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Evidence for the existence of IL-4 and IFNγ secreting cells in the T cell repertoire of naive mice

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1. Summary

The kinetics with which IgE responses develop in vivo following immunization of experimental animals indirectly support the existence of IL-4-secreting T cells as a normal component of the T cell repertoire. At the same time, studies of IL-4-secreting cell frequencies directly ex vivo have argued that T cells with the potential to become IL-4 secretors exist in vivo, in the form of precursors requiring stimulation and 4 – 12 days of culture as well as restimulation with mitogen or Ag before they become detectable as lymphokine-secreting cells. We demonstrate here that intravenous administration of low doses of anti-CD3 mAb 145-2C11 results in IL-4 production within 60 min of stimulation as demonstrated by Northern analysis of mRNA and a sensitive, selective bioassay (CT.4S cell proliferation) of biologically active IL-4 protein. Production of IL-4 is paralleled by IFNγ synthesis, displaying similar kinetics. These findings, consistent with the presence of mature cells capable of IL-4 and IFNγ synthesis in the T cell repertoire of naive mice, are supported by the observation that stimulation of spleen cells from naive mice with anti-CD3 mAb in vitro for 12 h also results in strong IL-4 and IFNγ mRNA and protein synthesis. The data support and extend those obtained through analysis of cytokine mRNA synthesis alone, thereby providing evidence that "fresh" T cells are indeed capable of producing IL-4 directly ex vivo and are consistent with the existence of IL-4-secreting cells as a normal component of the T cell repertoire of naive mice.

Key words: IL-4; IFNγ; Cytokine expression; T cell repertoire; IgE

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Abbreviations: TGFβ, transforming growth factor beta; TCR, T cell receptor.

2. Introduction

Much attention has focused on the role played by IL-2 and IL-4 in the regulation of immune responses in vivo [1 – 4]. However until recently, efforts to detect IL-4-secreting cells directly ex vivo have indicated that while IL-2 synthesis was readily induced in freshly derived murine spleen cell populations, IL-4 synthesis was undetectable without in vivo priming as well as 4 – 12 days of in vitro culture and restimulation with Con A, anti-CD3 or antigen [5 – 7]. Similarly, although limiting dilution analyses of cells obtained from mice immunized with antigen in complete Freund's adjuvant (CFA) displayed a high frequency of IL-2 secretors, IL-4-secreting cells were undetectable by this method without in vivo priming, 5 days in vitro culture with antigen, 7 days rest and another round of in vitro restimulation [8]. Indeed, even polyclonally activated in vitro cultures are reportedly unable to produce IL-4 in the absence of protracted periods of culture and restimulation [9, 10]. Taken together, these data were seen to indicate that IL-4-secreting T cells exist in vivo only as precursors which require stimulation, several days of culture and restimulation with antigen or polyclonal acti-

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vators in order to obtain IL-4 production [5 – 10].

In contrast, studies of IgE production in response to immunization with protein antigens in appropriate adjuvants [11, 12] or to polyclonal activators such as goat anti-mouse IgD [13], have provided indirect evidence that IL-4 secretion occurs very soon after immunization without a requirement for extended periods of antigenic or polyclonal stimulation.

Recent work, using sensitive approaches towards detection of IL-4 gene expression addresses this apparent paradox. PCR analysis of the pattern of cytokine mRNA production which is elicited following primary in vivo sensitization with picryl chloride demonstrated the induction of both IL-2 and IL-4 mRNA synthesis [14]. Independently, evidence was obtained that intravenous administration of anti-CD3 mAb leads to rapid and sequential activation of IL-2, IL-4 and other cytokine genes, with significant mRNA production observable within 60 min of stimulation [15] or, using in situ hybridization, 4 – 24 h after Con A stimulation in vitro [16]. It is important to note, however, that in each of these reports the authors have made it clear that the relationship between mRNA production and protein production remains speculative [14 – 16]. Given the related systems in which cytokine mRNA production is not representative of protein production, such as murine TNFα [17], TGFβ [18], human IL-1α and IL-1β [19], the possibility of translational regulation is relevant and needs to be addressed.

We demonstrate here that polyclonal stimulation of spleen cells of naive mice with anti-CD3 mAb 145-2C11 in vivo, or in vitro with immobilized anti-CD3, results in the production of IL-4 and IFNγ mRNA and biologically active protein. These findings demonstrate that "fresh" T cells are indeed capable of producing IL-4 directly ex vivo and are consistent with the existence of mature IL-4-secreting cells as a normal component of the T cell repertoire of naive mice.

3. Materials and Methods

3.1. *Animals*

C57Bl/6 mice (6 – 12 weeks old) were purchased from Charles River, Canada (St. Constant, PQ) and were used in strict accordance with the guidelines issued by the Canada Council on Animal Care. Mice were monitored for antibodies to mycoplasma, Sendai virus and rodent coronaviruses including MHV by ELISA (Murine ImmunoComb, Charles River).

3.2. *Polyclonal in vivo stimulation*

Mice were stimulated in vivo by i.v. administration of 20 μg of anti-CD3 mAb 145-2C11 [20, 21] (provided by Dr. J. Bluestone, Univ. of Chicago). Injection of normal hamster IgG under these conditions was previously shown to be without effect on cytokine mRNA production under these conditions (our unpublished data, and [15]). At times from 0.5 – 6 h later, independent groups of anti-CD3 injected and control uninjected mice were killed, their spleens removed and used for Northern analysis (see below) or in vitro culture at 7.5×10^6/ml (2 ml/well) in complete medium (RPMI-1640 containing 10% fetal calf serum (Gibco, Burlington, ON), 1% penicillin, streptomycin, fungizone, 1% L-glutamine (Flow Laboratories, Mississauga, ON) and 5×10^{-5} 2-mercaptoethanol (Kodak, Rochester, NY) overnight at 37 °C in the absence of in vitro stimulation.

3.3. *Polyclonal in vitro stimulation*

Spleen cells obtained from naive C57Bl/6 mice were cultured (7.5×10^6/ml; 2 ml/well) in anti-CD3 mAb pre-coated, 24-well plates in complete medium for 3 – 24 h at 37 °C. Culture supernatants were then harvested for analysis of biologically active cytokine and cells were harvested for RNA extraction and analysis by Northern blotting.

3.4. *Northern analysis of IL-4 and IFNγ mRNA synthesis*

Plasmid DNAs containing murine IL-4 and IFNγ inserts (provided by Dr. T. Mosmann, DNAX Research Institute, Palo Alto, CA) were transformed and cloned in *Escherichia coli* strain DH5'a (Gibco). cDNA inserts were purified by digestion with *Xho*I and *Bam*HI restriction endonucleases (Pharmacia, Montreal, PQ), respectively.

Total cellular RNA was isolated by a guanidini-

um isothiocyanate cesium chloride gradient centrifugation procedure as described [22] from C57Bl/6 spleen cells before or after in vitro culture as indicated at Results. 30 μg of total RNA per lane (as determined by A_{260} units) was electrophoresed in 1.5% agarose gels under denaturing conditions. Following staining with EtBr to confirm the integrity of the RNA samples used, gels were destained and blotted onto Hybond-N nylon membrane (Amersham, Canada). Following cross-linking to the membrane by UV irradiation, hybridization of the Northern blot with ^{32}P-labelled cDNA probes and autoradiography were carried out as described [23].

3.5. *Bioassays*

IL-4 production was determined using the IL-4 responsive cell line CT.4S essentially as described [24]. These cells are highly responsive to IL-4 and make only modest responses to IL-2 even at high concentrations (100 U/ml IL-2). Briefly, 10^4 CT.4S were cultured for 48 h in tissue culture supernatants obtained as described above prior to pulsing with 1 μCi [^{3}H]thymidine/well. Cultures were harvested 8 – 16 h later and [^{3}H]thymidine incorporation was determined by liquid scintillation counting. Internal standards of rmIL-4 (provided by Dr. R. Tepper, Dana-Farber Cancer Institute, Boston, MA or Dr. W. Paul, NIAID, NIH, Bethesda, MD) were included as positive controls, and IL-4-deficient medium as negative controls, in each assay. The specificity of this assay for determination of IL-4 was assured by parallel analysis of supernatants in the presence of purified anti-IL-4 mAb 11B11 at concentrations sufficient to neutralize the activity of >100 U/ml of rmIL-4.

IFNγ production was determined using inhibition of WEHI-279 cell growth essentially as previously described [25]. Briefly, 10^4 WEHI-279 cells/well were cultured for 48 h in 96-well flat-bottom plates in the presence of 100 μl tissue culture supernatant. Internal standards of IFNγ-containing mouse Con A-stimulated spleen cell supernatants (provided by Dr. X. Yang, Univ. of Manitoba, Winnipeg, Canada) were included in each assay. The specificity of the assay was assured by parallel analysis of supernatants in the presence of purified XMG 1.2 mAb (hybridoma provided by Dr. T. Mosmann) at concentrations sufficient to neutralize the activity of >100 units of IFNγ as defined against WHO-NIAID international reference reagent Gg02-901-533 (provided by Dr. C. Laughlin, NIAID, NIH, Bethesda, MD).

4. Results

4.1. *Detection of IL-4 and IFNγ mRNA following anti-CD3 stimulation in vivo*

Naive C57Bl/6 mice were injected (i.v.) with anti-CD3 mAb 145-2C11 and killed 1, 3 or 5 h later. Northern analysis of total RNA extracted from spleen cells of these mice indicated rapid induction of cytokine mRNA production (Fig. 1). IL-4 and IFNγ mRNA were detected within 60 min of anti-CD3 administration to naive mice but were undetectable in RNA prepared from the spleens of unstimulated mice. mRNA expression for these cytokines was transient following in vivo stimulation, rarely being observed later than 3 h after treatment with anti-CD3 mAb.

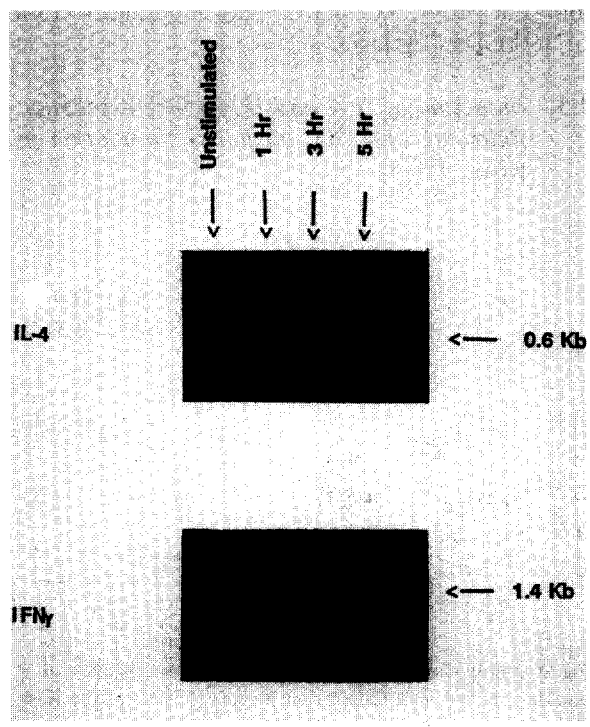

Fig. 1. Expression of IL-4 and IFNγ mRNA following in vivo stimulation of naive mice with anti-CD3 mAb. Northern analysis was carried out as described in Materials and Methods.

4.2. *IL-4 and IFNγ cytokine production following anti-CD3 stimulation in vivo*

To determine if IL-4 and IFNγ mRNA production represents synthesis of biologically active cytokine, spleen cells obtained from independent groups of anti-CD3-injected and unstimulated control mice were killed 0.5 – 6 h post-injection. These cells were cultured overnight in the absence of additional stimulation. Supernatants obtained from these cultures were examined for IL-4 and IFNγ production. As demonstrated in Fig. 2, IL-4 mRNA production was followed by synthesis of biologically active IL-4 protein. IL-4, indicated as anti-IL-4 (mAb 11B11) neutralizable stimulation of the IL-4 dependent cell line CT.4S, was rapidly and transiently induced following polyclonal stimulation. Cultures of cells obtained from mice killed at or later than 3 h following in vivo stimulation with 145-2C11 failed to produce detectable IL-4 under these conditions.

Similarly, IFNγ production, measured as inhibition of WEHI-279 cell growth, was rapidly induced and short-lived, peaking 1.5 h after administration of anti-CD3 (Table 1).

4.3. *Induction of IL-4 and IFNγ gene expression following anti-CD3-mediated stimulation in vitro*

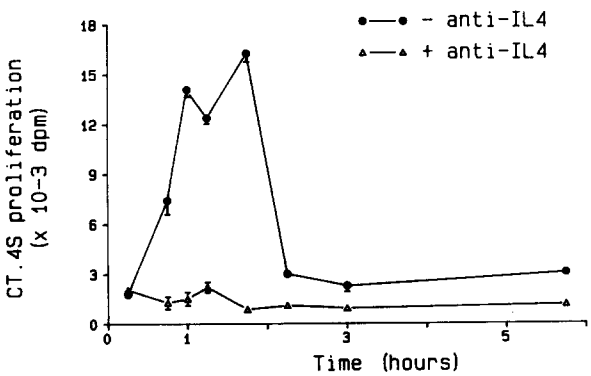

Fig. 2. In vivo stimulation with anti-CD3 mAb results in IL-4 protein production. Proliferation (mean ± S.D.) of the IL-4-dependent CT.4S cell line in response to supernatants harvested from spleen cell cultures established 0.75 – 6 h following i.v. injection of 20 μg anti-CD3 mAb, as described in Materials and Methods. Data are presented from one of five experiments.

TABLE 1

Synthesis of biologically active IFNγ following in vivo stimulation with anti-CD3.

In vivo stimulus		In vitro stimulus	WEHI-279 proliferation[a]
Unstimulated	0 h	None	265800 ± 25989
Anti-CD3	0.5 h	None	202655 ± 9972
	0.75 h	None	12744 ± 2510
	1.5 h	None	9200 ± 1545
	3 h	None	132300 ± 4529
	6 h	None	195419 ± 9464

[a]dpm ± standard deviation.

As an alternative approach to determining if functionally competent IL-4-secreting cells are a component of the normal in vivo T cell repertoire, spleen cells from naive C57Bl/6 mice were cultured for varying periods of time with immobilized anti-CD3 mAb. As seen from the data in Fig. 3, IL-4 and IFNγ mRNA was detectable within 12 h of

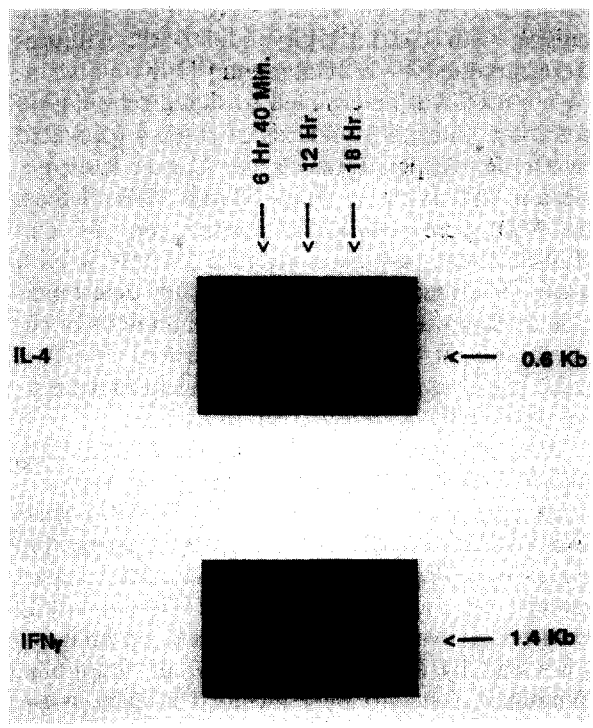

Fig. 3. Expression of IL-4 and IFNγ mRNA following in vitro stimulation with immobilized anti-CD3 mAb. Northern analysis was carried out as described in Materials and Methods.

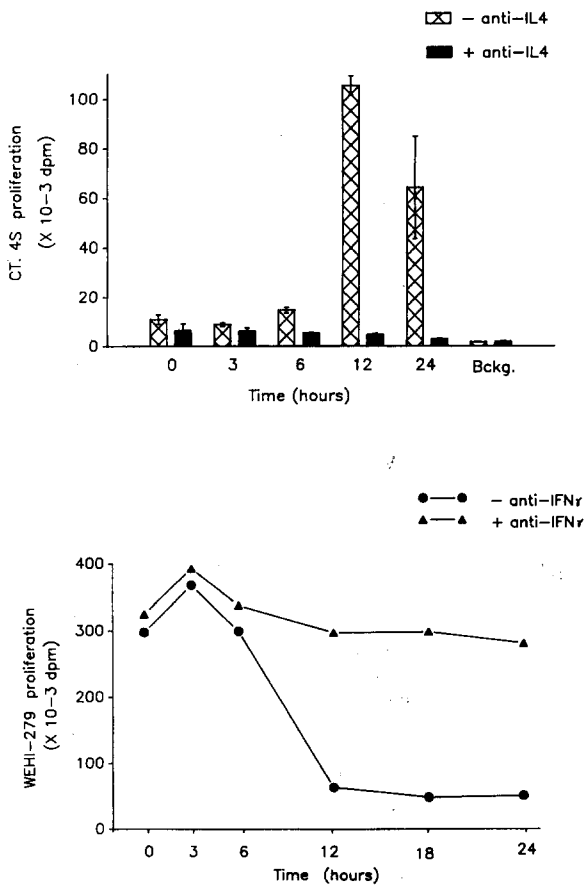

Fig. 4. In vitro stimulation of spleen cells from naive mice with immobilized anti-CD3 results in IL-4 and IFNγ protein production. Assays for the production of biologically active cytokine were carried out in the presence/absence of neutralizing, cytokine-specific mAbs as described in Materials and Methods.

anti-CD3 stimulation in vitro. Similarly, biologically active IL-4 and IFNγ protein synthesis peaked 12 h after anti-CD3-mediated stimulation in vitro (Fig. 4).

5. Discussion

We demonstrate here that in vivo activation of T cell populations through the TCR-CD3 complex results in IL-4 and IFNγ mRNA synthesis within 1 h of stimulation of naive mice. Overnight culture of spleen cells from these animals, in the absence of additional stimulation, results in synthesis of biologically active IL-4 and IFNγ protein. Independently, polyclonal in vitro activation of naive spleen cells was shown to initiate IL-4 and IFNγ mRNA and protein synthesis within 12 h of culture. The results argue that IL-4 secretion does not require multiple cycles of priming and restimulation as previously reported [5 – 10], but rather that cells capable of IL-4 secretion constitute a normal component of the naive murine T cell repertoire.

Our ability to detect IL-4 mRNA and protein production under circumstances unsuccessfully used by previous investigators [5 – 10], is likely attributable to two factors. Until recently, most assays of IL-4 (protein) activity relied upon markedly less sensitive bioassays such as HT-2 or CTLL-2 cell proliferation in the presence of anti-IL-2/IL-2 receptor mAbs [8] or induction of MHC Class II antigens on resting B cells [6]. In this report we employ CT.4S, a highly sensitive murine IL-4-responsive cell line [24] which detects biologically active IL-4 in picogram amounts.

The second major difference between our own and previous studies lies in the selection of experimental conditions. Powers et al. [8] found IL-4-secreting cells to be rare or undetectable following immunization with a variety of protein antigens emulsified in complete Freund's adjuvant. However, the inhibitory effects of CFA on the development of IgE responses in vivo are well established [26]. Induction of IgE synthesis, an IL-4-dependent process [4, 12, 27], suggests that the previously selected conditions may have been counterproductive for stimulation of IL-4 synthesis. This hypothesis is supported by our recent demonstration that administration of protein antigens in $Al(OH)_3$ adjuvant, a well-established adjuvant for eliciting IgE responses [11], results in antigen-specific induction of IL-4 responses in OA ($Al(OH)_3$) or ragweed extract ($Al(OH)_3$) immunized mice while naive mice, or mice immunized with OA or ragweed extract in CFA, generate strong IL-2 and IFNγ responses but no detectable IL-4 following primary antigen-specific restimulation in vitro (X. Yang, K. T. HayGlass, manuscript submitted).

Taken together, these findings indicate the importance of the in vivo and in vitro conditions selected for examination of the cytokine synthesis patterns present within T cell repertoire.

As in a previous report [15], cytokine gene expression following this mode of in vivo stimulation was rapid but transient. Cytokine mRNA, as de-

tected by Northern blotting, was detectable for only a few hours under these conditions. It should be noted that these results do not argue for or against the existence of Th2-like cells in vivo. Such an extrapolation will require analysis of IL-4/IFNγ cytokine secretion by individual, normal (i.e., not cloned) T cells directly ex vivo. Such studies are currently in progress using reverse transcription-PCR analysis of cytokine mRNA production.

This study does not address the potential role of IL-1 or IL-2 in activation of IL-4-secreting cells following TCR-mediated stimulation. Recent work suggests that these cytokines may play an essential role in clonal expansion of IL-4-secreting T cells [8, 15, 28]. Experiments to assess the role of such cytokines by examining the kinetics of IL-1/IL-2 synthesis relative to IL-4/IFNγ synthesis [15] are contingent upon the T cell repertoire of such mice being in a truly resting state. However, the extent to which anti-CD3 stimulation results in activation and IL-4 synthesis by resting naive cells vs. cells previously primed by environmental antigens is difficult to determine. The observation that naive and germ-free mice exhibited no difference in the kinetics of IL-4 mRNA induction (IL-4 protein was not examined) [15] was taken to argue that IL-4 gene expression was not attributable to environmental antigen pre-priming. However, use of polyclonal activators to elicit cytokine gene expression makes it difficult to assess the concern of environmental antigens with certainty, even in nominally "germ-free" mice. Comparison of IL-4 production in naive (with respect to a particular antigenic signal) vs. allergen-primed mice is more likely to lead to a greater degree of certainty in terms of the relative contribution of naive vs. memory T cells to IL-4 synthesis in vivo and the role played by IL-2 and other cytokines in their clonal expansion.

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