A Repetitive Peptide of *Leishmania* Can Activate T Helper Type 2 Cells and Enhance Disease Progression

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**Summary**

Leishmaniasis provides a biologically relevant model to analyze the heterogeneity of CD4⁺ T cells and may lead to answering the major question of the mechanism for the preferential induction of T helper type 1 (Th1) and Th2 cells. Using synthetic peptides corresponding to the tandemly repeating regions of *Leishmania* proteins, we have identified an epitope that can preferentially induce the disease-exacerbating Th2 cells in susceptible BALB/c mice. Lymph node cells from BALB/c mice immunized subcutaneously with the octamer (p183) of the repeating 10-mer peptide EAEEAARLQA proliferated strongly against the peptide as well as the soluble antigen extract (SolAg) of *Leishmania major*. The proliferative T cells are CD4⁺, major histocompatibility complex class II restricted, and secrete interleukin 4 (IL4) but little or no IL2 and interferon γ when stimulated with the peptide in vitro. T cells from BALB/c mice with progressive disease, but not from BALB/c mice cured of the infection, recognized this epitope. BALB/c mice injected subcutaneously with p183 developed significantly exacerbated disease when subsequently challenged with *L. major*. Furthermore, subcutaneous injection with p183 prevented the subsequent induction of resistance against *L. major* by intravenous immunization with soluble antigen. The T cell response to p183 is H-2d restricted. Immunization of the genetically resistant B10.D2 mice with p183 also produced strong T cell responses and exacerbated disease when challenged with *L. major*.

It is now generally accepted that murine CD4⁺ T cells can be functionally divided into two subpopulations. Principally, Th1 cells secreting IL2 and IFN-γ mediate the classical delayed-type hypersensitivity, whereas Th2 cells secreting IL-4 and IL-5 help IgE synthesis (1, 2). In experimental murine leishmaniasis, a disease caused by the protozoa parasites of *Leishmania* species, it is now clear that Th1 cells are host protective (3, 4), whereas Th2 cells can lead to disease exacerbation (5, 6), and the outcome of the infection is determined by the balance of Th1 and Th2 cells (7). Thus, IL-4 produced by Th2 cells can neutralize the macrophage-activating and leishmanicidal effect of IFN-γ produced by Th1 cells (8).

From a genomic *Leishmania major* DNA expression library, Wallis and McMaster (9) selected two clones by using polyclonal antibodies raised against *L. major* membranes. The clones encoded proteins containing regions of tandemly repeated peptides of 14 and 10 amino acids, respectively. Regions of repetitive peptides are a characteristic of many malaria proteins, and this feature has been implicated in immune evasion (10). We therefore decided to investigate the immunogenicity of the tandemly repeated peptides of leishmanial parasite. Results reported here demonstrate that the octamer of the 10-residue peptide (EAEEAARLQA) preferentially induces Th2 cells and promotes disease progression in the genetically highly susceptible BALB/c mice infected with *L. major*. This finding may help to define the molecular requirement for the preferential induction of Th2 cells.

**Materials and Methods**

*Mice.* Female BALB/c, CBA, C57BL/10, B10.S mice, 8–12 wk old, were bred and maintained at the Wellcome Research Laboratories, Beckenham, Kent, UK. BALB.b, BALB.k, and B10.D2 mice of the same age were obtained from Olac Ltd. (Bicester, Oxon).

*Peptide Synthesis.* Solid-phase peptide synthesis using established procedures in "Tea bags" was performed as described by Houghten (11). Peptides were assembled on Pmp-gly resin (Applied Biosystems, Inc., Foster City, CA) or p-methylbenzydrylamine resin, depending on whether free peptides or peptide amides were required. The octamer configuration was according to Tam (12). Cleavage of the finished peptides from the resins was achieved with trifluoromethanesulphonic acid (TFA), as described by Bergof et al. (13), and the resulting peptides were extracted twice with ether before lyophilization from solutions in 10% acetic acid. Purity was

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1 Abbreviations used in this paper: LNC, lymph node cells; SolAg, soluble antigen extract of promastigotes; TFA, trifluoromethanesulphonic acid.
each peptide gave a single peak indicating a homogeneous product. Reversed-phase HPLC of each peptide gave a single peak indicating a homogeneous product. PAGE confirmed that all peptides migrated at their expected molecular weight and were resolved with standard markers (low molecular weight kit for peptides with molecular weights between 2,510 and 16,950; Sigma Chemical Co., St. Louis, MO). Amino acid analysis was consistent with the proposed structures (Fig. 1). Although molar values for p183 were >10% at variance with the expected value, for glutamic acid/glutamine, sequencing of peptide 183 indicated that glu/gln residues did not couple quantitatively, possibly due to steric hindrance in the octamer structure. This was seen with more than one batch of p183 and suggests that the peptide chains in this octamer contain deletion variants in the glu/gln residues.

Parasites. The maintenance, cultivation, and isolation of the promastigotes stage of the parasite *L. major* (LV39) have been described in detail (15). Parasites used for immunization were exposed to γ irradiation (150,000 rad) from a 137Cs source. After irradiation, the parasites suspension (2 × 106 of PBS) was kept at 4°C and used within 7 d. The parasites thus prepared are non-invasive when injected subcutaneously (in 0.05 ml PBS) in the left hind footpad with 1–3 × 106 (BALB/c) or 107 (B10.D2) stationary phase promastigotes. Infection was also given in the rump with 2 × 107 organisms in 0.1 ml PBS. Some BALB/c mice were irradiated with 550 rad from a 137Cs source 4 h before infection in the rump. The lesions that developed were measured with a dial calliper. In some experiments, mice were killed and the footpads removed. The parasite loads in the footpads were estimated by a method described by Heinz et al. (6). Briefly, the footpad tissue was homogenized, and supernatants containing the released parasites were serially diluted in Schneider's medium (Gibco Laboratories, Paisley, UK) containing 30% FCS. Quadruplicate cultures were incubated at 28°C for 3 d and then pulsed with 1 μCi of [3H]thymidine (Amersham Corp., Arlington Heights, IL). Cultures were harvested 18 h later. The results were expressed as the highest dilution (log10) at which viable parasites are detectable (2 SD over background cpm of cultures without parasites).

Proliferative T Cell Response. Draining lymph node cells were removed from mice and made into single cell suspension at 4 × 106 cells/ml in Clicks EHA medium supplemented with 0.5% fresh normal mouse serum. The cell suspension (100 μl) was placed into microtitre wells (96-well flat-bottomed microtitre plates; Nunc, Roskilde, Denmark), and antigen (100 μl) was added at the induced concentration per well: peptides, 5–0.05 μg; soluble antigen extract of promastigotes (SolAg) 10–104 organisms equivalent; SRBC, 10–104. [3H]Thymidine incorporation into DNA during the last 4 h (high specific activity thymidine, 1 μCi/well; Amersham Corp.) of a 4-d culture at 37°C and 5% CO2 was determined as a measure of proliferation. Cultures were set up in triplicate, and the results are expressed in mean cpm ± 1 SEM. SolAg was prepared by five cycles of freezing and thawing of 2 × 108/ml of stationary phase promastigotes in PBS. The material was then centrifuged at 8,000 g for 10 min at 4°C, and the supernatant was filtered (0.45-μm filter; Gelman Scientific, UK) and stored at −70°C. The protein concentration was 0.76 mg/ml/2 × 106 organisms, as determined by a BCA assay (Pierce Chemical Co., Rockford, IL). SRBC were kept in Alsever's solution at 4°C, and a suspension at the required concentration was made freshly after three washes with PBS.

Lymphokine Assays. Lymph node cells (107/ml) from immunized mice were stimulated for 24–48 h in vitro with 106 organisms/ml of SolAg, and the lymphokine activities in the cell-free culture supernatants were determined as follows: IL-2 and IL-4 activities were determined using CTL cell line (American Type Culture Collection, Rockville, MD) mAb anti-IL-2 (S4B6, 50 μg/ml) and anti-IL-4 (1B11, 5 μg/ml) were used to distinguish the two lymphokines. The CTL cells (105/50 μl) were cultured in 96-well flat-bottomed plates (Nunc) with several dilutions of the supernatant (100 μl) and mAb (50 μl) for 36 h at 37°C and 5% CO2. The cultures in triplicates were pulsed with 1 μCi/well high-specific activity [3H]thymidine for the last 4 h before harvesting and radioactivity incorporation was counted. The results are expressed as U/ml by reference to standards of rIL-2 and rIL-4 (Genzyme, Boston, MA). The IFN-γ activity in the supernatant was analyzed by a solid-phase immunoradiometric assay using a laboratory reference standard of recombinant MuIFN-γ derived from Chinese hamster ovary (CHO) cells calibrated in U/ml as described previously (16).

Results

Antigenicity of the Peptides. Six peptides corresponding to the tandemly repeating regions of proteins encoded by *L. major* genes (9) were synthesized: p178, p179, and p180 correspond to a repetitive peptide of 14 amino acids (Gln Glu Thr Ser Ala Asn Val Ala Asp Thr Glu Thr Leu), while p181, p182, and p183 correspond to another repetitive peptide of 10 residues (Glu Ala Glu Glu Ala Ala Arg Leu Glu Ala). p178 and p180 are peptide amides, p179 and p182 have an extra glycine residue on the COOH termini, and p180 and p183 are of the octamer configuration as detailed in Fig. 1. Genetically susceptible BALB/c mice developed resistance to leishmanial infection when they were immunized intravenously or intraperitoneally with lethally irradiated parasites or soluble parasite antigen. In contrast, they developed exacerbated disease if the immunization was administered subcutaneously (15). This has been attributed to the preferential induction of the protective Th1 cells by the intravenous route and the activation of the disease-promoting Th2 cells by the subcutaneous route (7). BALB/c mice were immunized intravenously or subcutaneously with the SolAg extract of *L. major* promastigotes together with *C. parvum*, and their lymphoid cells were tested against the peptides for proliferative response. Mesenteric lymph node cells (LNC) and spleen cells from mice immunized one to four time intravenously with SolAg in a range of adjuvants (*C. parvum*, Al(OH)3, MDP, saponin, and liposomes) produced little or no response against any of the peptides tested, but showed significant proliferation against SolAg (data not shown). In contrast, LNC from subcutaneously immunized mice developed significant response to all the six peptides (Fig. 2). The response was antigen specific as no proliferation was detected against SRBC. The proliferative response of the LNC against the peptides ranged from 30 to 60% of that stimulated by the SolAg derived from the whole organism, indicating that the peptides could be major immunogenic components of the SolAg preparation when presented via the subcutaneous route.
Figure 1. The amino acid sequence and the theoretical configurations of the octamer peptides (180 and 183).

LNC were also obtained from BALB/c mice with progressive L. major infection and from BALB/c mice recovered from the infection as a result of sublethal γ irradiation just before infection with L. major (17). The former LNC population is known to contain predominantly Th2 cells secreting IL-4 and IL-5, whereas the latter are mainly Th1 cells producing IL-2 and IFN-γ upon specific antigen stimulation in vitro (18, 19). When these cells were cultured with the peptides in vitro, only p183 elicited a positive response, which was only found with the LNC from mice with progressive disease (Table 1). None of the peptide was recognized by the LNC from mice recovered from the infection.

Immunogenicity of the Peptides. BALB/c mice were then injected subcutaneously with 100 μg of the peptides in CFA. LNC were tested 7 or 14 d later for proliferative response in vitro against the peptide as well as SolAg. Only p183 showed a strong response against itself and a significant proliferation against the SolAg (Fig. 3, a and b). LNC from p183-primed mice also responded to p181 and p182 to a degree similar as that of SolAg (data not shown). The response is antigen specific in that the LNC did not recognize SRBC or p180, the octamer configuration of the 14 residue repeat.

Table 1. The Proliferative Response of LNC from Infected and Cured BALB/c Mice against Peptides and SolAg

| LNC from BALB/c Mice | SolAg | p178 | p179 | p180 | p181 | p182 | p183 |
|----------------------|-------|------|------|------|------|------|------|
| With progressive disease† | 52 ± 2 | <1.0 | <1.0 | <1.0 | 2.1 ± 0.1 | 3.1 ± 0.2 | 21.2 ± 3 |
| Recovered from infection§ | 83 ± 5 | <1.0 | <1.0 | <1.0 | <1.0 | <1.0 | <1.0 |

* SolAg = 10^6 organism equivalent/well. Peptides = 5 μl/well. Figures = mean ± 1 SEM of triplicate cultures, after subtracting background cpm in cultures without antigen (2.5 ± 0.1).
† BALB/c mice infected in the shaved rump with 2 x 10^7 L. major promastigotes 90 d previously, with mean lesion size of 7.5 ± 1.0 mm and progressing.
§ BALB/c mice were irradiated with 550 rad and infected in the rump 4 h later with 2 x 10^7 promastigotes. LNC were obtained 125 d later when lesions had completely healed.
Characterization of T Cells Induced by p183. The proliferative response of LNC from mice immunized subcutaneously with p183 and CFA was inhibited by anti-CD4 antibody (YTS 191) and anti-I-Ad antibody (MKD 6), but not by anti-CD8 antibody (YTS 169) or anti-I-A' antibody (Y3P) (Fig. 4). The response induced by p183 is therefore due to class II-restricted CD4+ cells. No cytotoxic T cell activity was detected when spleen or LN cells from peptide-or L. major-immunized mice were tested against a tumour (P815) cell line coated with various concentrations of the peptides in a standard cytotoxic T cell assay (5 d in vitro stimulation followed by a 5–18-h assay, data not shown). The LNC from BALB/c mice immunized 7 d previously with p183 or p180 in CFA were cultured for 24–48 h with 0.5 µg/ml of p183 in vitro. The culture supernatants of cells from mice immunized with p183 and CFA were found to contain high levels of IL-4 but little or no IL-2 or IFN-γ (Table 2). IL-2, IL-4, or IFN-γ were not detectable in any of the nonproliferating cultures. These results therefore indicate that p183 induces predominantly Th2 cells when administered via the percutaneous route.

The Effect of p183 on Disease Progression. Since among the six peptides tested, only p183 can induce significant immunological responses in vivo, experiments were therefore carried out to test its ability to influence the course of infection in BALB/c mice. When BALB/c mice were injected four times intravenously with SolAg, they developed substantial resistance to a challenge infection (Fig. 5). However, no significant protection was induced by similar intravenous immunization with p183 with or without C. parvum. In contrast, mice injected subcutaneously with p183 with or without C. parvum consistently developed significantly exacerbated lesions after challenge infection compared with unimmunized controls (Fig. 5). These results suggest that p183 may be a disease-exacerbating epitope when administered via the percutaneous route. This is confirmed by the result of the following experiment. BALB/c mice were given four weekly subcutaneous injections of p183 with C. parvum or C. parvum alone, and then were immunized four times intravenously with a 2 × 10^7 organism equivalent of SolAg. The resistance developed in the intravenously immunized mice was not affected by prior C. parvum injection alone, but was completely abrogated by the prior subcutaneous injection of p183 + C. parvum (Fig. 6). Disease exacerbation by subcutaneous immunization is leishmanial specific, as reported previously (15). p183 induced little or no delayed-type hypersensitivity to itself or to SolAg (data not shown).

![Figure 3](image3.png) Induction of specific proliferative T cell response in BALB/c mice by peptide 183. BALB/c mice were immunized subcutaneously in the flanks with: (a) 100 µg of p183 emulsified in CFA; or (b) CFA alone. Draining lymph node cells were harvested 7 d later and stimulated in vitro against graded doses of p183, p180, SolAg, or SRBC. The results are expressed as [3H]thymidine incorporation in cpm, after subtracting background response in the absence of antigen (1,900 cpm). p180 is included here to show that the response is not due to the recognition of the octamer configuration.

![Figure 4](image4.png) BALB/c mice were immunized subcutaneously in the flanks with 100 µg p183 in CFA. Draining LNC were harvested 7 d later and stimulated in vitro with graded doses of p183 in the presence of various antibodies as indicated. Antibody (10 µg/well) was added at the start of the culture. All antibodies were ammonium sulphate precipitated ascitic fluids: anti-CD4 (YTS 191) and anti-CD8 (YTS 169) were from Dr. H. Waldmann, Cambridge University, anti-I-A<sup>d</sup> (MKD 6) and anti-I-A<sup>b</sup> (Y3P) were from Dr. J. Tite, Wellcome Biotech. The results were expressed as in Fig. 2.
Table 2. Lymphokine Production by LNC from BALB/c Mice Immunized with Peptides

| Immunization | IL-2 | IFN-γ | IL-4 |
|--------------|------|-------|------|
| p183 + CFA   | 12   | 3     | 240  |
| CFA          | 10   | 2     | <1.0 |
| p180 + CFA   | 5    | 2     | <1.0 |

BALB/c mice were injected subcutaneously with 100 μg of peptide in CFA, and the draining LNC were cultured with μg/ml of p183. The supernatants were harvested 24 h later and assayed for lymphokine activities as described in Materials and Methods.

Genetic Restriction of p183. Several strains of inbred mice (BALB/c, BALB.b, BALB.k, B10.D2, CBA, B10, and B10.S) were tested for their ability to respond to the six peptides. The mice were immunized with the peptides in CFA, and the LNC was stimulated by graded concentrations of the peptides in vitro. The results in Table 3 clearly demonstrate that the response to p183 is H-2d restricted. The other five peptides were not able to induce a detectable response in all the strains of mice tested (data not shown).

Effects of p183 in the Genetically Resistant B10.D2 Mice. Since B10.D2 (H-2d) is genetically resistant to L. major infection and is responsive to p183, it is of interest to test whether p183 can also influence the disease progression in this strain of mice. Groups of B10.D2 mice were given four weekly subcutaneous or intravenous injections of p183 and C. parvum and then challenged in the footpads with L. major. Table 4 shows that the mice injected subcutaneously with p183 and C. parvum developed significantly exacerbated disease compared with control mice immunized with C. parvum alone. p183 administered intravenously had little or no effect. It

Table 3. Genetic Restriction of T Cell Response to p183

| Antigen in vitro | Strain | H-2 | p183 | p182 | p180 | SolAg |
|------------------|--------|-----|------|------|------|-------|
|                  | cpm × 10^5 |
| BALB/c           | d      | 54.8 ± 5.2 | 7.7 ± 0.8 | <1.0 | 5.2 ± 0.5 |
| BALB.b           | b      | <1.0   | <1.0  | <1.0 | <1.0 |
| BALB.k           | k      | 1.0    | <1.0  | <1.0 | <1.0 |
| CBA              | k      | <1.0   | <1.0  | <1.0 | <1.0 |
| B10              | b      | <1.0   | <1.0  | <1.0 | <1.0 |
| B10.S            | s      | <1.0   | <1.0  | <1.0 | <1.0 |
| B10.D2           | d      | 40.3 ± 3.2 | 3.7 ± 1.5 | <1.0 | 3.5 ± 0.8 |

Mice were injected subcutaneously with 100 μg of p183 in CFA and LNC were harvested 7 d later and stimulated in vitro with 5 μg/well of peptide or 10^6 organisms/well of SolAg.

* Figures are the mean of triplicate cultures ± 1 SEM, after subtracting background cpm in cultures without antigen (3.1 ± 0.2).
Meager, and J. P. Tite, manuscript submitted for publication.

Peptides representing single Th epitopes can induce both Th1 local cytokines (23-25). There is now evidence that synthetic Th2 clones (Gao, X.-M., F. Y. Liew, K. Bottomly, A. Meager, and J. P. Tite, manuscript submitted for publication). Data reported in the present study show that certain epitopes (e.g., p183) can nevertheless preferentially induce a subset of Th cells. However, such a bias is likely to be due to the mode of antigen presentation rather than repertoire selection, because p183 can only induce Th2 cells via the subcutaneous route. Immunization with p183 by the intravenous route provoked little or no host response. Other factors, such as the MHC gene products, may also influence the induction of CD4+ T cell subsets. 1-A' mice were found to selectively activate Th1 cells, whereas 1-A+ mice exhibited selective activation of Th2 cells in response to collagen IV (26).

The crucial role of APC is also underlined by the fact that only the octamer configuration of the peptide can induce a significant immune response. The monomers (p181, p182), although recognizable by T cells from mice immunized with SolAg in vitro (Fig. 2), were not able to induce a T cell response. The mechanism by which the octamer p183 is processed and presented to T cells leading to the preferential activation of Th2 cells is unknown. During infection, T cells from the susceptible mice only were activated, demonstrating the complexity of this process. Normally, susceptible BALB/c mice and DBA/2 mice (unpublished results) contain Th2 cells recognizing p183 during the course of infection. In contrast, T cells from BALB/c mice that had been rendered resistant to L. major infection by prior sublethal irradiation did not respond to p183 or its monomeric forms (Table 1). Similarly, the highly resistant B10.D2 mice also did not produce a detectable T cell response to p183 during L. major infection, although they responded vigorously to p183 when immunized subcutaneously with p183 plus CFA (Table 3).

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The T cell response to p183 is H-2d restricted. However, mice of other H-2 haplotypes with the BALB/c background are also susceptible to L. major infection. BALB.b and BALB.k mice, although less vulnerable to L. major infection compared with BALB/c mice, nevertheless develop disseminated disease with fatal outcome (27). Thus, the induction of Th2 cells by p183 epitope is but one of several mechanisms capable of influencing the outcome of leishmanial infection.

In malaria, it has been proposed that regions of repetitive peptides may play a role in immune evasion and thus contribute to chronic infection (28, 29) by over stimulating B cells with otherwise too low an affinity for the antigen to be activated. This may result in the failure of the host to select B cell clones capable of synthesizing higher affinity antibody. The demonstration here that a tandemly repeating peptide of Leishmania, a distinct protozoa that also causes chronic infection, induces disease exacerbation suggests that some of these molecules may serve a general function by conferring a selective advantage to protozoa parasites. The finding here also suggests that they do so by preferentially inducing Th2 cells, which secrete IL-4 neutralizing the macrophage-activating function of IFN-γ produced by Th1 cells (8).

### Table 4. Effect of p183 Immunization on Disease Development in B10.D2 Mice

| Immunization        | Lesion size (mm) |
|---------------------|-----------------|
|                     | Week 2 | Week 5 | Week 10 |
| p183 + C. parvum (s.c.) | 1.5 ± 0.2 | 5.8 ± 0.7 | 3.2 ± 0.6 |
| p183 + C. parvum (i.v.) | 1.1 ± 0.1 | 2.5 ± 0.2 | 1.0 ± 0.1 |
| C. parvum alone (s.c.) | 1.3 ± 0.1 | 2.0 ± 0.1 | 1.3 ± 0.1 |
| C. parvum alone (i.v.) | 1.0 ± 0.1 | 1.9 ± 0.2 | 1.0 ± 0.1 |
| Control             | 0.5 ± 0.1 | 2.8 ± 0.2 | 1.2 ± 0.1 |

Mice were immunized weekly with 100 µg p183 + 100 µg C. parvum or C. parvum alone and challenged in the footpad with 10⁶ promastigotes 7 d after the last immunization.

* Lesions were measured at various times post-infection. Figures are mean ± 1 SEM, n = 5; those underlined are significantly different (p < 0.05) from controls.

should be noted that LNC from the resistant B10.D2 mice obtained at various stages of the infection did not produce a significant proliferative response to p183 in vitro (data not shown), although they responded vigorously to SolAg.

### Discussion

Data presented in this report suggest that the octamer of a tandemly repeating peptide from L. major could induce exacerbated disease when administered subcutaneously to genetically susceptible or resistant strains of mice in the murine leishmaniasis model. Furthermore, evidence was also presented that the peptide did so by preferentially inducing Th2 cells, which secrete IL-4 but little or no IL-2 and IFN-γ (1). This finding extends the earlier observation that in the cutaneous leishmanial system, Th2 cells are disease-promoting (6, 7, 20); it may also provide a molecular model for the selective induction of these cells.

Scott et al. (20) reported that Th1 and Th2 cells were separated by two noncrossreacting fractions of L. major antigen extract. Jardim et al. (21) showed that a synthetic peptide (PT3), corresponding to a conserved region of the major surface glycoprotein (gp63) of Leishmania, could activate Th1 type cells and induce a host-protective response in BALB/c mice. We also found that synthetic peptides covering a similar region of the gp63 molecule could preferentially stimulate Th1 cells in vivo and protect CBA mice against L. major infection (22). This implies that Th1 and Th2 cells may have different antigen-recognition repertoires. Others have argued that Th1 and Th2 cells are derived from a common precursor cell and the eventual differentiation into these two subsets is influenced by accessory signals such as local cytokines (23-25). There is now evidence that synthetic peptides representing single Th epitopes can induce both Th1 and Th2 clones (Gao, X.-M., F. Y. Liew, K. Bottomly, A. Meager, and J. P. Tite, manuscript submitted for publication).
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