IL-13 where *n* = 5) are presented in Fig. 3. Priming with dexamethasone, IL-4 or dexamethasone plus IL-4 enhanced IL-10 production 16-, 7- or 68-fold, respectively. Analysis of T cells staining positive for

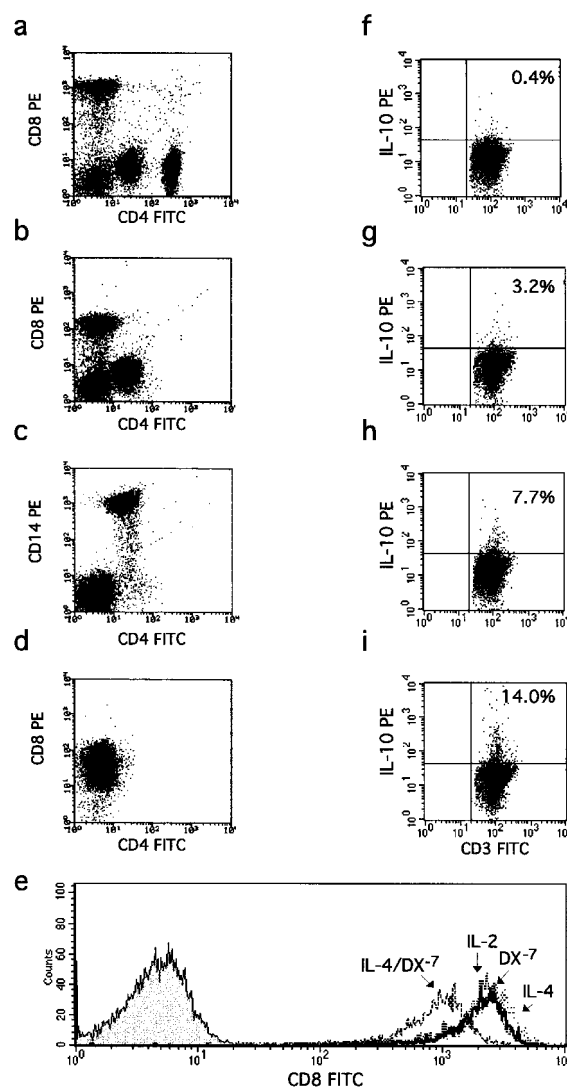


Fig. 1. Representative FACS profiles of human CD8⁺ or CD8⁺ + APC populations before and after culture. Cell purity at day 0: total PBMC (a) were used to isolate CD8⁺ T cell populations containing APC (b) by CD4⁺ T cell depletion. The remaining CD4^{low}-expressing cells were predominantly CD14⁺ monocytes (c). Pure, APC-free, CD8 populations (CD8) were purified by positive selection (d). Cell purity following culture: at 7 days of culture CD8 populations stimulated with anti-CD3 and IL-2 alone (IL-2) or plus IL-4 or/and dexamethasone (IL-4; DX-7; IL-4/DX-7) were stained with isotype control (shaded histogram) or specific antibody to confirm that cells remained CD8⁺ in culture (e). To detect intracellular IL-10 production in secondary restimulation cultures, CD8⁺ T cells were primed for 7 days with anti-CD3 plus IL-2 alone (f) or together with 10⁻⁷ M dexamethasone (g), IL-4 (h) or dexamethasone plus IL-4 (i), washed and restimulated for a further 48 h with anti-CD3 and IL-2 alone prior to staining to detect co-expression of CD3 and IL-10. Cell purity data are representative of five or more experiments, IL-10 data are representative of three experiments.

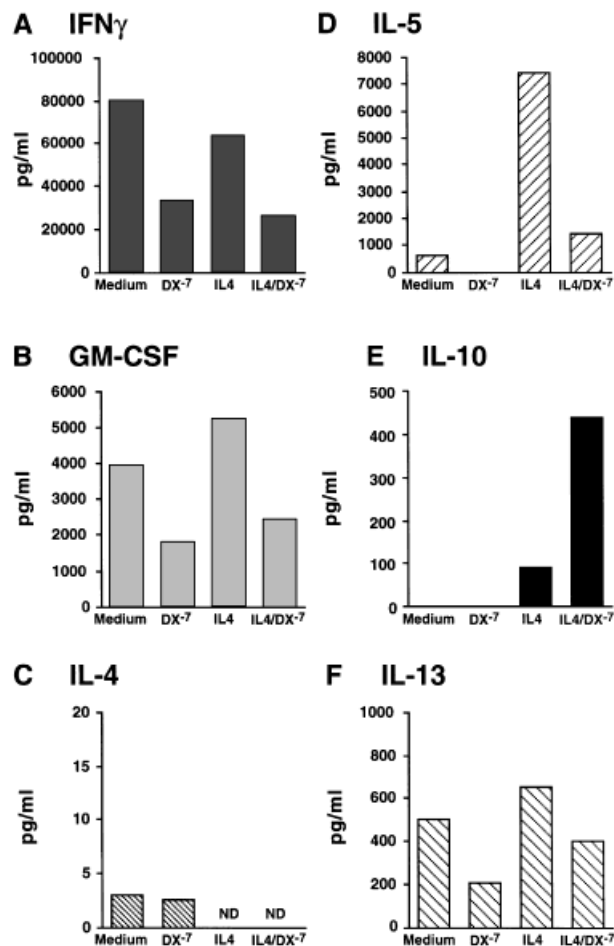


Fig. 2. Dexamethasone and IL-4 act in synergy to stimulate high IL-10 production by CD8⁺ T cells. Purified CD8⁺ T cells (1×10^6) were stimulated with anti-CD3 and 50 U/ml IL-2 (Medium), plus 10^{-7} M dexamethasone (DX-7), plus 50 U/ml IL-4 (IL-4), or with IL-4 and dexamethasone (IL-4/DX-7). Culture supernatants were harvested at the optimal time of 24 h for analysis of IFN- γ (A), GM-CSF (B) and IL-5 (D) or 72 h for the analysis of IL-4 (C), IL-10 (E) and IL-13 (F) production by ELISA. Data are presented as mean pg/ml/ 10^6 cells cytokine from duplicate tests. Standard deviations were <10% in all cases. The data are representative of four experiments. ND = not determined due to the presence of exogenous IL-4 in culture.

IL-10 by flow cytometry in these cultures demonstrated that all IL-10⁺ cells were CD3⁺, and that these IL-10⁺ CD3⁺ T cells increased from 0.4% to 14% following priming in the absence or presence of IL-4 plus dexamethasone, respectively (Fig. 1f, i). Use of reverse transcriptase (RT)-PCR demonstrated increased levels of IL-10-specific mRNA in CD8⁺ T cells primed in the presence of dexamethasone and IL-4 (Fig. 4). This result was confirmed using a ribonuclease protection assay (data not shown). A comparable profile of cytokine production,

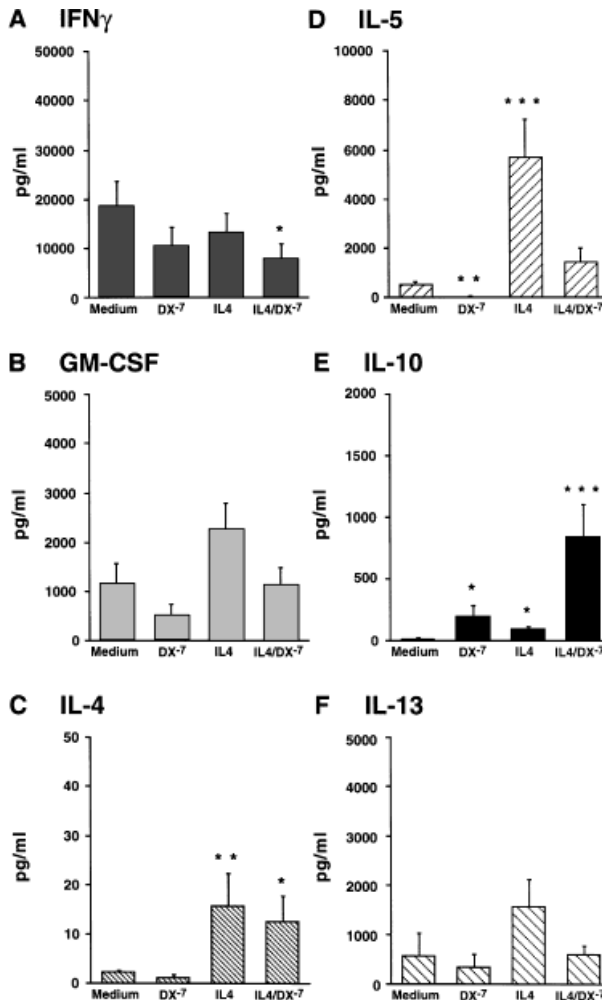


Fig. 3. Priming with dexamethasone leads to the generation of a stable phenotype producing high levels of IL-10. Purified CD8⁺ T cells were cultured with anti-CD3 and 50 U/ml IL-2 plus combinations of 50 U/ml IL-4 and 10^{-7} M dexamethasone as indicated, for 7 days, washed and recultured at 10^6 /ml with anti-CD3 and IL-2 alone for 48 h. Supernatants were analyzed for the indicated cytokine content as described for Fig. 2. Data are presented as mean $\pm$ SEM of cytokine production by purified CD8⁺ T cells isolated from eight individuals (with the exception of IL-13 which is the mean of five). Statistical analysis of cytokine values between treatment groups was performed using the Mann Whitney test. All treatment groups were compared to stimulation with anti-CD3 and IL-2 alone. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

including high IL-10 production, was observed in tertiary cultures, even though IL-4 and steroid were only present during the 1st week of culture (data not shown). IL-4 synthesis in secondary culture was low in cells primed with anti-CD3 plus IL-2 alone. Priming with IL-4 led to a 7-fold increase in IL-4 synthesis in secondary culture, which was only slightly inhibited by dexamethasone. Synthesis

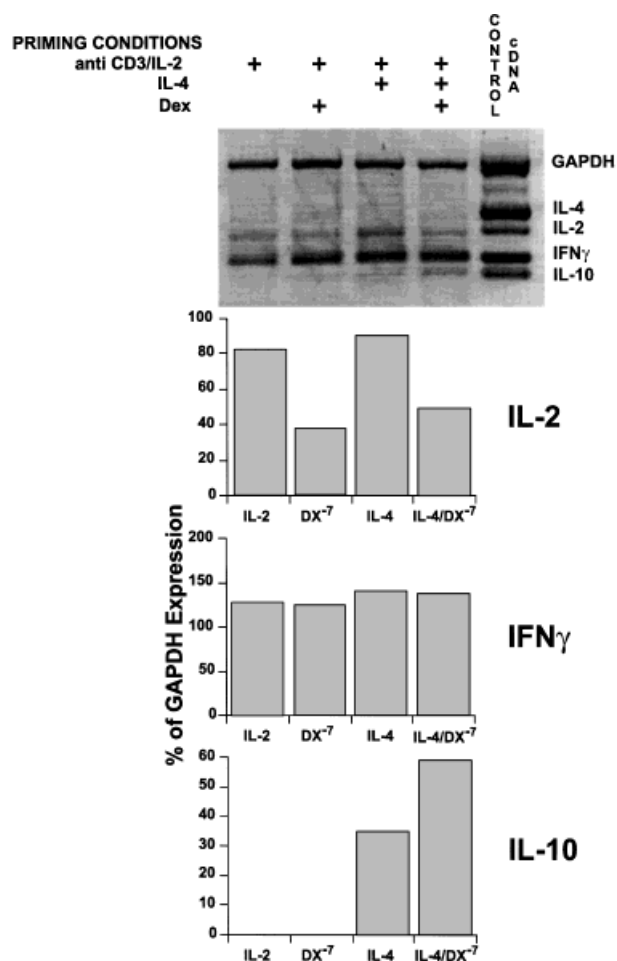


Fig. 4. Dexamethasone and IL-4 enhance expression of IL-10-specific mRNA by CD8⁺ T cells. (A) RT-PCR analysis of IL-2, IL-4, IL-10 and IFN- γ mRNA from purified CD8⁺ T cells after restimulation with anti-CD3 and IL-2 for 18 h. Cells had previously been cultured for 7 days with anti-CD3 and IL-2 in the presence or absence of 50 U/ml IL-4 and 10^{-7} M dexamethasone as indicated. The control lane shows control RNA supplied with kit. The levels of IL-2 (B), IFN- γ (C) and IL-10 (D) were measured by densitometry and are expressed as a percentage of the GAPDH expression. The data is representative of two experiments.

of IFN- γ , GM-CSF, IL-5 and IL-13 showed comparable profiles to those observed in primary cultures.

Preliminary studies suggest that this effect of glucocorticoids is specific to IL-10, since synthesis of a second anti-inflammatory and immunosuppressive cytokine, TGF- β 1, was not enhanced by dexamethasone. TGF- β 1 production was not significantly altered by the four different treatments: mean pg/ml $\pm$ SEM of total (latent and active) TGF- β 1 produced from three individuals following priming with anti-CD3 + IL-2 was 483 ± 453 ; plus 10^{-7} M

dexamethasone, 553 ± 108 ; plus 50 U/ml IL-4, 611 ± 144 ; plus dexamethasone and IL-4, 660 ± 380 .

These data demonstrate that glucocorticoids act directly upon human CD8⁺ T cells to generate a phenotype characterized by high IL-10 production.

2.3 The effect of accessory cells on CD8⁺ T cell cytokine profile generation: induction of high levels of IL-10 production, but diminished levels of IL-4 and IL-5

Under physiological conditions of activation, T cells require signals derived from APC. The development of T cells producing discrete cytokine profiles characteristic of the Th1 and Th2 phenotype has been shown to be dependent on signals derived from the APC and the T cells themselves early in immune responses [15]. The effects of glucocorticoids on cytokine production by purified CD8⁺ T cells or CD8 + APC populations (CD4-depleted PBMC; Fig. 1) was, therefore, compared. When CD8⁺ T cells were primed in the presence of APC (Fig. 5), the levels of IFN- γ , GM-CSF, IL-10 and IL-13 secreted in secondary restimulation cultures (containing anti-CD3 and IL-2 alone) were generally greater than those in cultures of purified CD8⁺ T cells (Fig. 3). However, this was not true for IL-4 or IL-5, which were both greatly reduced. Indeed, IL-4 was frequently undetectable in the presence of APC. These results suggest that both dexamethasone and APC influence the CD8⁺ T cell phenotype induced, leading to production of high levels of IL-10 but reduced amounts of IL-4 and IL-5.

T cells from all donors tested, cultured for 7 days in the presence of APC, steroid and IL-4, showed enhancement of IL-10 production in secondary cultures. In six of eight donors optimal priming for IL-10 production occurred at 10^{-7} M dexamethasone, while two of eight individuals responded optimally at 10^{-6} M steroid (Fig. 6). At no dose tested was there any inhibition of IL-10 production to below that in the absence of steroid. Inhibition of IL-5, IL-13 and IFN- γ was dose dependent and greatest at 10^{-6} M dexamethasone (Fig. 6). IL-4 production by CD8⁺ T cell plus APC populations was not detected in this donor (data not shown).

2.4 Comparison of IL-4 and glucocorticoid mediated up-regulation of IL-10 production by CD4⁺ and CD8⁺ human T cells

The regulation of IL-10 production by IL-4 and steroids in human CD4⁺ and CD8⁺ T cell subsets was compared. Both CD4⁺ and CD8⁺ T cells produced maximal amounts

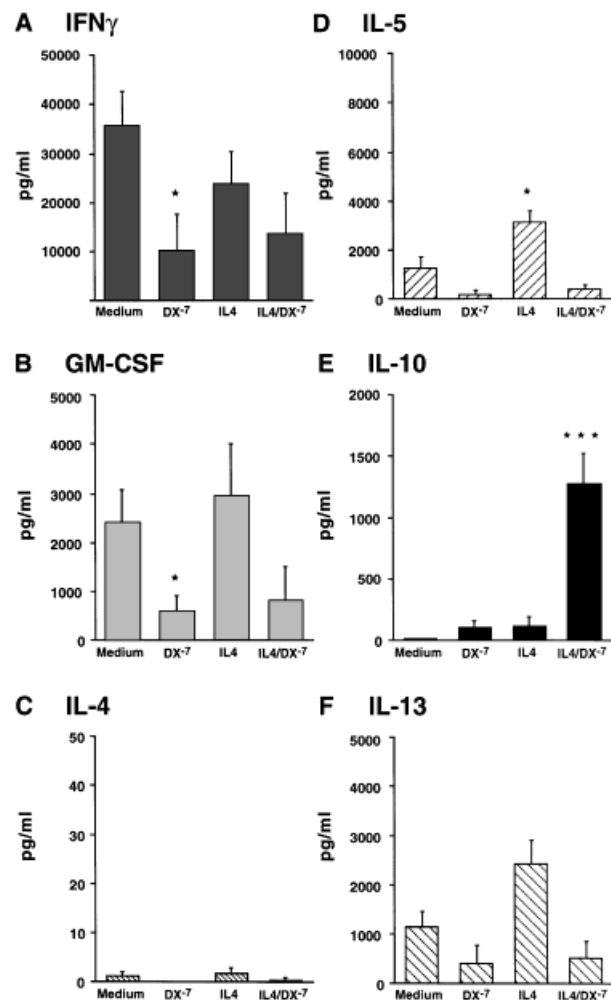


Fig. 5. Priming of CD8⁺ T cells in the presence of APC maintains IL-10 levels but inhibits production of Th2 cytokines. CD8⁺ T cells were cultured in the presence of APC and stimulated as described for Fig. 3. Data represent mean $\pm$ SEM cytokine production by cells isolated from six individuals (with the exception of IL-4 and IL-13 which are the mean of five individuals). Statistical analysis as for Fig. 3.

of IL-10 when primed with anti CD3 plus IL-2, IL-4 and dexamethasone and then restimulated with anti-CD3 and IL-2 alone. However, overall CD4⁺ T cells generally showed much higher levels of production of IL-10. Stimulation with anti-CD3 and IL-2 alone induced a high level of IL-10 synthesis and addition of dexamethasone to priming cultures induced a smaller increase in IL-10 release in comparison to CD8⁺ T cell cultures. Peripheral blood CD4⁺ T cell populations contain a higher percentage of antigen-experienced cells in comparison to CD8⁺ T cell populations [16]. To address, therefore, whether the high level of IL-10 induced by anti-CD3 and IL-2 alone was due to higher levels of antigen-experienced

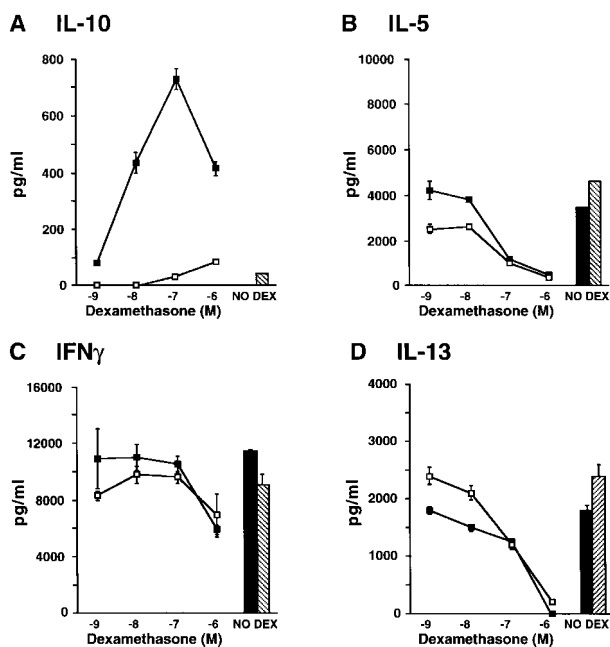


Fig. 6. Optimal steroid concentration for inducing IL-10 production. CD8 + APC were stimulated with anti-CD3 and IL-2 in the absence (open squares/solid bars) or presence (closed squares/hatched bars) of 50 U/ml IL-4 and the indicated concentrations of dexamethasone for 7 days and then restimulated with anti-CD3 and IL-2 alone. Culture supernatants from secondary cultures were assayed for (A) IL-10, (B) IL-5, (C) IFN- γ and (D) IL-13 content at 48 h by ELISA. Representative of experiments using eight donors.

cells, IL-10 production by CD4⁺ CD45RA⁺ (naive) versus CD4⁺ CD45RO⁺ (antigen experienced) T cells was compared. The latter made large quantities of IL-10 following priming with anti-CD3 and IL-2 alone, while IL-10 production by CD4⁺ CD45RA⁺ cells under these conditions was below the level of detection (Fig. 7B). Higher numbers of antigen-experienced CD45RO⁺ T cells within CD4⁺ T cell compared to CD8 T cells preparations may, therefore, contribute to the observed differences in magnitude of IL-10 synthesis between CD4 and CD8 T cells. A further difference between the two T cell subsets was the lack of synergy between IL-4 and dexamethasone in inducing IL-10 synthesis by CD4⁺ T cells (Fig. 7A) in many donors. It is possible that small amounts of endogenous IL-4 produced by the CD4⁺ T cells brought about this process in CD4⁺ T cells [17]. In the experiments described above with CD8⁺ T cells, high IL-10 production, together with low IL-4 and IL-5 synthesis, was observed when APC were also present in priming cultures. APC also down-regulated IL-4 and IL-5 synthesis by CD4⁺ T cells, but did not affect IL-10 levels (Fig. 7C). Thus, the development of CD4⁺ T cells producing high levels of IL-10 and reduced IL-4 and IL-5 was also

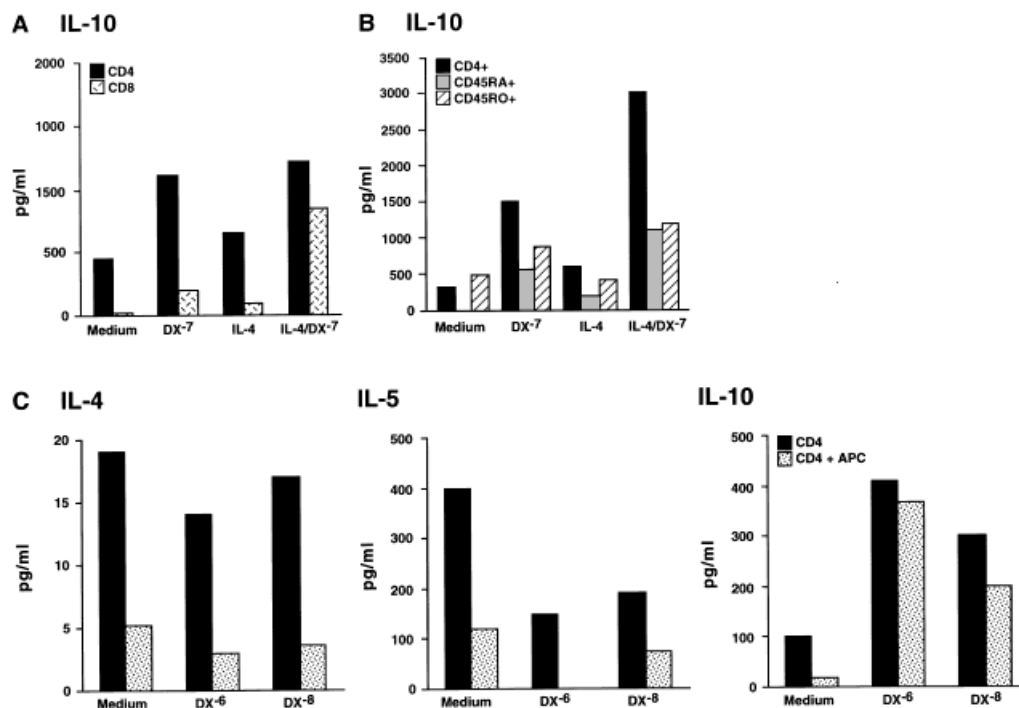


Fig. 7. Comparison of cytokine production by CD4⁺ and CD8⁺ human T cells. (A) The capacity of purified human CD4⁺ (solid bars) or CD8⁺ T (hatched bars) cells to synthesize IL-10 following priming with anti-CD3 and IL-2 in the presence or absence of 50 U/ml IL-4 and 10⁻⁷ M dexamethasone was compared. IL-10 production was measured at 48 h in secondary cultures stimulated with anti CD3 and IL-2 alone. Data are presented as mean from seven and eight individuals, respectively; (B) IL-10 production by CD4⁺, CD4⁺CD45RA⁺ (naive) and CD4⁺CD45RO⁺ (antigen experienced) T cell populations in secondary restimulation cultures was compared following culture as described in (A); (C) CD4 (solid bars) or CD4 + APC T cell (stippled bars) populations were stimulated with anti-CD3 plus IL-2 in the presence or absence of 10⁻⁶ and 10⁻⁸ M dexamethasone for 7 days and then restimulated with anti-CD3 and IL-2. Culture supernatants were assayed for IL-4, IL-5 and IL-10 production at 48 h. Data representative of two individuals.

observed following priming of human CD4⁺ T cells with dexamethasone in the presence of APC.

2.5 Inhibition of T cell activation by IL-10^{high}Th2^{low} T cell lines

IL-10 has well-defined inhibitory effects on human CD4⁺ T cell activation [18] and APC function [19]. It was, therefore, of interest to investigate whether IL-10-secreting T cell lines generated following priming with IL-4 and dexamethasone exhibit regulatory function and inhibit T cell function in a manner comparable to recombinant IL-10. Supernatants were generated from these cells and assessed for their capacity to regulate cytokine production and expression of the T cell activation marker CD25. Freshly isolated CD4⁺ T cells plus accessory cells were stimulated with soluble anti-CD3 in the presence of medium, IL-10 or culture supernatant from CD4⁺ and CD8⁺ T cell lines derived under the four standard treat-

ments described above (Table 1). The culture supernatants inhibited IL-2 receptor alpha chain (CD25) expression and IL-2 and IFN- γ synthesis, as determined by flow cytometry, in a manner comparable to recombinant IL-10. The level of inhibition observed was proportional to the amount of IL-10 detected in the respective culture supernatants by ELISA, with the highest level of inhibition occurring with supernatants derived from IL-4 plus dexamethasone primed T cell lines. Furthermore, the inhibitory activity of both IL-10 or supernatants from IL-4 plus dexamethasone primed CD4⁺ T cells, could be reversed by the addition of neutralizing anti-IL-10, but not isotype control, antibodies (Table 1).

3 Discussion

This study shows that priming of human CD4⁺ and CD8⁺ T cells in the presence of corticosteroids and IL-4 leads to the synthesis of high levels of the anti-inflammatory

Table 1. Supernatants from IL-4 plus dexamethasone primed T cells inhibit the activation of freshly isolated CD4⁺ T cells

SN source ^{a)}	IL-10 (pg/ml) ^{b)}	% positive ^{c)}		
		IFN- γ	IL-2	IL-2R
Medium only	n.d.	4.54	5.9	47.5
CD4	75	4.09	6.03	41.7
CD4(IL-4/Dex)	650	2.29	3.22	20.8
CD8	n.d.	5.48	6.3	32.6
CD8(IL-4/Dex)	25	3.6	5.4	26.1
IL-10	3200	0.96	0.47	14.5
Medium only	n.d.	2.4		
CD4(Dex)	200	1.87		
CD4(IL-4)	100	1.39		
CD4(IL-4/Dex)	750	0.84		
IL-10	3200	0.07		
Medium	n.d.			
+CAb		5.3		
+anti-IL-10		5.13		
CD4(IL-4/Dex)	750			
+CAb		3.02		
+anti-IL-10		7.17		
IL-10	3200			
+CAb		2.68		
+anti-IL-10		6.39		

a) Supernatants (SN source) were generated from CD4⁺ or CD8⁺ T cell lines primed with anti-CD3 plus IL-2 in the absence or presence of IL-4 and dexamethasone, as indicated, for 7 days and then restimulated for 48 h with anti CD3 and IL-2 alone.

b) SN were assayed for IL-10 content by ELISA. Values shown represent final concentration in culture. n.d. = not detectable.

c) SN were added, at a final dilution of 50 %, to 0.5–1 ml cultures of freshly isolated CD4⁺ T cells plus accessory cells (10⁶/ml) and stimulated with 1 μ g/ml soluble anti-CD3. The effects of the respective SN versus 100 U/ml (equivalent to 3,200 pg/ml) IL-10 on IL-2R (CD25) expression and IL-2 and IFN- γ synthesis by the CD4⁺ T cells was assessed at 48 h by flow cytometry. In some cultures neutralizing anti-IL-10 or isotype control antibody (CAb) were added at a final concentration of 1 μ g/ml. Data are expressed as % cytokine or cytokine receptor positive CD4⁺ T cells from three experiments. Antibody isotype control values have been subtracted and were $\leq$ 0.5 % in all cases. Freshly isolated unstimulated CD4⁺ T cells were typically <1 % IL-2R⁺, <0.2 % IL-2⁺ and <0.2 % IFN- γ ⁺, respectively.

cytokine IL-10 and reduced Th2 cytokines, which are responsible for regulating the allergic response. This phenotype is maintained in secondary and tertiary cultures no longer containing IL-4 or steroid. The production of undetectable or low levels of IL-4 and IL-5 is critically dependent upon the presence of APC in priming cultures. Culture supernatants from T cell lines generated in the presence of dexamethasone and IL-4 exhibited regulatory activity and inhibited IL-2 receptor expression and IL-2 and IFN- γ production. This activity was abrogated in the presence of neutralizing anti-IL-10 antibody.

Glucocorticoids represent a common and highly effective treatment in inflammatory conditions such as asthma. In atopic asthma, therapy with corticosteroids has been shown to result in reduced mRNA expression for IL-4 and IL-5 in cells in bronchoalveolar lavage fluid (BALF) [9, 20] or bronchial biopsy specimens [21]. Nasal biopsy samples from patients with allergic rhinitis similarly show decreased IL-4 mRNA following topical administration of fluticasone [22]. Despite these encouraging reports, concern exists regarding the possible consequences of long-term chronic treatment with corticosteroids and their potential to drive CD4⁺ T cell differentiation towards a Th2 phenotype.

The original studies suggesting that treatment of CD4⁺ T cells with glucocorticoids can promote Th2 cytokine production were performed in mice [23], and were followed by experiments using rat [24] and human cells [10]. Differences exist between these studies which may relate to the use of different species. The present discussion, therefore, will focus on studies with human cells. Two distinct experimental approaches were taken that both resulted in promotion of a Th2 phenotype. In the first dexamethasone pretreatment of human monocytes enhanced their capacity to stimulate IL-4 and IL-10 and inhibit IFN- γ and IL-2 production by CD4⁺ T cells [11]. In the second approach flow cytometry-sorted, human, naive CD4⁺ CD45RO⁻ T cells were stimulated by plate-bound anti-CD3 and IL-2 in the absence of APC. If corticosteroids were present during restimulation of these effector cells, they enhanced the production of IL-4. Restimulation of effector cells generated from a CD4⁺ CD45RO⁺ memory T cell population with corticosteroids did not enhance IL-4 production [10]. In the present study, the simultaneous co-culture of T cells with APC was critical for the generation of a phenotype of high IL-10 and low IL-4 and IL-5 production.

The mechanism whereby APC influence T cell phenotype is unclear and may represent glucocorticoid-dependent and -independent effects. Several properties of APC are known to influence cytokine secretion by T cells [15]. For

example, IL-4 and IL-5 production is down-regulated by IL-12 [11]. More recently, in two distinct experimental systems the presence of ICAM-1 or ICAM-1 plus ICAM-2 was found to exert a profound inhibitory effect on IL-4 production [25, 26]. Although glucocorticoids inhibit both IL-12 production and ICAM expression by APC, if inhibition is only partial, as in the case of ICAM-1 [27], these mechanisms may contribute to the observed T cell phenotype.

The regulation of cytokine synthesis by human CD4⁺ and CD8⁺ T cells was compared. Under all but optimal conditions of stimulation, namely priming with both IL-4 and dexamethasone, CD4⁺ T cells synthesized much greater amounts of IL-10 than autologous CD8⁺ T cells. Furthermore, CD4⁺ T cells from many donors did not require the addition of IL-4 in the priming culture for maximal induction of IL-10, and one cannot rule out the involvement of endogenously produced IL-4. Indeed, a recent study demonstrated that blocking of IL-4 action with anti-IL-4 receptor antibodies inhibited the PGE₂-mediated enhancement of IL-4 and IL-10 production by naive CD4⁺ T cells. This occurred even though no exogenous IL-4 was added to these cultures [17]. This data implies that endogenous IL-4 synthesis by CD4⁺ T cells can regulate phenotype development, supporting our theory. Although CD8⁺ T cells were able to synthesize large quantities of IL-5 and IL-13 following priming with IL-4, under all stimulation conditions large amounts of IFN- γ could also be detected. This can antagonize Th2 development and B cell synthesis of IgE and may inhibit eosinophil recruitment [28, 29]. Consideration of IFN- γ levels is, therefore, also likely to be important when evaluating the role of CD8⁺ T cells in allergy [8].

The synthesis of large amounts of IL-10, but low amounts of Th2 cytokines is predicted to be immunosuppressive and indeed inhibition by supernatants from T cells primed in the presence of IL-4 and dexamethasone could be demonstrated. IL-10 inhibits a number of monocyte and macrophage functions, including those associated with antigen presentation for T cell activation. It both inhibits activation and induces anergy in human CD4⁺ T lymphocytes [18], but may also be an important growth factor for CD4⁺ T regulatory cells [30] and, in synergy with IL-2, for some CD8⁺ T cell populations [31]. Despite these diverse effects, *in vivo* studies with IL-10 suggest it is likely to benefit the patient. IL-10 secretion by BALF cells is significantly reduced in allergic asthma [32]. Clinical trials show it is well tolerated and may ameliorate the symptoms of a number of inflammatory conditions, including those associated with endotoxemia [33], inflammatory bowel disease [34] and rheumatoid arthritis [35]. Thus, the capacity of either conventional therapy [36–39] or immunotherapy [40] to promote IL-10 synthe-

sis is predicted to be highly beneficial to the patient.

A large body of work has historically proposed the existence of subsets of immunoregulatory T cells. These prevent induction of harmful immune responses to innocuous antigens introduced into the body at mucosal surfaces, such as the gut or respiratory tract, as well as limiting damage to the host by potentially autoreactive T cells. Studies in animal models and with human cells suggest the likelihood of several different populations with distinct antigen specificities acting through a variety of effector mechanisms (reviewed in [15, 41]). The most comparable cells to the ones described in the present system are those defined from mice and humans which secrete IL-10 and TGF- β , but little or no IL-4 and inhibit antigen-specific immune responses *in vitro* and *in vivo* [30]. The present studies suggest that a similar type of T regulatory subset may also be induced during therapy of the host with glucocorticoids. In our system, increased IL-10 production was accompanied by down-regulation of IL-5 production. In contrast, the T regulatory cells described by Groux et al. [30] showed high levels of IL-5 and IL-10 production but decreased IL-2 and IL-4 synthesis. A further subset of immunoregulatory human T cells, called Th3, are characterized by the production of high levels of TGF- β [42]. They are proposed to regulate induction of oral tolerance and clearly differ from the cell phenotype described herein. The capacity of glucocorticoids to differentially modulate cytokine production by human T cells, leading to increased IL-10 synthesis but reduced production of the classic Th2 cytokines associated with allergy, imply distinct signaling pathways may regulate their synthesis. Since IL-10 inhibits APC function and induces anergy in CD4⁺ T cells, these *in vitro* effects are consistent with the known clinical effects of glucocorticoids in inflammatory disease.

4 Material and methods

4.1 Cell purification

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood, freshly collected into sodium citrate, by centrifugation on a Lymphoprep (Nycomed, Birmingham, UK) density gradient. Donors (8 male, 2 female) were between 28–49 years old (mean 32.7 $\pm$ 5.8 years), healthy at the time of study and not taking glucocorticoid medication at least 2 weeks prior to inclusion in the study. Four of the ten donors were atopic as defined by the presence of clinical symptoms and at least one positive skin test reaction (3 mm greater than diluent control) to a panel of allergens (*Dermaphagoides pteronyssinus*, birch, grass and cat). PBMC were positively selected for CD8⁺ or CD4⁺ T cells using antibody-coated magnetic beads and Detach-a-beads [Dynal (UK), Wirral, GB] according to the manufacturer's

guidelines. Alternatively, PBMC were depleted of CD4⁺ or CD8⁺ T cells by negative selection using antibody-conjugated magnetic beads to give CD8⁺ + APC or CD4⁺ + APC populations, respectively. In some experiments CD4⁺ T cells were further subdivided into naive CD45RA⁺ and antigen-experienced CD45RO⁺ populations using specific antibodies (PharMingen) and negative selection with magnetic beads as previously described [43]. APC consisted predominantly of CD4^{low}, CD14⁺ monocytes (13–28%), although a minor population (1.3–5%) of CD4^{low}, CD14⁺ cells is likely to contain DC (Fig. 1). Isolated populations were washed and resuspended at 1×10^6 /ml in RPMI 1640 (Life Sciences, Abingdon, GB) containing 10% heat-inactivated FCS (PAA Laboratories, Oxford, GB), 2 mM L-glutamine and 50 µg/ml gentamycin (both from Life Sciences).

Cell purity was assessed by flow cytometry using a FACScan (Becton Dickinson, Abingdon, GB) and Lysis 2 software. Anti-CD4 (Leu-3a) and CD14 (Leu-M3) antibodies were purchased from Becton Dickinson; CD3 (UCHT1), CD8 (UCHT4), isotype-matched control IgG₁ (MOPC21) and IgG_{2b} (MOPC141) antibodies were from Sigma (Poole, GB).

4.2 Cell culture and stimulation

Purified T cells or T cells plus accessory cells (T + APC) at 1×10^6 in 1 ml were stimulated in 24-well tissue culture plates pre-coated with 1 µg/ml OKT-3 antibody (purified in-house) and previously determined optimal concentrations of human IL-2 (Eurocetus, Harefield, GB) with or without IL-4 (NBS, Hatfield, UK) and dexamethasone as indicated (Sigma). Cells were incubated for up to 7 days at 37°C/5%CO₂ with a 50% change of media on day 3. On day 7 cells were washed and resuspended at 1×10^6 /ml and cultured with anti-CD3 and IL-2 alone. Supernatants for analysis of cytokine production were harvested as indicated. At 24 h all stimulation cultures were between 72–75% viable as determined by double staining with propidium iodide and annexin V (AMS Biotechnology, Whitney, GB). After 7-day culture mean cell recoveries from 1×10^6 cells from all treatment groups were not significantly different. From purified CD8⁺ T cells, cell recovery was between 1.2×10^6 and 1.3×10^6 and from CD8⁺APC between 0.9×10^6 and 1.3×10^6 cells.

In experiments to assess effects on T cell function, freshly isolated CD4⁺ T cells plus accessory cells were prepared as described above, and stimulated for 36 h with anti-CD3 alone, or plus 100 U/ml IL-10 (R&D Systems, Abingdon, GB) or 50% culture supernatants from T cell lines as indicated. Cells were then stained for co-expression of CD4 and the T cell activation marker CD25 (Becton Dickinson). Alternatively immunofluorescence staining and flow cytometric analysis was also used to assess IL-2 and IFN-γ-producing cells in these cultures. All reagents for this were purchased from PharMingen and used according to the manufacturer's instructions. Briefly, Cytofix/Cytoperm Plus (with GolgiStop)

containing monensin was added to the above cultures for the final 16 h of culture to block intracellular transport processes, thereby resulting in accumulation of cytokine within the cell and enhancing the cytokine staining signal. Live cells were then stained for the T cell-specific surface marker CD4, fixed and permeabilized prior to incubation with specific antibody for detection of intracellular IL-2 and IFN-γ.

4.3 ELISA

An ultrasensitive IL-4 ELISA was purchased from BioSource Europe (Watford, GB) and had a lower limit of detection of 0.8 pg/ml IL-4. All other cytokines were measured using commercially available matched antibody pairs and following manufacturers instructions. IFN-γ, TGF-β1 and IL-13 antibody pairs were purchased from R&D Systems; IL-10, IL-5 and GM-CSF from PharMingen (Abingdon, GB). Cytokine concentrations were determined by reference to standards purchased from R&D Systems. The lower limit of detection of the ELISA to IFN-γ was 50 pg/ml, for GM-CSF 100 pg/ml, IL-5 200 pg/ml, IL-10 50 pg/ml, IL-13 100 pg/ml and TGF-β1 50 pg/ml. Cytokine levels from different stimulation regimes were statistically analyzed using the Mann Whitney test. In some experiments T cells were also assessed for intracellular expression of IL-10 by flow cytometry using reagents purchased from PharMingen using methodology described above. The only difference between the two protocols was firstly pretreatment during the last 5 h of culture with 1 ng/ml PMA (Sigma) and 400 ng/ml ionomycin (Calbiochem, Beeston, GB) prior to staining, and secondly, T cells were double stained with anti-CD3 and anti-IL-10.

4.4 Cytokine specific RT-PCR and ribonuclease protection assays

Total RNA was isolated from 1×10^6 cells by the guanidinium thiocyanate-phenol/chloroform (Sigma) extraction method using standard techniques [44]. Samples were reverse transcribed using 'You prime first strand beads' (Amersham/Pharmacia, St. Albans, GB). cDNA was then cleaned using one phenol and one phenol/chloroform extraction and resuspended in 100 µl of RNA-free water. A Multiplex PCR kit for IL-2, IL-4, IL-10 and IFN-γ mRNA was purchased from Maxim Biotech (San Francisco, CA) and used according to the manufacturer's guidelines. Briefly, 10 µl of cDNA was amplified by PCR in the presence of primers and 2.5 U Taq polymerase (Roche, Lewes, GB). The cycling conditions were 2 cycles of 96°C for 1 min and 58°C for 4 min, respectively, followed by 28 cycles at 94°C for 1 min and at 58°C for 2.5 min. A final step of 70°C for 10 min was performed. Products were run on a 2% agarose gel containing ethidium bromide (Sigma). The gel was visualized using an Eagle Eye imager (Stratagene, Cambridge, GB) and relative copies of mRNA calculated using the Q-Gel-1d program (Kendrick Labs Inc., Madison, WI). Expression of cytokine mRNA was

also analyzed by multiprobe ribonuclease protection assay (Becton Dickinson-PharMingen). Briefly template DNA (hCK-1) was used to generate ³²P-labeled riboprobes of defined length. Total RNA (10 µg) was added to 2.1×10⁵ cpm of riboprobe for 18 h at 56°C, then treated with RNase A and T1. RNase-resistant duplexes were extracted by phenol chloroform and resolved on 5% sequencing gels.

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