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Counter-Regulation of T Helper 1 Cell Proliferation by Nitric Oxide and Interleukin-2

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It was reported previously that cloned Th1 cells, but not Th2 cells, raised to malaria antigens, produce nitric oxide (NO) when activated with specific antigen or mitogen. Furthermore, NO inhibits the proliferation of, and production of interleukin-2 (IL-2) and interferon-γ by, Th1 but not Th2 cells. By dose-response analysis, I demonstrate here that Th1 cells produce optimal levels of IL-2 and a proliferative response, and no detectable NO, when stimulated with relatively low concentrations of antigen or mitogen *in vitro.* As the antigen/mitogen increased, however, high levels of NO were produced, accompanied by a concomitant reduction in IL-2 secretion and T cell proliferation. At the highest concentrations of antigen/mitogen examined, addition of recombinant IL-2 reversed the NO-mediated downregulation of T cell proliferation. These results suggest that NO may serve as a self-regulatory molecule preventing the over-expansion of Th1 cells. At the other extreme, exogenous IL-2 may act to counter-regulate the suppressive effect of high concentrations of NO on Th1 cell proliferation, thereby maintaining homeostasis.

Nitric oxide (NO), derived from molecular oxygen and the guanidino nitrogen of L-arginine in a reaction catalysed by NO synthase (NOS), is a crucial mediator for a variety of biological functions (1-4). Thus, it is involved in vascular and muscular relaxation, platelet aggregation and central and peripheral neurotransmission. Most mammalian cells constitutively produce a low level of NO through the constitutive enzyme NOS. A number of cell types, including macrophages, neutrophils, hepatocytes and endothelial cells, when activated by immunological stimuli such as interferon-γ (IFN-γ), tumour necrosis factor-α (5, 6), migration inhibitory factor (7) or lipopolysaccharide (8), may be induced to produce in large amounts NO, which is an important effector in the destruction of pathogens and tumours and in potentially damaging immune responses (9). However, the precise role of NO in immune regulation remains to be explored. A number of reports (10-12) showed that NO can inhibit the proliferation of spleen cells *in vitro.* The mechanism of such inhibition is unknown. We have addressed this question using a panel of cloned T helper type 1 (Th1) and type 2 (Th2) cell lines specific for malaria antigens. The Th1 cells produce interleukin-2 (IL-2) and IFN-γ but no detectable IL-4 or IL-10, whereas the Th2 cells produce IL-4 and IL-10 but not IL-2 or IFN-γ (13). Both the Th1 and Th2 cells can protect mice against malaria infection (14). Th1 cells protect, in part at least, via the L-arginine:NO pathway, whereas Th2 cells protect by enhancing a specific IgG1 antibody response. We demonstrated recently (15) that the cloned Th1 cells but not the Th2 cells express high levels of inducible NOS (iNOS) and produce large amounts of NO when activated with specific antigens or with the T cell mitogen concanavalin A (Con A) *in vitro,* consistent with an earlier report of NO synthesis by cloned CTLL and HT2 cell lines (16). In addition, NO inhibits Th1 but not Th2 cells. Thus, the proliferation of, and IL-2 and IFN-γ production by, the cloned Th1 cells *in vitro* were progressively and completely inhibited by the presence of the NO donor S-nitroso-N-acetyl penicillamine, which has now been confirmed in activated human Th1 cells (17). The proliferation of, and IL-4/IL-10 production by, the similarly activated Th2 cells were not affected in this murine system (15). The interaction of NO with Th1-type but not with Th2-type reactivities (15) is supported by the recent finding (18) that IL-12 gene expression by macrophages is regulated by NO. Previously, it has been shown that NO may inhibit Th1 cell proliferation by blocking secretion of IL-2, an autocrine mediator of T cell growth (15, 19). Thus, the production

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Abbreviations used: APC, antigen-presenting cells; Con A; concanavalin A; IFN-γ, interferon-γ; IL-2, interleukin-2; NO, nitric oxide; NOS, nitric oxide synthase; NO_{2-}, nitrite; pRBC, parasitized red blood cells.

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of NO by Th1 cells may perform a self-regulating function, preventing the overexpansion of Th1 cells. Here, it is shown that the suppression of Th1 cell proliferation by high concentration NO may be lifted by exogenous IL-2. It is postulated that NO and IL-2 counterregulate Th1 cell proliferation, such that under normal circumstances, it comes under strict control, thereby maintaining homeostasis and preventing an overexpansion of Th1 cells that has been implicated in a range of autoimmune diseases.

MATERIALS AND METHODS

Materials. Culture medium was RPMI-1640 (Gibco) containing FCS (10%), L-glutamine (2mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and 2-mercaptoethanol (50 μM). Con A was obtained from Sigma. The parasitized red blood cell (pRBC) preparation used was a crude homogenate of *Plasmodium chabaudi chabaudi*-infected RBC prepared as described previously (13). The lysed RBC pellet following repeated freeze-thawing was resuspended in PBS, mixed vigorously and the extract microcentrifuged for 1 h at 4°C. The supernatant collected was used as pRBC lysate in the experiments described and has negligible haemoglobin content (19).

T cell clones and culture. Four T cell clones (WEP 996, 997, 998 and 999) were established as described previously (13). They were derived from inbred NIH mice infected with *P. c. chabaudi* following limiting dilution and long term culture with lysates of pRBC. The surface phenotype of each clone was reconfirmed as $CD3^+$, $CD4^+$, $CD8^-$, $TcR\alpha\beta^+$, $TcR\gamma\delta^-$ by flow cytometry (FACScan, Becton Dickinson) prior to this study, using a method described in detail previously (20), which excluded any residual antigen-presenting cells (APC). All the clones are specific for malaria antigens, producing IL-2 and IFN-γ but no detectable IL-4 or IL-10, and helping IgG2a synthesis. They have maintained a stable cytokine secretion profile through five years experimentation to date as reported previously (13-15).

Cells were routinely incubated at 2×10^5 cells/ml as 10 ml volumes in 25 ml tissue culture flasks (Nunc) in the presence of either 1 μg/ml Con A or 200 μg/ml pRBC lysate. For cultures using pRBC, APC (naive syngeneic spleen cells, depleted of RBC by ammonium chloride lysis and irradiated to 30 Gy) were added at 2×10^6 cells/ml. Cultures were maintained at 37°C, 5% CO_2 for up to 6 days, washed and either subcultured (2×10^5 cells/ml, with either 1 μg/ml Con A alone, or with 200 μg/ml pRBC lysate plus 2×10^6 cells/ml APC) for a further 6 days, or incubated in medium alone (2×10^5 cells/ml, without stimulation) for 72 h.

For all experiments, cells which had been rested for the previous 72 h were used. Viable cells were collected by Ficoll gradient centrifugation, washed 2-3× and cultured in a total volume of 200 μl in 96-well flat bottom microtitre plates (Nunc) at 2×10^5 cells/ml in culture medium containing graded concentrations of Con A or pRBC lysate. For experiments using pRBC lysate, APC were added at 2×10^6 cells/ml. In some experiments, graded concentrations of rIL-2 (Genzyme) were included in the culture medium, as indicated in the text or figure legends. T cell proliferation was assayed after 48 h (Con A) or 72 h (pRBC) stimulation by incorporation of $[^3H]$-thymidine (13). At the time of cell harvesting, supernatants were collected and measured for nitrite (NO_{2-}), IL-2 and IFN-γ. Irradiated spleen cells (30 Gy) did not produce detectable levels of NO_{2-} under these culture conditions in the presence of exogenously added IFN-γ and lipopolysaccharide (15).

Anti-NOS treatment. In some experiments, prior to activation with pRBC lysate or Con A, T cells were incubated with MAb to NOS for 12 h (the previously determined optimal incubation period), washed thoroughly, before routine incubation as described above. In order for the MAb to access the intracellular NOS, T cells were reversibly permeabilized by 3 min exposure to an isoosmotically-buffered solution of 1 haemolytic unit of the pore-forming protein streptolysin O (Sigma) followed by recovery in PBS containing 0.001M cholesterol (polyoxyethanyl-cholesteryl sebacate; Sigma), by adaptation of a previously described method (21), conditions which left the cell membrane intact and which did not significantly reduce cell viability. The MAb used were: anti-neuronal (n) NOS; anti-iNOS; and anti-endothelial (e) NOS (all from Calbiochem-Novabiochem), each of which does not cross-react, and which recognises the corresponding NOS isoform in the mouse. The MAb to iNOS was raised against the enzyme extracted from murine macrophages. However, sequencing of the cDNA of iNOS from the Th1 clones used herein has shown that it is identical to the macrophage iNOS, and that the MAb used recognises Th1 cell iNOS by Western blotting (15).

Nitrite measurement. NO_{2-} levels were determined by the Griess method (22). Levels of NO_{2-} reflect NO production *in vitro* by cultured cells (23).

Cytokine ELISA. Two-site sandwich ELISAs were performed to quantify cytokines in cell culture supernatants. For IL-2, S4B6 (provided by R. K. Grencis, University of Manchester, U.K.) and a polyclonal monospecific rabbit anti-mouse IL-2 Ab (provided by F. Y. Liew, University of Glasgow) were coating and detecting Ab (15). IFN-γ was detected using R4-6A2 (provided by S. Landolfo, University of Torino, Italy) for capture and AN-18 (provided by G. L. Spitalny, Trudeau Institute, NY) for detection (20).

RESULTS

Antigen/mitogen dose dependency of IL-2 and NO synthesis. Th1 cells were cultured with increasing concentrations of pRBC lysate or Con A, and the proliferative response and the quantities of IL-2, IFN-γ and NO_{2-} in the culture supernatants were determined. IL-2 was detectable in the supernatant of cells cultured with 5 μg/ml of pRBC lysate or 0.01 μg/ml Con A, and peaked at 200 μg/ml of pRBC lysate or 1 μg/ml Con A. Thereafter, the levels of IL-2 declined progressively with increasing concentrations of antigen or mitogen (Table 1). In contrast, NO was not produced above the background level until above 200 μg/ml of pRBC lysate or 1 μg/ml Con A, and the level increased progressively with increasing concentrations of pRBC lysate or Con A. IFN-γ concentrations and T cell proliferation paralleled the production of IL-2 (Table 1). The dose response of NO synthesis in the antigen- or mitogen-activated Th1 cells examined was therefore distinct from those for the production of IL-2 and IFN-γ, and for T cell proliferation.

Reversal by IL-2 of the NO-mediated inhibition of Th1 cell proliferation. I investigated whether the NO-mediated inhibition observed when Th1 cells are incubated with the NO-releasing agent S-nitroso-N-acetyl penicillamine (15, 17) can be reversed by the addition of exogenous IL-2. At the highest concentrations of antigen/mitogen examined, 400 μg/ml pRBC lysate and 10 μg/ml Con A, addition of rIL-2 reduced levels of NO_{2-} in culture supernatants in a dose-dependent manner (Fig. 1). Concurrently, Th1 cell pro-

TABLE 1

Effects of Graded Concentrations of Antigen or Mitogen on IL-2 and NO_2-Production by, and Proliferation of, Th1 Cells

pRBC(μg/ml) Con A (μg/ml)	0 0	5 0.01	20 0.05	100 0.1	200 1	300 5	400 10
IL-2	<0.5	1.5 ± 0.3	18.7 ± 1.4	24.5 ± 1.8	29.4 ± 1.3	9.0 ± 1.8*	6.5 ± 1.1*
(U/ml)	<0.5	0.9 ± 0.3	16.0 ± 2.1	22.1 ± 1.6	28.7 ± 1.5	15.8 ± 1.4*	7.3 ± 1.2*
NO_2^-	9.4 ± 1.5	10.3 ± 1.7	11.5 ± 2.4	9.9 ± 1.7	14.8 ± 2.6	128.4 ± 18.5†	164.6 ± 21.7†
(μM)	10.8 ± 2.0	14.1 ± 2.1	15.0 ± 1.9	13.3 ± 1.4	16.0 ± 2.3	145.1 ± 22.6†	210.1 ± 28.5†
IFN-γ	<0.5	1.0 ± 0.3	4.3 ± 0.4	9.3 ± 0.5	12.6 ± 0.3	6.0 ± 0.3‡	3.4 ± 0.4‡
(ng/ml)	<0.5	1.6 ± 0.4	6.7 ± 0.6	11.4 ± 0.7	13.5 ± 0.3	8.8 ± 0.4§	3.3 ± 0.5‡
Proliferation	<0.1	1.5 ± 0.2	7.6 ± 1.0	18.5 ± 1.5	24.8 ± 1.9	13.0 ± 1.1§	7.5 ± 0.7‡
(cpm × 1000)	<0.1	3.5 ± 0.6	9.1 ± 0.8	17.7 ± 1.3	21.5 ± 1.6	12.4 ± 1.6§	4.5 ± 0.9‡

Note. Values (mean ± 1 S.D., n = 4) are pooled from 4 replicate experiments, each using one or more cloned Th1 cells WEP 996-999.
* $P < 0.01$, †$P < 0.001$, vs values for 100-200 μg/ml pRBC or 0.1-1 μg/ml Con A.
§ $P < 0.01$, ‡$P < 0.005$, vs values for 200 μg/ml pRBC or 1 μg/ml Con A.

liferation increased with increasing concentrations of rIL-2 (Fig. 1), indicating that addition of rIL-2 reversed the NO-mediated downregulation of Th1 cell proliferation. The inverse relation between levels of NO_{2-} and proliferation was apparent across a broad range of antigen (5-400 μg/ml pRBC lysate) or mitogen (0.01-10 μg/ml Con A) concentrations (Table 1). However, the reversal by IL-2 of the suppression of Th1 cell proliferation by NO was evident only at the highest concentrations of stimuli tested (400 μg/ml pRBC lysate and 10 μg/ml Con A; Fig. 1), when NO_{2-} was maximal, and was not apparent at those concentrations of antigen (200 μg/ml pRBC lysate) or mitogen (1 μg/ml Con A) known to induce optimal proliferation of the cloned Th1 cells examined (Table 2), at which NO_{2-} levels were already low (Table 1).

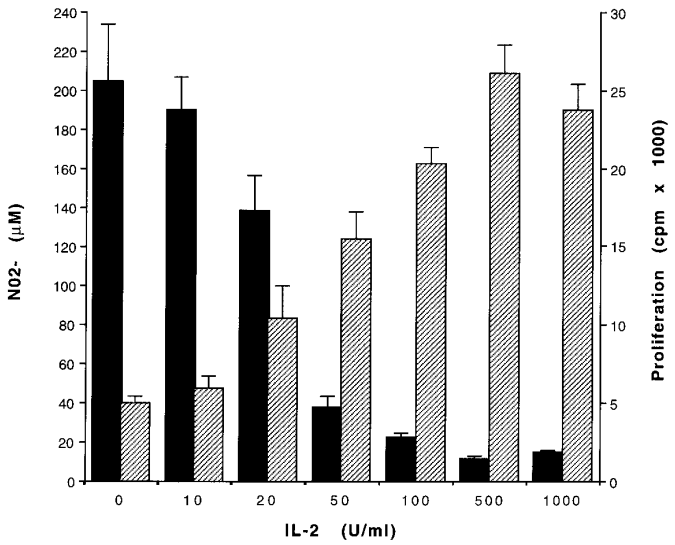

FIG. 1. Reversal of the NO-mediated inhibition of proliferation of Th1 cells by IL-2 at high mitogen concentrations. Cloned Th1 cells WEP 999 were cultured with 10 μg/ml Con A and increasing concentrations of rIL-2, and the level of NO_{2-} (■), and the proliferative response (▨), were determined. Each bar represents mean ± 1 S.D., n =3. Data are representative of 3 separate experiments. Similar results were obtained with all other Th1 clones tested. Similar kinetics of downregulation of NO synthesis by exogenous IL-2 were observed when the same malaria-specific Th1 cells were cultured at 400 μg/ml pRBC lysate with a range of concentrations of rIL-2.

Restoration of Th1 cell proliferation by anti-iNOS Mab treatment. In order to confirm the involvement of NO in the inhibition of Th1 cell proliferation, and the identity of the isoform of NOS involved, cultures of cloned Th1 cells were established at high antigen or mitogen concentrations following preincubation with MAb to either nNOS, iNOS or eNOS (NOS I, II or III, respectively). MAb to mouse iNOS blocked iNOS activity *in vitro,* as determined by a progressive and complete inhibition of production of NO_{2-} in cultures to which graded concentrations of anti-iNOS had been added (Fig. 2). However, MAb to nNOS or eNOS, neither of which show any cross-reactivity with iNOS, did not inhibit NO synthesis in the assay system used, as the levels of NO_{2-} detected were not significantly affected by exposure of Th1 cells to any of the concentrations of anti-nNOS or anti-eNOS tested.

DISCUSSION

The importance of NO as a mediator of immune function has recently come to light (15, 24). A key role for NO in the protective immune response has also been indicated by the susceptibility of mutant mice lacking iNOS to infection with *Leishmania major* (25). The precise role of NO in immune regulation, however, remains elusive.

The substantial literature on NO suggests that,

TABLE 2

Effects of IL-2 on NO_{2-} Production by, and Proliferation of, Th1 Cells

pRBC (μg/ml)		0	5	20	100	200	300	400
Con A (μg/ml)		0	0.01	0.05	0.1	1	5	10
NO_{2-}	Control	10.1 ± 1.6	10.7 ± 1.8	11.4 ± 2.2	10.8 ± 1.9	14.4 ± 1.8	131.4 ± 20.2*	172.6 ± 25.1*
(μM)		10.4 ± 2.2	14.9 ± 2.4	14.7 ± 1.7	12.7 ± 1.6	15.9 ± 2.4	141.7 ± 19.3*	203.9 ± 23.7*
	+ rIL-2	9.7 ± 1.4	11.0 ± 2.1	10.9 ± 1.5	12.8 ± 1.7	15.8 ± 2.2	17.0 ± 2.7†	13.6 ± 1.8†
	(500 U/ml)	10.5 ± 1.7	13.5 ± 1.8	14.6 ± 2.0	13.6 ± 1.5	16.4 ± 2.6	17.3 ± 2.1†	14.1 ± 2.5†
Proliferation	Control	<0.1	1.9 ± 0.4	7.2 ± 1.2	18.1 ± 2.0	25.1 ± 1.7	14.2 ± 1.5§	6.8 ± 1.0‡
(cpm × 1000)		<0.1	3.3 ± 0.7	8.4 ± 1.0	17.5 ± 1.5	22.0 ± 1.6	12.5 ± 1.3§	5.0 ± 0.8‡
	+ rIL-2	<0.1	1.6 ± 0.6	13.9 ± 1.5	25.9 ± 1.9	32.6 ± 2.1	32.1 ± 2.3†	29.6 ± 1.8†
	(550 U/ml)	<0.1	2.5 ± 0.7	15.1 ± 1.3	24.7 ± 2.4	30.8 ± 1.8	30.2 ± 1.9†	26.5 ± 2.4†

Note. Values (mean ± 1 S.D., n = 4) are pooled from 4 replicate experiments, each using one or more cloned Th1 cells WEP 996-999. Similar results were attained using IL-2 at final concentrations of 100 and 1000 U/ml.

*$P < 0.001$, vs values for 5-200 μg/ml pRBC or 0.01-1 μg/ml Con A.

†$P > 0.05$, vs values for 100-200 μg/ml pRBC or 0.1-1 μg/ml Con A.

§ $P < 0.01$, ‡$P < 0.005$, vs values for 200 μg/ml pRBC or 1 μg/ml Con A.

among its many properties, it is microbicidal but in excess it can cause pathology (3). This double-edged sword highlights the tightly controlled regulation of the production of NO that must exist under physiological conditions for the immune system to function appropriately. Wei *et al.* (25), in their experiments with *L. major*, suggest that a low concentration of endogenously generated NO stimulates Th1 cell proliferation, whereas a higher concentration may prevent the possibly deleterious overexpansion of Th1 activities. We have performed a similar investigation using a murine model of malaria, *Plasmodium chabaudi,* the results of which support and extend this notion. The initial findings showed that Th1, but not Th2, cells can express high levels of iNOS (15) and that at higher concentrations, NO may also inhibit their excessive proliferation (15, 19). Here, a series of experiments was undertaken using cloned malaria-specific T cells (13-15, 20) which provides evidence for the way in which NO may regulate Th1 cell proliferation. The way in which these findings relate to other antigenic systems (26) and to freshly isolated lymphocytes is currently under investigation.

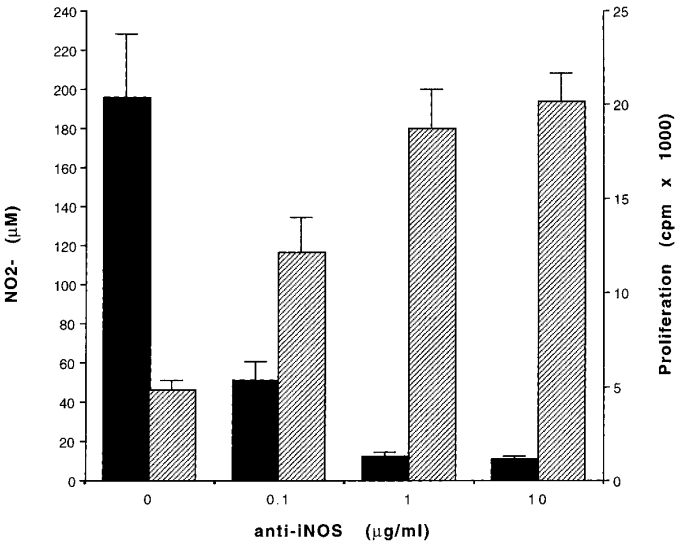

FIG. 2. Reversal of the NO-mediated inhibition of proliferation of Th1 cells by anti-iNOS MAb treatment at high mitogen concentrations. Cloned Th1 cells WEP 999 were cultured with 10 μg/ml Con A and increasing concentrations of anti-iNOS, and the level of NO_{2-} (■), and the proliferative response (▨), were determined. Each bar represents mean ± 1 S.D., n =3. Data are representative of 4 separate experiments. Similar results were obtained with all other Th1 clones tested. Similar kinetics of downregulation of NO synthesis following incubation with anti-iNOS were observed when the same malaria-specific Th1 cells were cultured at 400 μg/ml pRBC lysate with a range of concentrations of the same MAb.

Data presented here demonstrate that NO produced in high concentrations by iNOS can inhibit Th1 cell proliferation, which may act by blocking the synthesis of IL-2, a major autocrine growth factor for Th1 cells (15, 19). As a T cell-potentiating effect of NO on human peripheral blood lymphocytes has been reported (27), a dual effect of NO on T cell proliferation is considered likely (28). At physiological concentrations, provided constitutively, NO may well be required for proliferation of T cells (and of other cell types), while at the higher concentrations produced by iNOS, NO inhibits cellular proliferation. In the present system, NO was produced by antigen-specific Th1 cells rather than by any macrophages in the APC, since irradiated APC do not produce detectable levels of NO under various stimulating conditions (15, 19). Further, Th1 cells mitogenically stimulated in the absence of any APC also secreted NO, while NO was not detectable in the superna-

tants of similarly cultured cloned Th2 cells (15). The activities of Th1 cells are likely to be regulated by NO produced by Th1 cells themselves, since as well as the findings of the present report, we have shown that the addition of the specific NO synthase inhibitor L-N^G-monomethyl arginine significantly enhanced the proliferation (unpublished) and IL-2 and IFN-γ production (15) by Th1 cells *in vitro*. The following scheme is therefore proposed: Th1 cells produce IL-2, which serves as an autocrine growth factor, and IFN-γ, which activates macrophages to produce NO. In addition, Th1 cells also produce NO themselves. When the local concentration of NO reaches a certain threshold level, it prevents further Th1 cell proliferation by feedback inhibition of IL-2 synthesis.

This presents an apparent paradox: if NO is produced by Th1 cells, and also inhibits Th1 cell proliferation, then how do Th1 cells manage to grow in the first place? This conflict is resolved by dose-response studies which show that Th1 cells produce optimal levels of IL-2 and a peak proliferative response, but little detectable NO, when stimulated *in vitro* with low concentrations of specific antigen or mitogen. As the concentration increases, however, high levels of NO are produced accompanied by a concomitant reduction in IL-2 secretion and thereby Th1 cell proliferation. Thus, the dose-response of IL-2 production and of NO synthesis in activated Th1 cells are distinct. This finding may explain the bell-shaped dose-response curves of antigen- or mitogen-induced T cell proliferation *in vitro* (29).

In conclusion, I suggest that NO may be a self-regulatory molecule, the production of which exerts paracrine control over the expansion, but not the normal functions, of the Th1 subset of $CD4^+$ T cells. Unrestricted growth of Th1 cells has been implicated in a range of immune pathologies (30-32). The mechanism of the inhibition of IL-2 and IFN-γ synthesis by NO is at present unclear. It is likely that the effect of NO is highly specific, since it has little or no effect on the secretion of IL-4 and IL-10 by activated Th2 cells (15). On the other hand, as uncontrolled production of NO may inappropriately prevent Th1 cell proliferation, data presented here also suggest that exogenous IL-2 may reverse the suppression of Th1 cell proliferation imposed by high concentrations of NO. In this way, IL-2 may counter-regulate the activity of NO, thereby optimalising the proliferation of Th1 cells within a given localised *in vivo* environment, and promoting the maintenance of homeostasis. An understanding of the regulatory mechanism(s) exerted by NO and IL-2 on cytokine synthesis by effector cells of the immune system, enabling the judicial local application of these immunoregulatory mediators, would be of considerable therapeutic value to the treatment of infectious, inflammatory and autoimmune diseases.

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