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Diversity of Cytokine Synthesis and Function of Mouse CD4$^+$ T Cells

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INTRODUCTION

The immune response is capable of invoking a variety of different effector mechanisms, each of which is particularly effective against a certain set of pathogens. The regulation of the type of effector mechanisms chosen during an immune response is of critical importance to the host, and thus it is not surprising that this aspect of the immune system is subject to precise and complex regulation. Over the past few years, it has become clear that subsets of T cells secreting distinct patterns of cytokines have a major role in the regulation of the effector functions induced against a particular infectious agent. Two major patterns of cytokine synthesis were initially recognized, and these two patterns appear to correlate with the induction of delayed-type hypersensitivity (DTH) and help for antibody synthesis, thus providing a possible explanation for the separate and often reciprocal regulation of these two responses. Recent information suggests that additional cytokine secretion phenotypes exist, and that there is extensive regulation of the differentiation and effector function of the various cell types.

CYTOKINE SECRETION PHENOTYPES OF TH CELLS

The TH1 and TH2 patterns

Originally defined in a panel of long-term mouse T-helper cell clones, these two phenotypes have been confirmed in strong mouse immune responses, and amongst

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human T-cell clones. TH1 but not TH2 cells secrete interleukin 2 (IL2), interferon-γ (IFNγ) and lymphotoxin (LT). In contrast, TH2 but not TH1 clones secrete IL4, IL5, IL6 and IL10, and express mRNA for the P600 gene (Mosmann et al. 1986, Cherwinski et al. 1987, Fiorentino et al. 1989, Brown et al. 1989). Both TH types express IL3, granulocyte-macrophage colony-stimulating factor (GM-CSF), preprometenkephalin, tumor-necrosis factor (TNF) and three members of the macrophage inflammatory protein family (Brown et al. 1989, Zurawski et al. 1986, Mosmann et al. 1986, Cherwinski et al. 1987). Most of these "common" cytokines are expressed in significantly greater amounts by TH1 clones, but IL3 is expressed at similar levels, and prepromet enkephalin is expressed more strongly by TH2 cells. Additional cytokines synthesized by these two cell types are still being discovered, and studies by two-dimensional gel electrophoresis (T. R. Mosmann, unpublished) suggest that a moderate number of additional secreted proteins have not yet been characterized. The large number of differences between the two cytokine secretion patterns (eight at present), and the substantial number of clones that fit these patterns, indicate that the TH1 and TH2 phenotypes represent distinct, precisely regulated patterns of gene expression.

Cytokine secretion by CD8$^+$ cells

In a large panel of alloreactive CD8$^+$ mouse T-cell clones, most of which were cytotoxic, stimulation by either Con A or antigen resulted in the secretion of a set of cytokines very similar to the TH1 pattern (Fong & Mosmann 1990). A major exception was the synthesis of IL2 by only some of the CD8$^+$ T-cell clones. IFNγ synthesis occurred at high levels and the synthesis of most of the other TH1 cytokines tended to be lower than the amount synthesized by TH1 cells. Lectin-stimulated normal mouse CD8$^+$ T cells also secreted small amounts of IL2, moderate amounts of IFNγ, but no detectable levels of IL4 or IL5. Although these results are all consistent with the production of only the TH1 pattern of cytokines by CD8$^+$ cells, some human CD8$^+$ clones secrete TH2-specific cytokines such as IL4 and IL5 (Paliard et al. 1988), and so it remains possible that additional CD8$^+$ cytokine patterns also exist in the mouse.

Additional TH cytokine secretion phenotypes

The TH1 and TH2 phenotypes were originally defined as very distinct cytokine secretion patterns that were expressed by large numbers of independently derived T-cell clones. Since that original definition it has become clear that a number of other cytokine secretion phenotypes are displayed by T-cell clones in tissue culture, and some of these phenotypes are stable and are obtained in a number of independent experiments. These are likely to constitute additional cytokine patterns and the full extent of the cytokine secretion diversity of T cells has not

yet been established. A phenotype known as TH0 (Firestein et al. 1989, Gajewski & Fitch 1988, Street et al. 1990) expresses most or all of the cytokines made by either TH1 or TH2 clones. Another relatively common pattern is the synthesis of IL2, IL4 and IL5, but not IFNγ (Street et al. 1990). Several other patterns have been observed but it is not yet clear how many of these represent genuinely different cytokine secretion phenotypes that occur *in vivo*, or whether some of these patterns represent *in vitro* artifacts. The full extent of the diversity of cytokine secretion *in vivo* will need to be determined at the single cell level using cells isolated from ongoing immune responses. Since the TH1 and TH2 phenotypes are found most easily after vigorous immunization of the animals used to derive the T-cell clones, it seems likely that the TH1 and TH2 cells represent phenotypes that have differentiated in response to strong and repeated stimulation during powerful immune responses, whereas the TH0 or the IL2, IL4, IL5 patterns may represent cells that are at an intermediate stage of differentiation or that have not been driven as hard by antigen stimulation.

Variability of the phenotype of T-cell clones derived in different laboratories

One of the frustrating aspects of the analysis of mouse T-cell clones is the unexplained differences between laboratories in the ability to grow different phenotypes of T-cell clone. Certain laboratories normally make TH1 clones, other laboratories with very similar methods produce TH2 clones, and some produce a mixture. Within our laboratory, the production of TH1 clones is normally dominant over TH2 clones, but occasionally we obtain predominantly TH2 clones. Table I shows an example of two pairs of experiments performed within a few months of each other indicating the variability that can occur in such cloning experiments. Some of the cytokines regulating the development of either TH1 or TH2 clones are now known. IFNγ tends to result in the isolation of TH1 cells, whereas IL4 drives the production of cells that produce large amounts of TH2 cytokines (Gajewski et al. 1989, Le Gros et al. 1990, Swain et al. 1990b).

TABLE I
Variability in the frequency of isolation of subtypes of T cell clones

		CD8+	TH1	TH0	TH2
C57Bl/6 anti-BALB/c	Exp. 1	0[a]	1	22	14
C57Bl/6 anti-BALB/c	Exp. 2	70	9	1	0
BALB/c anti-CBA/J	Exp. 3	0	0	16	42
BALB/c anti-CBA/J	Exp. 4	0	12	3	5

[a] Limiting dilution allospecific cloning experiments were carried out over a period of a few months with the strain combinations described, using non-immunized mice. The number of each type of clone isolated in each experiment is indicated.

However, these are not the only influences since we have been unable to reproducibly obtain TH1 clones or TH2 clones by combinations of IFNγ, IL4 and antibodies against these cytokines. Although it has been suggested that cloning in IL2 alone will give rise selectively to TH2 clones, we have used recombinant IL2 as the only exogenous cytokine in a large number of cloning experiments and often obtained exclusively TH1 clones by this protocol. Thus it appears that there are other regulatory influences on the differentiation of T cells into the TH1, TH2 and other phenotypes. The differences in the cloning efficiencies of different subtypes between labs may be attributed partly to methological differences, but a large part of the variation may be due to the ongoing immune responses in the mice at the time of sacrifice. This is supported by the results of cloning experiments carried out on strongly immunized mice, in which the clones could be enriched for the predicted cytokine secretion patterns after strong *in vivo* immunization with immunogens known to induce strongly TH1- or TH2-biased responses (Street et al. 1990).

Cytokine secretion by normal T-cell populations

Cytokine secretion by populations of normal spleen cells has also provided considerable information on the potential diversity of cytokine secretion phenotypes. Using spleen cells from relatively "clean" mice, most investigators find that, when stimulated with ConA, such cells produce large amounts of IL2 and low amounts of any other cytokines examined. IFNγ is normally detected at low to moderate levels, although even this cytokine is undetectable in certain experiments. Synthesis of TH2-specific cytokines, such as IL4 and IL5, is very low and occasionally undetectable and even synthesis of the shared cytokines, such as IL3 and GM-CSF, is normally undetectable. This pattern is clearly not consistent with any mixture of TH1 or TH2 cells and, in fact, is quite different from the pattern of any T-cell clone so far described in tissue culture. This suggests the existence of another cytokine secretion phenotype secreting mainly or exclusively IL2 that is expressed only by cells that have not recently been stimulated. This might explain why this pattern has not yet been seen in *in vitro* T-cell clones, which may be more representative of recently stimulated effector T lymphocytes. Although the synthesis of cytokines other than IL2 by normal spleen cells is very low or undetectable, the synthesis of these other cytokines can be rapidly induced following immunization with various strong immunogens. The pattern of cytokines obtained is characteristic of the immunogen; for example, infection by the helminth *Nippostrongylus brasiliensis* (Nb) induces strong production of IL4 and IL5, but not IFNγ, whereas immunization with *Brucella abortus* (Ba) greatly increases the amounts of IFNγ that can be produced in response to subsequent stimulation, but does not significantly affect IL4 and IL5 levels (Street et al. 1990).

The CD44 surface antigen marker has been used to obtain more definition of the normal cells secreting different cytokine patterns. CD44 is expressed at low levels by cells that have not yet been exposed to antigen and at higher levels by stimulated cells and long-term resting, memory cells (Budd et al. 1987a). When normal mouse spleen cells are separated on the basis of the level of expression of the CD44 marker and stimulated with polyclonal activators, both $CD44^{hi}$ and $CD44^{lo}$ populations of $CD4^+$ cells produce similar amounts of IL2. However, synthesis of other cytokines is detectable only in the $CD44^{hi}$ population (Budd et al. 1987b) (T. R. Mosmann, J. H. Schumacher and R. Budd, unpublished). After immunization with Nb, the greatly increased ability to synthesize TH2 cytokines is initially present in both $CD44^{lo}$ and $CD44^{hi}$ populations but after several days this ability is retained only by the $CD44^{hi}$ population. Although this parasite is eliminated from the mouse gut about 4 days after infection, the immune response, measured by the ability to secrete high levels of IL4 and IL5 on restimulation, is maintained for a period of several weeks. Over this time period the increased IL4 and IL5 production is found only in the $CD44^{hi}$ population. This response gradually declines over several weeks, suggesting that the cells that are able to produce high levels of cytokines other than IL2 are produced rapidly in response to an infection and then have a lifetime of several weeks thereafter. The majority of cytokine synthesis for non-IL2 cytokines in the $CD44^{hi}$ population is produced by the small cells indicating that there may be a population of short-term memory cells that are small, resting lymphocytes with the ability to secrete large amounts of cytokines other than IL2 when restimulated. However, this population may not be equivalent to long-term memory cells since large numbers of mouse T lymphocytes from normal mice are $CD44^{hi}$ and yet the predominant cytokine synthesized by this population is only IL2. These data are all consistent with the model in which naive T cells express only IL2 when first stimulated and then immediately differentiate into cells producing large amounts of other cytokines. This occurs both *in vivo* in the experiments described above and has also been shown *in vitro* in short-term stimulation cultures in which cells producing large amounts of cytokines other than IL2 can be generated in as little as 4 d after primary stimulation in culture (Swain et al. 1990a, Salmon et al. 1989). When present during the first few days of culture, IL4 induces a cell population that can secrete relatively large amounts of IL4 on subsequent restimulation (Le Gros et al. 1990, Swain et al. 1990b). IFNγ encourages the production of cells producing more IFNγ and selectively leads to the isolation of TH1 clones during limiting dilution cloning experiments (Gajewski et al. 1989). It is also suspected, although not yet proven, that different types of antigen-presenting cell may have a major role in influencing the choice of the type of TH cell that will develop.

During a primary response, the source of cytokines that might influence subsequent differentiation is not clear. Two possible sources are cell types that can produce cytokines and yet are present in relatively large numbers compared to

antigen-specific, naive T cells. These two cells are mast cells which produce the TH2 panel of cytokines (Plaut et al. 1989) and natural-killer (NK) cells which produce IFNγ. Antigens which are able to activate mast cells, either via antigen-specific or non antigen-specific mechanisms, might induce the production of TH2 cytokines, thus resulting in a bias towards a TH2-like response. Alternatively, antigens such as viruses, which induce activation of NK cells, might preferentially lead to a TH1-like response.

Model of T-cell subset differentiation

The information described above can be assembled into a plausible model of T-cell subset differentiation (Fig. 1). This model suggests that the mature T cell, which has matured in the thymus and migrated to the periphery, is competent to respond to antigen but secretes only IL2 on first contact with antigen plus antigen-presenting cells. This cell then differentiates, under the regulation of different cytokine and APC signals, into a variety of different cytokine secretion phenotypes, such as the TH0 pattern, the IL2, IL4, IL5 pattern and possibly several others. These represent cytokine secretion patterns obtained in situations of short-term stimulation. In very strong sustained immune responses characterized by repeated stimulation, for example during parasite responses, the TH1 and TH2

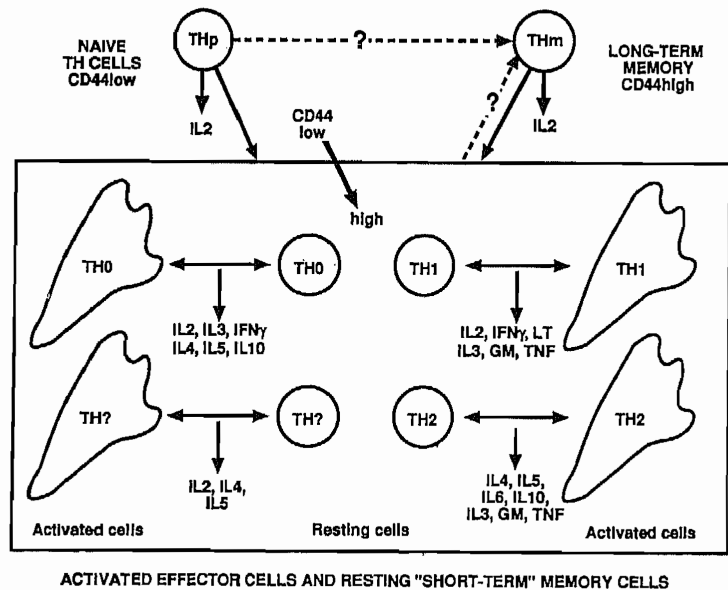

Figure 1. Cytokine secretion phenotypes of T-helper ($CD4^+$) cells.

phenotypes can become dominant. The short-term and long-term stimulated cells in these high cytokine secretion groups are probably the major effector cells of immune responses. Their high production levels of a large number of cytokines make them very potent stimulators and regulators of other aspects of the immune response and many of these cytokines have direct activities on infected cells. It is likely that these cells are relatively short-lived, as least in the high cytokine secretion state. Although short-term memory cells appear to retain the ability to secrete the secondary cytokines upon restimulation, the population as a whole appears to lose this ability after several weeks and it appears likely that the long-term resting, memory T cell produces only IL2 when first restimulated. This model suggests that short-term memory and long-term memory T-helper cells may represent distinct phenotypes. The lineage relationships of these various cells are not clear. The various effector TH phenotypes may all be derived directly by differentiation from the THp precursor cell in one step or, alternatively, it is possible that extreme phenotypes, such as TH1 or TH2 patterns, are derived from intermediate phenotypes. The origin of the long-term memory cells is equally unclear. These cells may be derived from the high cytokine secretion effector phenotypes by loss of the ability to synthesize cytokines other than IL2 or, alternatively, long-term memory cells could be derived directly from the THp cell without passing through a high cytokine secretion stage. CD44 antigen expression appears to be low or absent on the THp population, depending on the mouse strain, but it is expressed at much higher levels on all subsequent populations. Thus, CD44 allows the separation of naive cells from long-term memory cells, but does not aid in the separation of short-term effector cells and short-term memory cells from long-term memory cells. Fig. 2 shows the same model of TH differentiation, including more detail on the possible influences on this differentiation process. The roles of IL4 and IFNγ are indicated and mast cells and NK cells are shown as potential sources of cytokines that may influence this process during the early stages of a primary response. Antigen-presenting cell influences are considered very likely but cannot yet be specified in detail.

Human TH subtypes

After the description of the TH1 and TH2 patterns amongst mouse TH clones, initial studies on human CD4$^+$ clones indicated that the cytokines did not fall neatly into the TH1 and TH2 patterns, but rather showed the TH0 pattern that has been subsequently described also for mouse T cells. After the initial lack of similarity between the two species, subsequent data has revealed that mouse and human T cells are, in fact, quite similar in their range of cytokine secretion patterns, and in both species the type of clone obtained depends very much on the nature of the antigen used. Human T cell clones specific for allergens tend to show the TH2 pattern (Wierenga et al. 1990) while human T cell clones specific

for antigens that give a DTH reaction tend to show the TH1 pattern. This has now been shown particularly clearly in the studies of Romagnani and collaborators in which a large number of T-cell clones were isolated from a single donor who was immune to PPD and also to a canine parasite, *Toxocara canis*. The great majority of the parasite-specific T-cell clones showed clear-cut TH2 patterns when synthesis of IL4, IL5, IL2 and IFNγ was measured, whereas the PPD-specific clones showed mostly the TH1 pattern (Del Prete et al. 1991). Thus the initial differences between mouse and human T-cell clone panels may have been due to the nature of the antigens used to derive the clones, and the vigorous immunization methods that are often used for mice but not humans. The final picture of T cell diversity in the two species may be quite similar.

FUNCTIONS OF TH1 AND TH2 CELLS

Since the TH1 and TH2 phenotypes have been defined most clearly and many *in vitro* examples of these two cytokine secretion patterns are available, most of our current information on the function of defined T-helper cell subsets concerns the TH1 and TH2 phenotypes. The *in vitro* functions of these two types of T-cell clone correlate quite well with the effector functions observed during extreme responses which show either strongly TH1- or TH2-biased cytokine patterns. However, in several other types of immune response which may not involve such long-term, extreme immune responses, certain other cytokine secretion phenotypes may be very important and the potential functions of these other phenotypes will also be discussed.

Help for B-cell antibody synthesis is provided readily by most TH2 clones *in vitro*, and *in vivo* immune responses that show strong TH2-like cytokine biases are normally correlated with high antibody levels. TH2 cytokines, such as IL4, IL5 and IL6, stimulate various aspects of B-cell growth and differentiation. The synthesis of IgE is greatly enhanced by TH2 cells since IL4 induces the switching of B cells to IgE secretion (Coffman & Carty 1986, Lebman & Coffman 1988). This effect is mediated at the DNA level; IL4 induces first the transcription of the ε constant region (Stavnezer et al. 1988) and subsequently the recombination of the VDJ region from the μ to the ε constant region. This activity of IL4 is inhibited by IFNγ, produced by TH1 cells, and so an IgE response is normally an indication of active TH2-like cells in the absence of TH1-like cells. Since there is excellent correlation between IL4 production and help for IgE, both *in vivo* and *in vitro*, it appears that IgE production can be used as a good marker for the existence of a strong TH2-like response. IL4 also induces switching to IgG1 production (Isakson et al. 1982), although IL4 is not the only cytokine that can induce this isotype of antibody.

TH1 clones can also provide help for B-cell antibody secretion but results with different T-cell clones are not as uniformly positive as those obtained with TH2

clones (Coffman et al. 1988). In particular, TH1 cells can kill B cells at high density and so help by TH1 clones is often best seen when only low numbers of the TH1 cells are present. Some TH1 clones do not help at all and this can sometimes be remedied by incorporating IL2 and anti-IFNγ in the assay medium. Thus the adjustment of the quantities of two of the TH1 cytokines can make a large difference in the ability of some TH1 clones to help B cells. Since IFNγ is a switch factor for the production of IgG2a (Snapper & Paul 1987), help by TH1 cells results in the secretion of larger amounts of IgG2a and reduced secretion of IgG1 and IgE.

Many functions mediated by TH1 cells are concerned with cytotoxicity and so a TH1-biased immune response may be most useful in dealing with intracellular pathogens. Some TH1 clones can kill target cells directly, apparently in a manner analogous to the mechanism used by $CD8^+$ cytotoxic T cells. Cytotoxicity can also be mediated via the secretion of LT and IFNγ, which synergize to selectively kill virus-infected cells (Wong & Goeddel 1986). These two cytokines also activate macrophages to increase expression of the Fcγ receptor (Warren & Vogel 1985), which in turn binds the increased amounts of IgG2a induced by IFNγ. Thus there is a general up-regulation of the ability of the macrophage to mediate antibody-dependent cell-mediated cytotoxicity, and intracellular cytotoxic mechanisms are also activated (Pace et al. 1983). TH1 clones mediate a strong delayed-type hypersensitivity reaction (DTH) when injected with antigen into mouse footpads (Cher & Mosmann 1987, Fong & Mosmann 1989). The TH1 cytokine secretion pattern is often associated with strong DTH reactions *in vivo* and thus it appears likely that TH1-like cells are mainly concerned with strong DTH reactions and cytotoxic responses.

While this generalization may be substantially correct, there are at least two features that require a more complex explanation. Firstly, DTH can occur in different forms, notably the Jones Mote reaction and tuberculin-type reaction. By the criteria of short kinetics, substantial edema and a predominantly granulocytic infiltrate, the DTH induced by TH1 clones is more similar to the Jones Mote reaction than to the tuberculin reaction (Fong & Mosmann 1989). Thus the cells inducing the tuberculin-type reaction are not yet known. Possibilities include TH1 cells in concert with another cell type, or one of the other T-cell phenotypes that have recently been described.

A second area of increased complexity is the problem of TH1 help for B cells. There appears to be little doubt that at least some TH1 clones can provide very good help to B cells and, in fact, the cell-cell interaction signal delivered by a helper T cell appears to be very similar, whether delivered by a TH1 or a TH2 cell (Coffman et al. 1988). This has been recently confirmed using membrane fragments, which appear to deliver similar activating signals for B cells whether derived from TH1 or TH2 cells (Hodgkin et al. 1990). However, during strong TH1-like responses *in vivo* involving high DTH levels

and large amounts of IFNγ synthesis, antibody production is often completely absent and in fact can be shown to be suppressed. One possible explanation of this apparent paradox is that TH1 cells may provide B-cell help only when present either in small numbers or in association with other helper cell types, such as the TH2 phenotype. At very high numbers of TH1 cells in the absence of any ameliorating influence by TH2 cells, TH1 cells may only induce DTH and inhibit antibody production.

Although few data are available to define the functions of other Th types, some speculations can be made based on their cytokine secretion patterns. In particular, the cell producing IL2, IL4, and IL5, but not IFNγ, is potentially a very strong helper cell that may be important in normal antibody responses both *in vivo* and *in vitro*. If the cell does not produce lymphotoxin, then it secretes several cytokines that are strongly enhancing for B-cell responses without some of the cytokines that can inhibit such responses. This interpretation is consistent with data showing that many of the T cells in limiting dilution experiments which provide good help for B cells also produce IL2 (Powers & Miller 1987).

The production of only IL2 after the initial stimulation of naive T cells is consistent with a model in which the major function of these cells when first activated is to proliferate, so that increased numbers of antigen-specific T cells are available for subsequent effector functions within the shortest possible time. Since the initial number of antigen-specific T cells is often extremely small, this expansion function may be more important than the immediate induction of any effector functions, such as B cell help or DTH. Once the numbers have been expanded over the first few days, then restimulation of this population may result in secretion of large numbers of different cytokines having strong effector functions in B-cell help and DTH. In the case of B-cell help, it has been suggested that a naive T cell cannot be stimulated by a resting or even an activated B cell (Lassila et al. 1988). If this is true, the naive T cell must be first stimulated by an APC such as a macrophage or dendritic cell, and only after expansion and differentiation will it recognize antigen presented by a B cell and be able to help that B cell to produce antibody.

The TH0 phenotype is more difficult to reconcile with our current knowledge of cytokine functions since several apparently antagonistic cytokines are produced by these cells. IFNγ and IL4 are mutually inhibitory in many assays and IL10 inhibits the production of IFNγ. Thus the mixture of cytokines produced by this cell does not fit well with any particular effector function. One possibility is that the TH0 cell *in vivo* represents a relatively transient differentiation state but that this state is more stable in tissue culture resulting in the isolation of an unusually large number of this type of clone. Other possibilities are that the TH0 cell may be responsible for tuberculin-type DTH reactions or that the TH0 is a good helper cell for particular isotypes of immunoglobulin.

CROSS-REGULATION OF IMMUNE RESPONSES

The various effector functions of the immune system are under tight regulation, much of which is mediated by the T-helper cell population. Since the cytokine patterns expressed by different TH cell populations are the major determinants of the functions of those T cells, the selective differentiation and activation of T-cell subsets is a dominant regulatory influence on the effector functions of the immune response. It is important to distinguish signals affecting the selective differentiation of T-cell subsets from the cytokines and APC which induce selective activation of only one or a limited number of mature T-cell subsets. Since TH1 and TH2 clones are readily available in tissue culture, there is currently more detailed information regarding the selective activation and proliferation of these types of T cell. The differentiation process is also under very strict regulation and useful information is beginning to emerge regarding the cytokines that affect this pathway.

The regulation of differentiation and activation of T-cell subsets is likely to be mediated both by APC and by cytokines. Some TH2 clones require IL1 as a co-factor for the induction of proliferation (Greenbaum et al. 1988, Kurt-Jones et al. 1987) and antigen-presenting cells derived from liver present antigen effectively to TH1 but not TH2 clones (Magilavy et al. 1989). IFNγ derived from TH1, CTL and NK cells inhibits the proliferation of TH2 clones (Gajewski & Fitch 1988, Fernandez-Botran et al. 1988). IFNγ also inhibits many of the functions of IL4 (Mosmann & Coffman 1989). Thus IFNγ can inhibit both proliferation and effector function of TH2 cells. In the opposite direction, if the complex results of Horowitz et al. (1986) are interpreted in light of current knowledge about the T-cell clones involved, a TH2 clone, D10, appeared to produce a cytokine that selectively inhibited the proliferation of TH1 clones. Since there was good evidence from *in vivo* responses that suppression of DTH could occur during a strong antibody response, we searched for activities in TH2 supernatants that could inhibit any aspect of TH1 cell growth or effector function.

Cytokine synthesis inhibitory factor – Interleukin 10

We found an activity in TH2 supernatants that inhibited secretion of cytokines by TH1 cells stimulated with spleen cells plus antigen. This activity did not affect TH2 cell stimulation and was not present in TH1 supernatants (Fiorentino et al. 1989). The activity had a delayed effect and cytokine production was not significantly inhibited during the first few hours of stimulation. Taken together with data showing that only TH1 stimulation involving spleen antigen-presenting cells was inhibited, it appeared possible that the action of IL10 was mediated indirectly through an effect on the antigen-presenting cell. This has recently been found to be the case since preincubation of a macrophage cell line with IL10 inhibited that

cell line's ability to subsequently present antigen to TH1 cells (Fiorentino et al. 1991). This effect did not appear to be due to inhibition of antigen processing since stimulation by APC and *Staphylococcus* Enterotoxin B, which apparently does not require processing, was also inhibited by IL10.

When it was confirmed that CSIF bioactivity could not be explained by any of the known cytokines secreted by TH2 cells, we initiated cDNA cloning by direct expression and isolated a cDNA clone that, when transfected into COS cells, encoded the biological activity measured in the CSIF bioassay (Moore et al. 1990). The sequence of this clone was distinct from all other known cytokines, and included an open reading frame coding for a 178 amino acid polypeptide, including the N-terminal 18 hydrophobic residues that probably represent a secretion leader sequence. Natural and recombinant IL10 comprise polypeptides of 16 kd (non-glycosylated) and 20 kd (glycosylated). From molecular sizing columns, the native molecular weight of IL10 is approximately 25 to 30 kd and so the molecule appears to be a homodimer.

A human IL10 cDNA clone was isolated by cross-hybridization with mouse IL10 oligonucleotides using a cDNA library constructed from a human TH0-like clone (Vieira et al. 1991). The sequence and biological activity of the human IL10 are very similar to those of the mouse clone. Human IL10 functions in mouse assays, whereas mouse IL10 does not appear to bind to the human receptor.

One surprising finding that emerged from these studies was that the human and mouse IL10 genes are highly homologous to an open reading frame in the Epstein Barr Virus (EBV) genome. Sequence homology is present only in the mature protein coding sequence and is not apparent in the 5′ or 3′ flanking regions or even in the leader sequence. The cloned and expressed BCRF1 gene codes for a molecule with very similar activities to the human and mouse IL10's, i.e. BCRF1 protein inhibits IFNγ synthesis by NK and T cells. Since IL10 inhibits IFNγ and LT synthesis and both of these cytokines would have very deleterious effects on the ability of EB virus to carry out the productive replication cycle, it appears that the EB virus has acquired the mammalian IL10 gene and retained it for the purpose of interfering with the immune response against EBV-infected cells.

Functions of IL10

With the availability of recombinant IL10, many other functions were discovered. In general, these activities are similar to those of IL4, even though in the initial assay used to isolate IL10 cDNA clones – the inhibition of IFNγ synthesis by TH1 cells – IL4 is in fact an antagonist of IL10 activity. IL10 and IL4 both inhibit the synthesis of IL1, IL6 and TNF by macrophages. The viability of small resting B cells in tissue culture is improved and IL10 also induces the expression of Ia antigens but not the Fcε receptor CD23 on small resting B cells (Go et al.

1990). IL10 maintains viability of mast cell lines in culture, and synergizes with IL3 and IL4 to induce high-rate proliferation (Thompson-Snipes et al. 1991). Thymocytes proliferating in response to IL2 and IL4 show increased proliferation if IL10 is added (MacNeil et al. 1990).

Since $CD8^+$ cytotoxic T cells secrete predominantly the TH1 cytokine pattern, we also tested the effects of IL10 on the synthesis of cytokines by $CD8^+$ T-cell clones and normal $CD8^+$ T cells. Using spleen cells as antigen-presenting cells, we found that IL10 was able to significantly inhibit the synthesis of IFNγ by various $CD8^+$ clones although the extent of inhibition was somewhat lower than that seen with TH1 cells (T.A.T. Fong and T. R. Mosmann, unpublished). Since IL10 inhibits the ability of macrophages to activate TH1 cells but does not interfere with the ability of B cells to perform the same function, it is possible that the type of cell presenting antigen to the $CD8^+$ cell is also important. Experiments are in progress to examine this point.

Cross-regulation of TH1 and TH2 responses during parasite infections

Although bacterial and viral infections tend to be resolved relatively quickly, resulting in either cure or death of the host, there are many parasitic infections that persist for long periods and involve high antigen loads. Under these circumstances, the immune system is stimulated strongly and chronically and a variety of extreme immune responses are seen. There are a number of parasites that provoke an extremely strong TH2-like response involving not only the TH2 cytokines, but also high IgE and eosinophil levels. There are also some parasites that induce a very strong DTH response with the absence of significant antibody or IgE production. Since these responses are strong and the entire response appears to show a relatively simple pattern, these are excellent model systems for investigating the regulation of TH1 and TH2 responses.

Nippostrongylus brasiliensis (Nb) infection induces high levels of parasite-specific and especially polyclonal IgE, and also high eosinophil levels. These two phenomena are due to IL4 and IL5 respectively, as shown by *in vivo* treatments with antibodies (Finkelman et al. 1986, Coffman et al. 1989). When spleen, lung or mesenteric lymph node T cells are obtained from infected animals and restimulated with polyclonal stimuli in tissue culture, very high levels of the TH2 cytokines IL4, IL5 and IL10 are produced and there is often a reduction in the ability of these cells to produce IL2 and IFNγ (N.F. Street and T. R. Mosmann, unpublished). We have recently tested whether IL10 is responsible for this inhibition. If anti-IL10 antibodies are added to the *in vitro* culture system during the stimulation of normal mouse splenocytes, there is a moderate enhancement of the amount of IFNγ secreted. However, if anti-IL10 antibodies are added to cultures from parasite-infected animals, there is a much larger increase in the amount of IFNγ produced, suggesting that the high levels of IL10 produced

during the culture period are suppressing the synthesis of IFNγ in the same culture. It is interesting that even the low amounts of IL10 produced under these circumstances by normal mouse spleen cells also appear to suppress IFNγ production since even the control cultures showed a moderate elevation of IFNγ synthesis after anti-IL10 treatment. These results are consistent with the information mentioned earlier in which purified CD4^{+} T cells stimulated without added APC synthesized increased amounts of IFNγ after Nb infection. The existence of a strong IFNγ response revealed by anti-IL10 antibody treatment is also consistent with cloning experiments in which we found that cells from Nb-infected mice showed a considerable number of TH2 and related phenotypes during short-term *in vitro* cloning, but also showed an increased number of TH1 clones compared to controls (Street et al. 1990). All of these results are consistent with the induction of a moderate TH1 response that is not normally seen because of suppression of TH1 cytokine synthesis by IL10.

During infection by *Schistosoma mansoni*, a strong immune response develops after the production of eggs by the parasite (Grzych et al. 1991). The egg antigens induce a very powerful TH2-like response involving high levels of production of IL4, IL5 and IL10. Antigen stimulation in culture does not induce significant levels of IFNγ secretion; however, if anti-IL10 antibodies are added to these cultures, high levels of IFNγ secretion are observed, suggesting that, as in the Nb system, the IL10 produced during the culture is able to substantially inhibit the synthesis of IFNγ in the same culture (Sher et al. 1991).

Additional cross-regulatory influences

In many experiments involving the stimulation of spleen cells in tissue culture, there is good evidence for additional types of regulation involving unknown cytokines. As the cell concentration in culture is increased, the synthesis of IFNγ is normally relatively constant with the increase in cell number, i.e. the amount of IFNγ produced per cell is not influenced strongly by cell culture density. IL10 synthesis, on the other hand, is very sensitive to culture density and in the same supernatants in which IFNγ shows a relatively linear relationship, IL10 shows high synthesis amounts per cell at low cell concentration and greatly depressed synthesis per cell at higher cell numbers (Fig. 2). The simplest interpretation of this result is that an inhibitor of IL10 synthesis is produced during the stimulation and that only at high cell concentrations does the concentration of the inhibitor accumulate to sufficient levels to inhibit IL10 synthesis. It is noteworthy that even in cultures where no sign of regulation appears in the dose-response curve, such as in the case of IFNγ synthesis in Fig. 2, it is still possible to show regulation of the synthesis of this cytokine by the addition of anti-IL10 antibodies. The levels of IFNγ were increased substantially at all cell concentrations, revealing that even the initial straight line response was subject to considerable regulation.

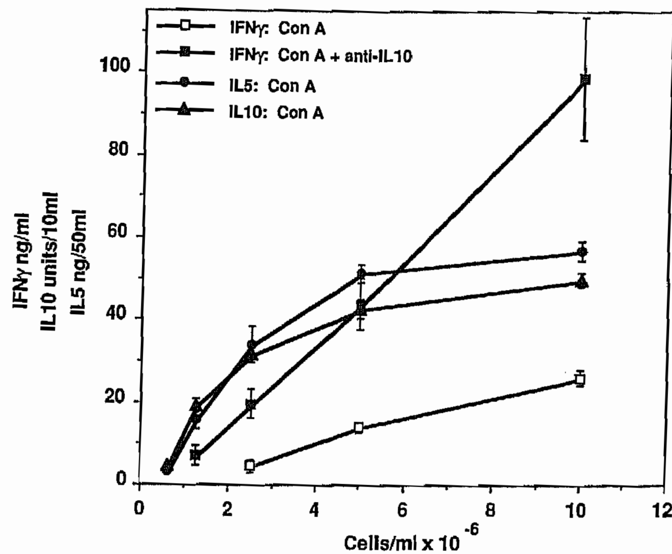

Figure 2. Regulation of IFNγ, IL5 and IL10 synthesis in spleen cultures. Spleen cells from 8-d Nb-infected mice were stimulated with 2 μg/ml Con A at the indicated cell concentrations, in the presence or absence of 10 μg/ml SXC1 anti-IL10 antibody. The cytokines in the 24-h supernatants were measured by ELISA.

The type of stimulation used for polyclonal stimulations in tissue culture can also have a strong influence on the patterns of cytokines obtained. This situation is made even more complex by the influence of prior immunization. For example, Con A stimulation was more effective than anti-CD3 + PMA for the stimulation of IFNγ secretion from cells from normal mice, but this preference was reversed when cells from *Brucella abortus* (Ba)-immunized mice were examined (Fig. 3). In the same anti-CD3-stimulated supernatants, the production of IL2 shows severe suppression at higher cell numbers with cells from Ba-immunized mice, whereas cells from normal mice do not show this effect (results not shown). These effects are currently unexplained, and strongly suggest further complexities of cytokine interaction and/or differential stimulation requirements of T-cell subsets that may be discovered in the future.

TH1 and TH2 cells and the allergic response

The cytokines produced by TH2 cells have a number of positive effects on the development of allergic responses (Fig. 4). IL4 induces B cells to switch to high-rate IgE production and IL3, IL4 and IL10 are all mast cell growth factors that synergize to induce the rapid growth of mast cell lines. IL5 is a major growth

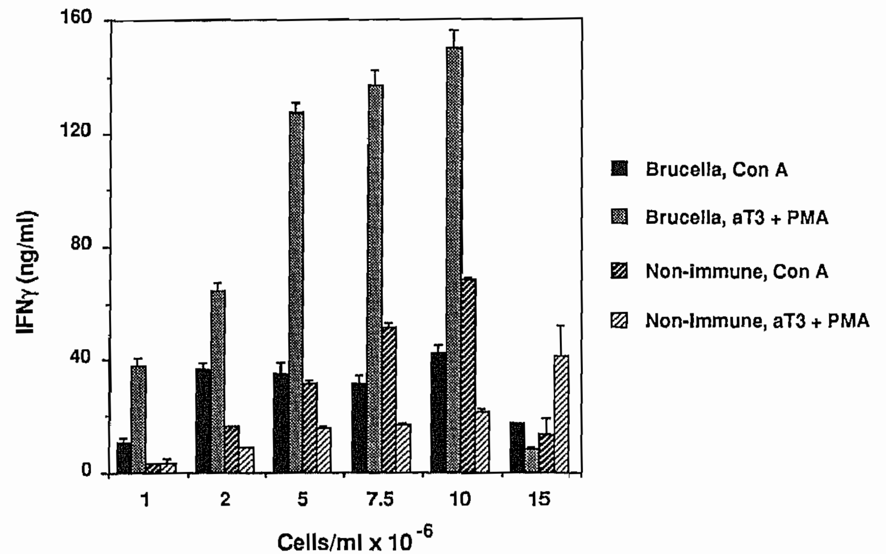

Figure 3. Optimum spleen cell stimulation conditions depend on the prior immunization history. Spleen cells from non-immunized or Ba-immunized mice were stimulated with Con A (2 μg/ml) or anti-T3 antibodies + PMA (10 ng/ml) at the indicated cell concentrations. Cytokines in the 24-h supernatants were measured by ELISA.

and differentiation factor for eosinophils and so the activation of TH2 cells should result in increased synthesis of IgE and also increased numbers of the two cell types that bind IgE to their cell surface and use it as an antigen receptor for subsequent response to antigen. IL4 encourages the differentiation of additional TH cells secreting IL4 and IL5, and so activation of TH2 cells will coordinately enhance several aspects of the allergic response. In contrast, TH1 cells produce IFNγ which is able to inhibit the allergic pathway in at least three stages: blocking the IL4-induced switching of B cells to IgE production; inhibiting proliferation of TH2 cells; and inhibiting the activation of mast cells. Since IL10 inhibits the production of IFNγ, it is apparent that a TH1 cell response can potentially inhibit many aspects of the allergic response, but only in the absence of large amounts of IL10. Thus, in a dominant TH2 response the production of IFNγ by TH1 cells will be suppressed and IFNγ will not be able to interfere with the allergic response. This pattern of cytokine regulation offers a plausible and internally consistent model for much of the regulation that occurs during an allergic response. Several aspects of this model have already been confirmed *in vivo* at least during the very powerful allergic responses that accompany parasite infections and so it appears likely that these pathways are a major and possible overriding part of the regulation of a general allergic response. The success of the cytokines of the different

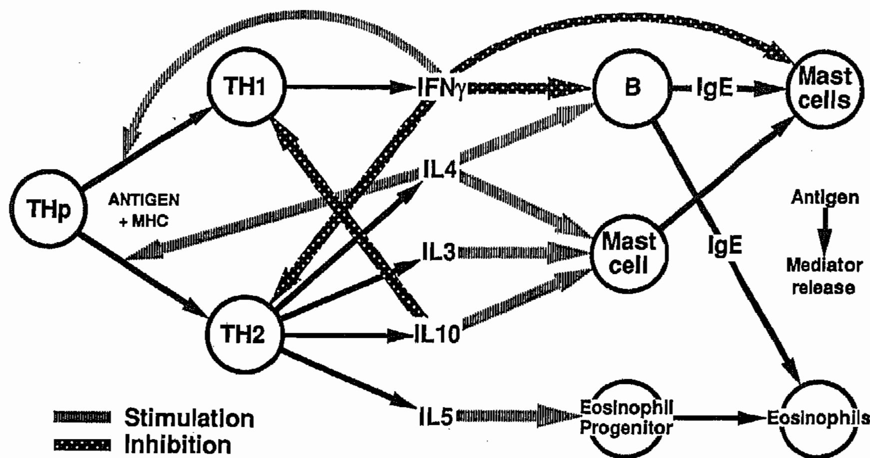

Figure 4. TH1 and TH2 regulation of allergic responses.

T-cell subsets in explaining a complex reaction, such as an allergic response, offers considerable encouragement that the cytokine patterns of T-helper cell subsets are major regulators of general immune responses and that further work along these lines will not only explain many aspects of immune regulation, but also offer future opportunities for therapeutic intervention in immune responses.

CONCLUSION

Unanswered questions

The diversity of TH subtypes is now clearly greater than two types, but the true extent of this diversity is not yet apparent. Future advances in this area will probably be obtained from single-cell studies, since *in vitro* clones may provide either an overestimate due to alterations arising *in vitro*, or an underestimate due to the lack of appropriate culture conditions for certain phenotypes.

Two related questions are the identity of the cell types that mediate the initial decision of the direction of TH cell differentiation, and the nature of the signals that induce selective differentiation. Several cytokines have already been implicated in the differentiation process, and it is very likely that different APC are also involved. Such APC subsets could directly influence the type of TH cell obtained, or the choice could be regulated by cytokines secreted by third-party cells, such as mast cells, NK cells or other T cells. Given the importance to the host organism of making the correct choice of immune effector functions, it is very likely that the mechanisms of this choice will be multiple, redundant and substantially interregulated.

Although interest has centered on the clear-cut cytokine secretion patterns of TH1, TH2 and similar cells, it also appears likely that quantitative variations may exist. If a cell reproducibly secretes, for example, 50-fold less of a particular cytokine than another cell, this may result in substantially different effector functions of the two cells. There is some evidence from T-cell clones that this may occur, and so the final number of T-cell types showing significantly different cytokine secretion patterns may be quite large. Another interesting quantitative question is whether T-cell activation is an "all-or-none" phenomenon, or whether different amounts can be released in response to quantitatively or qualitatively different signals. This is particularly important because of the suggestions of directional release of cytokines (Poo et al. 1988, Kupfer et al. 1991). TH cells, when maximally stimulated, secrete sufficient amounts of cytokines, such as IL3, IL4 and IL5, to stimulate more than 1000 target cells, and so directional release would not be expected to be significant unless smaller amounts of cytokine were secreted under some circumstances.

A final question relates to the possible existence of memory for effector function as well as specificity. Specificity is preserved in both T- and B-cell memory populations because of the stable rearrangement of a particular pair of antibody or T-cell receptor genes. In the B-cell memory population, memory for effector function is retained by the relatively stable rearrangement of the expressed immunoglobulin heavy chain gene to a particular constant region gene, thus establishing the effector function of the antibody induced during a secondary response. Recent evidence suggests that long-term memory TH cells may produce predominantly or exclusively IL2 when first restimulated. Under many circumstances, it would be useful for the memory response to re-express the same set of effector functions as those expressed during the primary response. It is possible that long-term memory cells are in fact uncommitted, so that the differentiation to effector phenotypes occurs under similar regulation to that of the primary response. Alternatively, the memory TH population, although expressing only IL2 when initially stimulated, nevertheless may still be committed to the production of a particular set of cytokines that will be expressed on repeated stimulation.

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