The Immunomodulatory Effect of Cyclophosphamide on Regulation of T-Cell Subsets and Antigen Presenting Cells

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Summary

The effect of cyclophosphamide (CYP) on the function of CD4 (Th1 and Th2) and CD8 T-lymphocytes and antigen presenting cells (APC) was studied. Experiments were performed in a rat allogeneic model using both *in vivo* and *in vitro* techniques. The results show that high doses of CYP inhibited all T-cell functions. By contrast, low doses of CYP (less than 25mg/kg) accelerated kidney allogarft rejection and affected CD4 T-helper rather than CD8 T-cells. At lower doses, CYP inhibited the activation of T-suppressor cell function but the activation of cytotoxicity. Low doses of CYP caused no significant effect on the stimulatory capacity of APC in MLR proliferation and suppressor assays. Lymph node cells from pre-treated animals produce abundant IL-2. In conclusion, CYP at lower doses preferentially inactivates Th2-cell dependent pathways and inducing Th1 type response. Therefore, CYP may selectively manipulate the cellular immune response. This finding could be improved new strategies for allergy treatment and development of cancer vaccine technologies. This report concerns the immunopharmacology aspects of CYP on the activation of Tc and Ts cells, which may be directed by different population of Th1 and Th2 cells.

Key words: cyclophosphamide, immunomodulation, T-cell subsets

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Introduction

Studies of T-cell clones have delineated two major sub-populations of $CD4^+$ T-cells in mice: Th1 and Th2 cells, which differ in their profile of cytokine secretion (Mosmann & Coffman 1989, Fiorentio *et al* 1989, Constant & Bottomly 1997). Th1 cells are involved in DTH, secreting IL-2 and IFN-γ, while Th2 cells are involved in the activation of B-cells, and produce predominantly IL-4, 5, and 10 (Romagnani 1999). Furthermore, Th1 cells activate effector mechanisms including cytotoxic T-cell (Tc), which destroy the graft and cause rejection (Fowler & Gress 2000). By contrast, Th2 cells activate Ts cells and are associated with acceptance of the graft (Fowler & Gress 2000). There is mutual antagonism between the Th1 and Th2 cells mediated by the lymphokines they produce (Romagnani 1999). The outcome of transplantation depends on the differential activation of the Th1 and Th2 cells.

Th1 and Th2 appear to have different susceptibility to immunomodulating agents (Matar *et al* 1998 and 2002, Shand 1979). Cyclophosphamide (CYP) is an alkylating agent with an interesting profile of immunopharmacological activity (Shand 1979). At lower doses, different subsets of T-lymphocytes have differential sensitivity to CYP, with suppressor T-cells (Ts) being the most sensitive (Matar *et al* 1998). It has been demonstrated in certain model systems that CYP enhances the immune response by selective elimination of the down-regulating suppressor mechanisms (Turk & Parker 1982, Matar *et al* 2000). Furthermore, CYP seems to possess selectivity for B-lymphocyte activation , which may reflect the inactivation of T-helper cells (Shand 1979).

The aim of the present study was to obtain further insight into these possible differentiation mechanisms, by studying the effect of CYP on antigen presenting cells (APC), $CD8^+$ and $CD4^+$ T-cells in a rat model of alloreactivity both *in vivo* and *in vitro*.

Materials and Methods

Animals. Male rats aged 8-12 weeks of inbred strains DA (Rtl[a]) and PVG. Rtl[u]7b were obtained from the Animal Unit of Manchester University Medical School, UK.

Administraion of cyclophosphamide. CYP (Endoxan) was dissolved in sterile saline immediately before use and recipient rats (PVG.Rtl[u]7b) were injected with the stated dose (6.25-100mg/kg) by the intraperitoneal route two days before kidney transplantation or the removal of lymph nodes for *in vitro* assays. There was a reduction of 30-70% in lymph node cellularity after CYP treatment.

Renal transplantation. Orthotopic transplants of the left kidney were performed using a microvascular technique. The renal artery, vein, and ureter were anastomosed end to end to the recipient using 10-0 microvascular sutures.

Preparation of cell suspension. Rat lymph nodes (axillary, cervical and mesentric) were aseptically removed and disaggregated Hanks balanced salt solution (HBSS). The cells were washed twice with HBSS. Viable nucleated cells were counted using trypan blue staining under light microscopy and adjusted to 5×10^6cells/ml with complete medium (RPMI 1640 with glutamine (GIBCO) and supplemented with 7% foetal calf serum, 5µM 2 Mercaptoethanol, 0.45mg/ml streptomycin, 0.45mg/ml penicillin G, and 0.9mg/ml kanamycin).

Fractionation of T-cells by magnetic particles. T-cell populations were separated into CD4^{+} and CD8^{+} T-cell subpopulations by a direct method for negative selection of lymphocytes from lymph node cells using immuno magnetic particles (Dynabead). Negative selections were performed using a cocktail of monoclonal antibodies against B-cells (OX-12), MHC class II (OX-6), the IL-2R (OX-39) and either CD4 (W3/25) or CD8 (OX-8). The purity of the isolated subpopulation was always>90% as assessed by using a FACScan (Becton Dickinson) flow cytometer. The isolated subpopulation of responder T-cells were maintained in complete medium for further experiments.

Mixed lymphocyte reaction (MLR). Single cell suspensions from lymph nodes were prepared in complete medium at a concentration of 5×10^6 cells/ml from CYP-treated and normal control PVG.RT1^{u}7b rats. These cells were the responder cells in the MLR. Stimulator cells were prepared from DA rats in the same manner and these were exposed to X-irradiate at 2000 rad (11min irradiation with a caesium source) to prevent further cell metabolism and growth. All assays were performed using a 1:1 stimulator to responder cell ratio in triplicate in 96 well flat bottomed microtitre plates with 200µl per well, and were incubated at 37°C with 5% CO_2 in a humidified incubator. On day 4 of culture, 1µCi of ^{3}H-thymidine (Amersham) was added to each well. On day 5 the cells were harvested on to a glass fiber filter paper and incorporated ^{3}H-thymidine was measured using a β-counter.Bulk mixed lymphocyte culture. A bulk mixed lymphocyte cultures were set up for the cytotoxicity assay and suppressor assays by mixing 10ml aliquots of each cell suspension type (responders and stimulators as above) in a 1:1 ratio into 25ml culture flasks and the cells were incubated for 4-6 days at 37°C with 5% CO_2 prior to further assay.

Cytotoxicity assay. This assay was a 4-h ^{51}Cr-release assay, based on the method of Brunner et al (1968).

Suppressor assay. Viable lymph node cells from day 5 bulk MLR cultures were separated on a ficoll-hypaque gradient and resuspended at 5×10^6 cells/ml in complete culture medium. They were then irradiated and added as modulator cells to a second fresh MLR performed in 96 well plates using responder (PVG.RT1^{u}7b), stimulator (irradiated DA) and modulator (irradiated PVG.RT1^{u}7b anti DA) cells in a 1:1 ratio. Triplicate MLR cultures were set up in flat-bottomed microtitre plates. All cells were added in 50µl volumes (Responder, Stimulator and Modulators) and the final well volumes were adjusted to 200µl with extra culture medium. Plates were then incubated and proliferation was quantitated by the incorporation of ^{3}H-thymidine added on day 4 of culture.

Production and assay of IL-2. Responder cells were obtained from normal or cyclophosphamide pretreated PVG.RT1^{u}7b rats. One ml of responder and stimulator cell suspensions were mixed together in a 1:1 ratio and incubated in 24 well tissue culture plates at 37°C with 5% CO_2 in a humidified incubator for 3 days. On the third day of culture the supernatants were harvested and dialysed against PBS. Supernatants were stored in aliquots at -70°C until they were assayed for IL-2 activity. An IL-2 dependent cell line, CTLL, was used to measure the activity of IL-2 released. 10µl volumes of lymphocyte culture supernatants, diluted 1/2, 1/8 and 1/32, were added in duplicate wells for each dilution into 60 -well Terasaki plates. 10µl of washed CTLL cells was then added to each well at 4×10^5 viable cells/ml in complete medium. The plate was inverted and incubated at 37°C with 5% CO_2 in a humidified atmosphere for 24h, then pulsed with 2µl (1:4 stock dilution) of ^{3}H-thymidine (final concentration was 1µCi) and reincubated for 18h. The culture was harvested and counted using a scintillation β-counter.

Statistical analysis. Results are expressed as Mean±SD of samples. The significance of the differences between means was evaluated by the Student's *t*-test.

Results

Outcome of renal transplantation. Treatment of PVG.RT1^{u}7b recipient rats with cyclophosphamide (12.5-50mg/kg) two days before transplantation with a DA kidney resulted in accelerated rejection, judged by histological criteria, gross morphology and recipient survival.

Effect of CYP treatment on the MLR. When cells from PVG.RT1^{u}7b rats pretreated with CYP were used as responder cells in the MLR the *in vitro* proliferate response was dependent on the dose of CYP administered to the rat. Low doses (6.25-25mg/kg) of CYP caused increased proliferation, but doses higher than 25mg/kg caused a significant decrease in proliferation. The increased responses from CYP-pretreated lymph node were significant different as compared to the untreated controls (Figure 1).
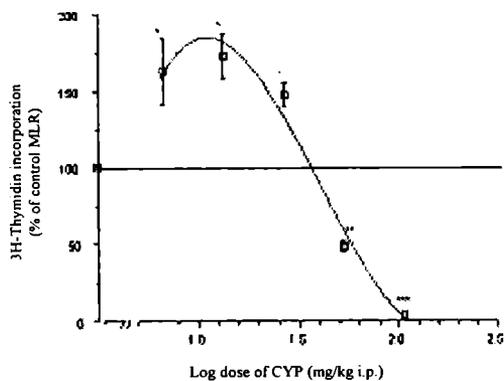
Figure 1. *Dose-response curve for the effect of CYP pre-treatment (i.p.) on the MLR. The results of 3-5 independent experiments were combined and are presented as percent of control response. Data expressed as Mean±S.D (% of control, n=3-5 experiments). ***P<0.005, **P<0.02 and *P<0.05 (unpaired Student's t-test).*

Effect of CYP pre treatment on the generation of cytotoxic T-cells. Pre-treatment of PVG.RT1^{u}7b rats with 6.25-25mg/kg CYP increased the generation of DA-specific cytotoxic T-cells in subsequent allogenic MLR (Table 1). There was no cytotoxic activity of lymph node effector cells form experimental or control rats against third party target cells.
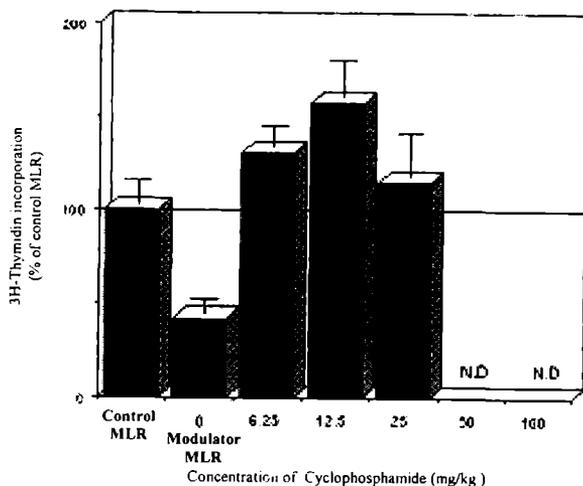
Table 1. *The effect of pre-treatment of PVG.RT1^{u}7b rat with CYP on the generation of cytotoxicity of unfractionated lymph node cells*

Dose (mg/kg) of CYP administered (i.p.) at day –2	Cytotoxic activity of lymph node cells	
	% of specific lysis of ^{51}Cr-labelled target	
	DA 100:1 (E:T)	LEWIS 100:1 (E:T)
0	31±6	-8±5
6.25	35±3	-11±3
12.5	48±8*	5±3
25	40±2*	-1±7
50	27±3	-9±1
100	24±10	-21±8

Effector cells (from day 5 lymph node MLRs, PVG.RT1^{u}7b X DA) incubated with ^{51}Cr-labelled DA-specific target or third party target cells (LEWIS) in a 4h ^{51}Cr-release assay. Data expressed as mean % lysis±S.D. (n=3 experiments), *P<0.05. (E:T=Effector:target ratio)

Effect of CYP pretreatment on the generation of suppressor T-cells. In the lymph node suppressor assay, the modulator cells from CYP-pretreated rats caused

an increase in proliferation in the assay when doses 6.25 and 12.5mg/kg CYP were administered, demonstrating an inhibition of T-suppressor (Ts) cells function. At higher concentrations of CYP (50 and 100mg/kg) complete inhibition of proliferation was observed compared with untreated cells (Figure 2).

suppressor T-cells from their lymph node cells, taken 2 days after treatment, in the MLR suppression was assayed in a second MLR culture to which the activated "modulators" (putative suppressor cells) were added, and in which proliferate response was quantiated by the addition of ^{3}H-thymidine. Control MLR cultures contained no "modulator cells". Other columns are proliferative response in the second MLR after the addition of "modulator cells" prepared from rats pre-treated with 0, 6.25, 12.5 or 25 mg/kg CYP. The results of three independent experiments are combined and are presented as % of control MLR proliferation ^{3}H-thymidine uptake. Values are Mean±SD (n= 3 experiments), ND: Not determined

Figure 2. *The effect of pre-treatment of rats with CYP (i.p.) on the generation of*

Effect of CYP on IL-2 production. Preteatment of rats with CYP at doses 12.5mg/kg were relatively sparing of IL-2 production while inhibition of IL-2 release in MLR cultures was observed at doses of 25mg/kg. At 100 mg/kg, CYP pretreatment completely abolished IL-2 production (Figure.3).

The Effect of CYP on antigen presenting cells. To examine the effect of CYP on the stimulatory capacity of APC, DA rats were treated with doses of CYP ranging from 6.25 to 100mg/kg two days before their lymph node cells were used as stimulator cells in the MLR assay. At lower doses (6.25 and 12.5mg/kg) there was no significant effect on the stimulatory capacity of APC, while higher doses were evidently detrimental to allo-stimulatory capacity. The ability of CYP-pretreated APC to stimulate suppressor cell activation was examined. Again, the lower doses

of CYP did not affect stimulator activity while 25mg/kg reduced APC function for suppressor cells as it did in the MLR proliferation (Table 2).
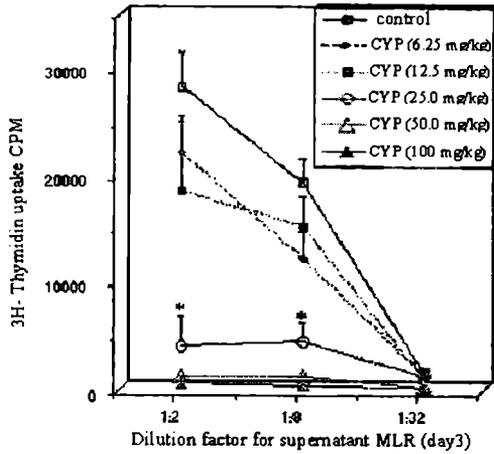

Figure 3. *The effect of pre-treatment of PVG.RTI^{u}7b rats with CYP (i.p) on the production of IL-2 by their lymph nodes, taken 2 days after treatment, in the allogeneic mixed lymphocyte reaction (MLR). The IL-2 in the MLR culture of three days supernate was measured in a bioassay. Data expressed as mean ^{3}H-thymidine uptake±SD (n=3 experiments), *P<0.05 compared to control MLR.*

Table 2. *Effect of pre-treatment of DA rats with CYP on the function of APC*

Dose (mg/kg) CYP administered (i.p.) at day –2	Proliferation assay Lymph node (% of control) ^{3}H-Thymidine uptake	Suppressor assay Lymph node (% of control) ^{3}H-Thymidine uptake
0.00 (control)	100 ± 12.2	100 ± 17.1
0.00 (Modulator)	----------------	64.6 ± 8.2
6.25	106 ± 10.5	86.2 ± 18.5
12.50	126.3 ± 17.6	91.3 ± 14.1
25.00	59.5 ± 18.1	48.7 ± 11.8
50.00	47.1 ± 5.8	N/D
100.00	15.0 ± 3.4	N/D

The results of 3 independent experiments were combined and are presented as % of control response. Pre-treatment with 6.25 or 12.5 mg/kg CYP abolishes the capacity of APC to stimulate Ts cells. Data expressed as mean ^{3}H-Thymidine uptake (% of control)±S.D. (n=3 experiments). N/D=Not determined.

Effect of CYP pretreatment on the proliferative activity of CD4 and CD8 T-cells. CD4 T-cells from rats pretreated with 12.5mg/kg cyclophosphamide gave a significantly higher response in the MLR (Figure 4).

Effect of CYP pretreatment on the generation of cytotoxicity by CD4 and CD8 T-cells. Normal CD4 or CD8 T-cells on their own do not generate good cytotoxic

activity in MLR while the mixture of normal CD4 and CD8 cells does lead to the activation of cytotoxic cells. The same is true for CYP-pretreated CD4 and CD8 T-cells. When CYP-treated CD4 T-cells were mixed with normal CD8 T-cells there was an increase in cytotoxicity while CYP-pretreated CD8 T-cells mixed with normal CD4 produced a lower activity (Table 3).
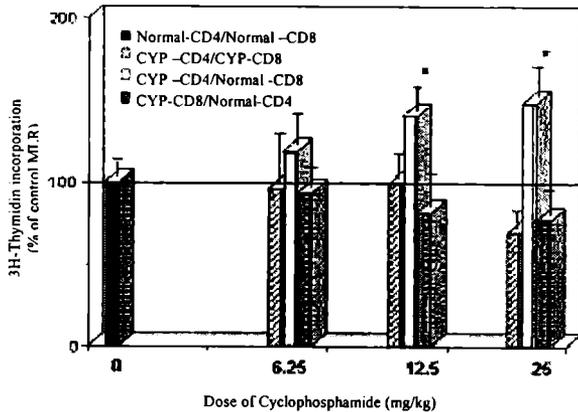

Figure 4. *Effect of cyclophosphamide pre-treatment on the MLR of in vitro fractionated lymph node cells. The results of three independent experiments were combined and are presented as % of control MLR proliferation ^{3}H-Thymidine uptake. Data expressed as Mean±S.D (% of control, n=3 experiments), *P<0.05 (unpaired Student 's t-test).*

Table 3. *The effect of pre-treatment of rats with cyclophosphamide on the activation of cytotoxic T-cells from lymph node cells, taken 2 days after treatment, in the allogeneic MLR*

Fractionated T-cells	Cytotoxic activity of lymph node cells % of specific lysis of ^{51}Cr-labelled target	
	50:1 (E:T)	100:1 (E:T)
Normal CD4$^+$+NormalCD8$^+$	38±5	43±3
Normal CD4$^+$ alone	5±1	7±9
Normal CD8$^+$ alone	-4±3	1±0
CYP-CD4$^+$ alone	1±6	3±2
CYP-CD8$^+$ alone	-3±4	9±8
CYP-CD4$^+$+CYP-CD8$^+$	35±2	30±9
CYP-CD4$^+$+Normal CD8$^+$	56±8*	78±15**
CYP-CD8$^+$+Normal CD4$^+$	13±7***	22±11*

Data expressed as Mean % lysis±SD (n=3 experiments). P values of the comparison between CYP pre-treated cells and the normal CD4$^+$+normal CD8$^+$ combination are as follows: *P<0.05, **P<0.02 and ***P<0.01 (unpaired Student's t-test), E:T=Effector :target ratio

Effect of CYP-pretreatment on the generation of suppressor cells by CD4 and CD8 T-cells. Suppressor cells were not detected in the suppressor cell assay when $CD4^+$ or $CD8^+$ T-cells were pretreated with CYP at the dose of 12.5mg/kg. Pretreatment with CYP at the doses of 6.25-12.5mg/kg completely prevented the generation of suppressor effectors within the $CD4^+$ subsets (Figure 5) and resulted in an augmentation of inducer activity in the presence of CYP.
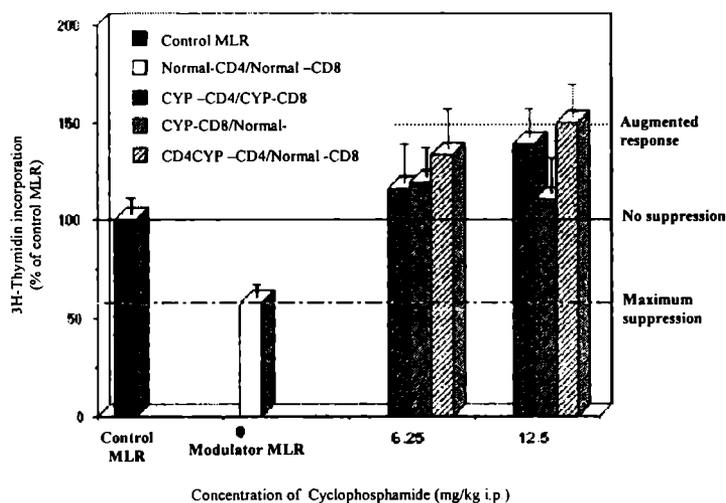

Figure 5. *Effect of cyclophosphamide on suppressor activity in lymph node fractionated MLR. The results of two independent experiments were combined and are presented as % of control MLR proliferation ^{3}H-thymidine uptake. Data expressed as mean ^{3}H-thymidine uptake±range of CYP pre-treated (% of control, n=2 experiments)*

Discussion

Cyclophosphamide (CYP) is an alkylating agent that was used widely in the management of graft rejection (Shand 1979, Matar *etal* 2002). CYP has been available since 1958 (Arnold *et al* 1958). More recently, it has been subjected to extensive investigation as an immunomodulatory drug.

The present study has shown that when low concentrations of CYP are used to pretreat rat, CYP appeared to augment the proliferation response in an allogeneic

MLR (Figures 1, 4), and to inhibit precursors of Ts cells from acquiring suppressor activity (Figures 2,5). There is substantial evidence for an augmentation of the immune response by low doses of CYP (Turk & Parker 1982, Ben-Efrain 2001, Matar *etal* 2002). Other investigators have reported that CYP pretreatment in mice decreased antibody production by spleen cells and low doses of CYP given to mice resulted in an enhanced delayed type hypersensitivity (DTH) response, thus indicating that there was an additional effect on T-cells (Himeno *et al* 1985, Jones *et al* 1987). Ben-Efrain (2001) also reported that low doses of CYP given to cancer patients resulted in enhanced DTH and reduction of suppressor cells activity in melanoma, some improvement in addition to use of melanoma vaccine and it was found to be beneficial for killing tumor cells.

The results in Table 1 show that the induction of cytotoxicity by T-lymphocytes (CTL) was increased at low doses of CYP. The induction of CTL is important in graft rejection, in resistance to viral infection, and in tumour growth (Mosmann *et al* 1997, Berd *et al* 2002). At doses of 6.25-12.5mg/kg CYP pretreatment had no significant suppressive activity (Figure 2). Furthermore, there was no significant inhibition of IL-2 release. Relatively sparing of IL-2 product was, however, sometimes observed. Li et al reported that $CD4^+$ T cell of mice treated with CYP produced IL-2 and IFN-γ and less IL-4 than those of untreated group.

The effect of CYP pretreatment on antigen presenting cells (APC) at these low doses were not significant on the stimulatory capacity of APC and even on their suppressive activities (Table 2). However, a higher concentration of CYP (50mg/kg) diminished both the T-cell proliferation response and the activity of suppressor T cells (Ts) and was also detrimental to allostimulatory capacity of APC. Since most studies are in agreement that CYP is one of the most potent inhibitors of Ts cells, we, therefore, investigated the effect of CYP on CD4 and CD8 fractionated T-lymphocytes. In this study, we found normal CD4 or CD8 cells on their own, with or without pre-treatment of CYP. This did not generate good response in terms of

proliferation and cytotoxic activity, while mixtures of both cells did lead to the proliferation activity and the activation of cytotoxic cells. The generation of cytotoxic T cell (CTL) increased when CYP-treated CD4 T-cells were mixed with normal CD8 T-cells while a decrease response was observed when CYP-treated CD8 T-cells were mixed with normal CD4 T-cells (Table 3). These results suggest that CYP could affect predominantly CD4 T helper cells. Whatever the exact mode of action of the drug, these data suggest that at least part of the immunomodulatory properties of CYP can be explained by the inhibitory effect of the drug on the induction of the immune response by APC, CD4 (Th1 and Th2) and CD8 cells. Lower doses of CYP are associated with an immunostimulatory effect and no morbidity when compared with higher dose (Sardi *et al* 1991, Matar *et al* 2002).

In conclusion, CYP at lower doses affect predominantly the CD4 T-cells and does not significantly affect IL-2 production. CTL activation is increased while Ts and B-cell activation is suppressed. Thus, CYP preferentially inactivates Th2 -cell dependent pathways and inducing Th1 type response. Therefore, CYP may selectively manipulate the cellular immune response. This finding could be improved new strategies for allergy treatment and development of cancer vaccine technologies. This report concerns the immunopharmacology aspects of CYP on the activation of Tc and Ts cells, which may be directed by different population of Th1 and Th2 cells. We have proposed that CYP may interfere with second messenger signaling pathways, through interactions in a subtle way with the cAMP-dependent protein kinase system. The intracellular and molecular mechanistic mode of action of CYP on the immune system are under manuscript preparation and open up a valuable probes of intracellular signaling pathways in T helper cells differentiations.

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