CD4 T helper cell response-choice between Th1 and Th2
Review of Literature


2.1 Background

2.1.1 T cell differentiation

During a primary immune response, naïve helper T cells (Th cells) produce IL-2 and proliferate. After entering the expansion phase, the progeny becomes competent to produce effector cytokines such as IFN-γ (a Th1 type cytokine) or IL-4, IL-5 and IL-13 (the Th2 cytokines). After primary activation, helper T cells express cytokines for several days (Assenmacher, M. et al. 1994.; Assenmacher, M. et al. 1998.). Later, the pattern of expression of the cytokines is memorised by Th cells since upon restimulation they express the cytokines that they had been instructed to express earlier during the primary response (Sornasse, T. et al. 1996.). Thus, T helper lymphocytes, by selective expression of cytokines, control immune reactions and call up distinct immune effector functions.

2.1.2 Functional characteristics of Th1 and Th2 T cells

The Th1 and Th2 T cell clones were initially characterised on the basis of their ability to produce distinct set of cytokines. Th1 cells produce IL-2, IFN-γ and TNF-β while Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 (Mosmann, T. R. et al. 1986.; Cherwinski, H. M. et al. 1987.).

In a Th1 cytokine response, activation of macrophages by IFN-γ results in destruction of intracellular parasites, virus infected cells (Murray, H. W. et al. 1985.) and tumor cells (Pace, J. I. et al. 1983.). IFN-γ also functions as a switch factor for the production of IgG2a by B cells (Snapper, C. M. et al. 1987.) and upregulates the expression of Fc receptors for IgG2a antibodies (Warren, M. K. et al. 1985.) leading to enhanced
antibody-dependent macrophage cytotoxicity. Th1 cells are also mediators of a strong delayed-type hypersensitivity reaction (Cher, D. J, and T. R. Mosmann. 1987.). Th2 cells are primarily involved in the clearance of the extracellular pathogens. IL-4, IL-5 and IL-6 promote B cell growth and differentiation. IL-4 greatly enhances the production of IgE by B cells (Coffman, R. L, and J. Carty. 1986.), induces switching to IgG1 production (Isakson, P. C. et al 1982.), upregulates the expression of IgE FcE receptors on B cells (Hudak, S. A. et al. 1987.) and MHC class II on macrophages (Zlotnik, A. et al. 1987.). IL-3 and IL-4 induce mucosal mast cell proliferation (Mosmann, T. R. 1988.) and IL-5 functions as a proliferative cytokine for eosinophils (Sanderson, C. J. et al. 1986.). Th1 and Th2 cytokines also produce many additional effects by influencing the expression of various adhesion molecules, chemokine receptors and other cell-surface molecules that alter leukocyte trafficking and homing responses (Carter, L. L. et al 1996.; Bradley, L. M et al 1996.).

2.1.3 Factors regulating Th1/Th2 cytokine phenotype

Since Th1 and Th2 type of responses are of utmost importance to the host to deal with different kind of pathogens, the selective activation or differentiation of the response is, therefore, subject to stringent regulation.

2.1.3.1 Cytokines

The role of cytokines in regulating T cell commitment has been vigorously studied. IL-4 has been shown to favour the growth/differentiation of Th2 cells and diminish the generation of Th1 cells. CD4 T cells from naive IL-4 deficient mice fail to produce Th2-
derived cytokines after *in vitro* stimulation (Kopf, M. et al. 1993.). The levels of IL-5, IL-9 and IL-10 from CD4 T cells obtained after *Nippostrongylus brasiliensis* infection are significantly reduced. IL-5 dependent helminth-induced eosinophilia is also reduced. In case of infection with *Leishmania major*, depletion of IL-4 by administration of neutralizing anti-IL-4 antibodies makes susceptible mice (BALB/c) resistant (Sadick, M. D. et al. 1990.; Sadick, M. D. et al. 1991.; Chatelain, R. et al. 1992.) and depletion of IFN-γ by either anti-IFN-γ monoclonal antibody (mAb) or gene disruption renders resistant mice susceptible to infection (Swihart, K. et al. 1995.; Wang, Z. E. et al. 1994.; Belosevic, M. et al. 1989.). Similarly, BALB/c mice genetically deficient in IL-4 are resistant to infection while the heterozygous counterpart develops smaller lesions with less ulceration and necrosis, indicating that the magnitude of IL-4 response determines the severity of the disease (Kopf, M. et al. 1996.). IL-10 also favours the generation of Th2 response but its effect on the cells during priming is less as compared to IL-4 (Hsieh, C. S. et al. 1992.; Macatonia, S. E. et al. 1993.).

In contrast to IL-4, IFN-γ directs the development of Th1 cells and inhibits the Th2 response. The role of IFN-γ has also been well studied in autoimmune mouse model of "experimental allergic encephalomyelitis" (EAE) (Kuchroo, V. K. et al. 1995.). By the use of exogenous Th2 cytokines, neutralising antibodies to TNF-α or soluble TNF receptor (TNF-R), the disease is reversed (Racke, M. K. et al. 1994.; Rott, O. et al. 1994.; Shaw, M. K. et al. 1997.; Mathisen, P. M. et al. 1997.). However, there are paradoxical results wherein injection of anti-IFN-γ antibody results in exacerbation of disease (Billiau, A. et al. 1988.; Duong, T. et al. 1992.) and administration of recombinant IFN-γ has been found to have a protective effect (Lublin, F. D. et al. 1993.; Voorthuis, J. et al. 1990.).
Moreover, EAE has also been induced in IFN-γ knockout and IFN-γ-receptor knockout mice (Ferber, I. A. et al. 1996.; Krakowski, M. et al. 1996.; Willenberg, D. O. et al. 1996.). These results together suggest that IFN-γ can actually act to suppress the development of EAE either during the evolution of encephalitogenic effectors or at a downstream event critical for the formation of the demyelinating plaques. In IFN-γ knockout mouse the Th1 cytokines are reduced thereby resulting in impaired type 1 response rather than a complete abrogation of Th1 response which could be due to the endogenous IL-12.

IL-12 has also been shown to promote the development of Th1 response (Magram, J. et al. 1996.; Trinicheri, G. 1997.). However, unlike IFN-γ, IL-12 directly favours Th1 response without any effect on Th2 response.

Of the regulatory cytokines, IL-12 is produced upon activation of the macrophages which in turn induces the production of IFN-γ by NK cells (Scott, P. 1993.; Seder, P. et al. 1991.). Mast cells and basophils contribute IL-4 during priming upon crosslinking of their FcRe (Seder, P. et al. 1991.; Plaut, M. et al. 1989.). Various reports suggest the role of NK1.1 CD4 T cells interacting with non polymorphic CD1 as the major contributors of IL-4 (Bendelac, A. 1995.). Thus, the preponderance of a particular cytokine in the microenvironment at the time of priming influences the cytokine commitment of T cells.

2.1.3.2 Antigen Presenting Cells

A CD4 T cell sees an antigen in the form of peptide-MHC class II complexes present on the plasma membrane of an APC. Each population of APC is assumed to deliver a primary signal through Ag associated with MHC class II, and distinct costimulatory signals,
through CD28-CD80/CD86, CD40/CD40L, and IL-1. The differences in the ability of
different APCs to promote growth of distinct Th subsets may be related to the expression
of costimulatory molecules at a given time (or under a given activation state) and the
intracellular processing (Weaver, C. T. et al. 1988.). According to some reports, B cells
are the primary APCs for Th2 clones (Gajewski, T. F. et al. 1991), while macrophages and
dendritic cells are the primary APCs for Th1 clones (Hsieh, C. S. et al. 1993.; Macatonia,
S. E. et al. 1993).

Besides differential levels of costimulation provided by different APCs, it has also been
suggested that there exists differences in the levels of peptide-MHC complexes as well as
the stability of these complexes.

2.1.3.2.1 Generation of peptide-MHC complexes

MHC class II molecules are assembled in the endoplasmic reticulum (ER) from an \(\alpha\) and a
\(\beta\) chain, which jointly form a peptide-binding groove. Invariant chain (Ii) trimerises in the
ER, which is used as a template for association with three class II \(\alpha-\beta\) heterodimers
(Cresswell, P. 1996.). The resulting trimer of class II \(\alpha-\beta-Ii\) heterodimers \((\alpha\beta Ii)3\) is
released from the ER. Following exit from the ER, Ii targets class II molecules to the
Golgi and trans Golgi network (TGN) and eventually to the endolysosomal compartments.

Here, the Ii chain is degraded, probably in a sequential manner, by proteases such as
cathepsin S (Reise, R. et al. 1996.). The shortest fragment of Ii found associated with
class II molecules is CLIP (Rudensky, A. Y. et al. 1991.; Chicz, R. M. et al. 1992.;
in class II maturation (Morkowski, S. et al. 1995.). The exchange of CLIP with the
antigenic peptides takes place in multivesicular and multilamellar structures in many cell types. These structures are termed as MIIC (for MHC class II containing compartment). These compartments originally identified in B cells (Sanderson, F. et al. 1994.), have also been identified in macrophages (Harding, C.V. and Geuze, H. J. 1992.; Harding, C.V. and Geuze, H. J. 1993.), dendritic cells (Kleijmeer, M. J. et al. 1995.; Nijman, H. W. et al. 1995.) and Langerhans cells (Kleijmeer, M. J. et al. 1994.; Mommaas, A. M. et al. 1995.). A distinct set of class II containing vesicles (CIIV) has also been identified in B cells (Amigorena, S. et al. 1994.). However, controversy still exists concerning the principal site of formation of peptide-MHC class II complexes.

Since lysosomes are the last subcellular organelle in the endocytic pathway, they are considered a dead end. However, certain vesicles of lysosomal nature (Griffiths, G. M. 1996.) have been shown to track along the microtubules before fusing with the plasma membrane and that MHC class II molecules may also follow a similar route.

2.1.3.2 Stability of peptide-MHC class II complexes

The half-life of peptide-MHC class II complexes in a given microenvironment influences the response of a T cell. Functional and biochemical assays have demonstrated that the lifetime of complexes are long and similar to that of MHC class II molecules themselves (Lanzavecchia, A. et al. 1992.). The half-life of peptide-MHC class II complexes is not decreased even in the presence of a large excess of peptides which may be better binders (Nelson, C. A. et al. 1994.). Moreover, the lifetime of the complexes depends on the length and the sequence of the peptide and correlates with the number and quality of peptide-MHC contacts made (Stern, L. J. et al. 1994.). Thus, different complexes between
peptides and MHC class II molecules vary greatly in the amount of time associated with the APC and in their immunogenic strength. Estimates of half-lives of peptide-MHC class II complexes on different cell types in vitro ranges between 20 and 50 h for human T cells and EBV-transformed B cells (Lanzavecchia, A. et al. 1992.). For mouse B lymphoma cells and peritoneal exudate cells half-lives of 1-6 h have been reported, where the short half-life of peptide-MHC class II complexes on the B lymphoma cells is suggested to be due to rapid recycling and peptide exchange (Harding, C. V. et al. 1989.). Antigen-pulsed dendritic cells can retain antigen for a day or more, which may be in part because of an increase in the amount of stability of peptide-MHC class II complexes (Pure, E. et al. 1990.; Inaba, K. et al. 1990.).

Half-lives in vivo are generally shorter than the respective half-lives in vitro (Muller, K. P. et al. 1993.) since they are limited by parameters such as the rate of MHC class II synthesis, the rate of MHC class II recycling, the stability of membrane bound peptide-MHC class II complexes, the turnover rate of APC in various organ microenvironments and the state of activation of APC. For instance freshly isolated Langerhans cells (LC) show a high rate of MHC class II synthesis and are very efficient in antigen processing, whereas culture in vitro leads to rapid and specific downregulation of MHC class II synthesis and concomitant loss of antigen presentation capacity (Pure, E. et al. 1990.; Kampgen, E. et al. 1991.). Splenic DCs are thought to be a population with a rapid turnover (Kupeic-Weglinski, J. W. et al. 1988.).
2.1.3.3 Dose of Antigen

The dose of foreign antigen can influence the differentiation of CD4 T cells. However, there has been quite a controversy regarding the direct correlation between the antigen dose and the cytokine profile of Th cells. Whereas earlier reports using parasites suggest that high doses favour Th2 response and conversely, low dose of antigen favours Th1 response (Bretscher, P. A. et al. 1992.; Bancroft, A. J. et al. 1994.), there is also some evidence for the reverse effect of antigen dose on the differentiation of naive CD4 T cells (Wang, L. F. et al. 1996.; Hayglass, K. T. et al. 1986.; Chaturvedi, P. et al. 1996.). Using DO11.10 TCR-αβ-transgenic CD4 T cells recognising ovalbumin (323-339) (Kupeic-Weglinski, J.W. et al. 1988.), it has been shown that midrange doses (0.3-0.6 μM) direct the development of Th1 cells whereas high doses such as >10μM and very low as <0.05μM lead to development of Th2 cells. In another study using αβ TCR-transgenic T cells specific for tobacco hornworm moth cytochrome c (pMCC) (Constant, S. et al. 1995.), it has been shown that low doses of antigen preferentially activate Th2 cells and high doses result in the activation of Th1 cells.

2.1.3.4 Affinity of peptide-MHC complexes/TCR

The affinity of peptide-MHC class II complex for TCR has been reported to regulate the cytokine profile wherein peptide-MHC class II complexes with high affinity tend to favour Th1 responses and low affinity interaction results in Th2 response (Pfeiffer, C. et al. 1995.). Affinity of peptide for MHC class II molecules also alters the cytokine profile. CD4 T cells primed with high affinity peptide are committed to differentiate into Th1 cells irrespective of the priming dose, whereas the differentiation of CD4 T cells primed with
low affinity peptide depends on the dose of immunisation (Chaturvedi, P. et al. 1995.). It has also been shown that mutation of a single amino acid residue in the TCR α chain complementarity determining region 2 of T cells from D10 TCR Tg mice, which reduces the response to the wild type peptide by 100 fold, switches the differentiation of naïve CD4 T cells from Th1 to Th2. However, when a mutation is made in the wild type peptide, the response as well as the differentiation to Th1 profile is restored (Blander, J. M. et al. 2000.).

2.1.3.5 Costimulation

Various studies have suggested a pivotal role for CD28-CD80/CD86 interaction in the activation of T cells. Signal transduction through CD28 synergizes with TCR signal transduction to augment both interleukin-2 (IL-2) production and proliferation of naive T cells (Thompson, C. B. 1995.). Signaling through CD28-CD80/CD86 pathway is complex because of the existence of two B7 family members (CD80 and CD86) and two CD28 family members (CD28 and CTLA-4) providing potential redundancy in the interactions between B7 and CD28 family members (Freeman, G. J. et al. 1993.). Monocytes constitutively express CD86 (Azuma, M. et al. 1993.; Nozawa, Y. et al. 1993.), whereas CD80 is induced after culture with interferon-γ (IFN-γ) (Freedman, A. S. et al. 1991.). On B cells, CD86 is rapidly expressed following activation, whereas CD80 expression appears significantly later (Freeman, G. J. et al. 1993.; Boussiotis, V. A. et al. 1993b.; Hathcock, K. S. et al. 1994.). CD86 is expressed at low levels on unstimulated dendritic cells and expression of both CD80 and CD86 is upregulated by granulocyte-monocyte colony stimulating factor (GM-CSF) (Hart, D. N. et al. 1993.).
In an EAE model, disruption of CD80 interaction using mAb to CD80 results in reduction in the severity of the disease and enhanced production of IL-4 while treatment with anti-CD86 mAb leads to aggravation of the disease, with high levels of type 1 cytokine, IFN-γ. Thus, it suggests the differential role of CD80 and CD86 in type 1 and 2 cytokine response respectively (Kuchroo, V. K. et al. 1995.).

In CD28 deficient mice development of T cells and B cells is normal (Shahinian, A. et al. 1993.). However, T cells derived from CD28 deficient mice have impaired responses to lectins, anti-CD3 and allo antigens. In addition, activity of T helper cells in CD28 deficient mice is reduced. In NOD (non-obese diabetic mouse) mouse strain with CD28 deficiency, T cells produce enhanced levels of Th1 type cytokine, IFN-γ and diminished Th2 type cytokine, IL-4 (Lenschow, D. J. et al. 1996.). Thus, the early differentiation of naïve diabetogenic T cells into the Th2 subset is dependent on CD28 signaling.

Recently, a third member of CD28 family has been identified and characterised-inducible costimulatory molecule (ICOS). ICOS is present on activated T cells and interacts with B7H/B7RP-1 expressed on B cells (Hutloff, A. et al. 1999.; Yoshinaga, S. K. et al. 1999.). Mice deficient in ICOS have impaired immunoglobulin class switching, including production of allergy-mediating IgE. ICOS deficient T cells primed in vivo and restimulated in vitro produce low levels of IL-4, but are fully competent to produce IFN-γ (Tafuri, A. et al. 2001.). ICOS deficient mice show greatly enhanced susceptibility to EAE, suggesting that ICOS has a protective role in inflammatory autoimmune diseases (Dong, C. et al. 2001.).

Besides, CD28/B7 signaling, CD40/CD40L interaction has also been reported to be important for the polarisation of helper T cells. CD40 is present on the professional APCs.
and interacts with its ligand, CD40L, which is induced on T cells upon activation. CD40 deficient mice are susceptible to *Leishmania major* infection and T cells from CD40 deficient mice make less IFN-γ than the wild type littermates, therefore failing to mount a Th1 response. Thus, CD40/CD40L interaction play an important role in the generation of Th1 response (Kamanaka, M. et al. 1996.; Campbell, K. A. et al. 1996.). In another study, infection of CD40L deficient mice with *Leishmania amazonensis* results in impaired T cell activation and reduced production of IFN-γ, lymphotoxin-tumor necrosis factor and nitric oxide, thus emphasising the role of cognate CD40/CD40L interaction in the generation of cellular immune response against intracellular parasite (Soong, L. et al. 1996.)

### 2.1.3.6 Adhesive Interactions

Adhesion molecules are situated to transduce information regarding the character of the apposing cell during the adhesion event. Although, the role of adhesion interactions has been extensively studied in T cell activation (Moingeon, P. et al. 1989.), their role in regulating helper T cell differentiation is not very well understood. CD2-LFA-3 adhesion mediates substantial augmentation of antigen-specific T cell responses and activation via CD2 affects integrin mediated adhesion (Shimizu, Y. et al. 1990.). Recently it has been shown that collagen binding integrins, α1β1 and α2β1, have an important role in inflammation. Mice treated with mAb to α1 and α2 show significantly reduced effector phase responses in models of both contact and delayed type hypersensitivity. Likewise, α1 deficient mice show impaired inflammatory responses in hypersensitivity and development of arthritis (de Fougerollas, A. R. et al. 2000.).
2.1.3.7 Chemokines

Numerous recent reports have extensively studied the role of chemokines in lymphocyte trafficking, priming and differentiation. They are broadly divided into two categories: inflammatory, which are induced or upregulated by inflammation, and constitutive, which are involved in housekeeping functions such as leukocyte migration. Various chemokines and their receptors have polarised expression on Th1/Th2 cells.


CCL3, CCL4 and CCL5 are expressed on multiple activated cell types including macrophages, DCs and lymphocytes and are ligands for CCR5 (Campbell, J. J. and E. C. Butcher. 2000.; Sallusto, F. et al. 2000.). Mice deficient in CCL3 show an increased susceptibility to viral challenge and decrease in viral clearance, via compromised Th1 responses (Cook, D. N. et al. 1995.). However, CCR5 deficicent mice show limited defects in cell-mediated immune responses (Zhou, Y. et al. 1998.), which could be because CCL3 also binds to CCR1. However, there are contrasting findings with CCR1 deficient mice, which show exaggerated Th1 responses (Topham, P. S. et al. 1999.).
In brief, Th2 cells show the expression of CCR2, CCR3, CCR4 while Th1 cells are characterised by the expression of CCR1, CCR2, CCR5, CXCR3 and CXCR5. However, the exact mechanism by which the interaction of chemokines with their receptors favour Th polarisation remains to be elucidated.

2.1.3.8 Transcriptional regulation of Th1/Th2 cytokine phenotype

Recently, the molecular basis for the Th polarisation has been analysed and several Th specific transcription factors have been identified that are selectively expressed in Th1 or Th2 cells. Proteins of the nuclear factor of activated T cells (NFAT) family cooperate with activator protein (AP-1) factors like Fos and Jun to induce IL-4 transcription (Rengarajan, J. et al. 2000). Although NFAT and AP-1 proteins are critical for IL-4 production, they are expressed in both Th1 and Th2 cells.

c-Maf, a proto-oncogene that codes for a leucine-zipper transcription factor, is specifically expressed in Th2 cells and strongly induces IL-4 production (Hodge, M. R. et al. 1996.; Ho, I-C. et al. 1996.). Mice deficient in c-Maf have impaired IL-4 production but the production of other Th2 cytokines is unaltered (Kim, J. et al. 1999.). Mice bearing the transgene for c-Maf show greater Th2 responses but Th1 T cell clones carrying transgene for c-Maf do not produce IL-4 (Ho, I-C. et al. 1998.).

GATA-3 is a zinc finger protein that is specifically expressed in Th2 cells. It is expressed during the process of Th2 differentiation and is undetectable in Th1 cells (Zheng, W-P. and R. A. Flavell. 1997.). GATA-3 has also been shown to regulate the expression of IL-5 and IL-13 and therefore, plays a broad role in the expression of Th2 cytokines (Zhang, D.
H. et al. 1997.). GATA-3 has also been shown to inhibit IFN-γ production and subsequent Th1 development (Ouyang, W. et al. 1998.).

T-bet is a member of T-box family of transcription factors and its expression correlates with the expression of IFN-γ. It is specifically regulated during Th1 differentiation pathway (Szabo, S. J. et al. 2000.). Transduction of T-bet into Th1 committed T cells converts them to Th1 polarised cells and represses the production of Th2 cytokines, IL-4 and IL-5.

The transcription factor IRF-1 has also been implicated in regulating Th1 differentiation. Mice lacking IRF-1 fail to mount Th1 responses and instead undergo Th2 differentiation (Taki, S. et al. 1997.). These mice also show defect in production of IL-12 by macrophages, hyporesponsiveness of CD4 T cells to IL-12 and defective development of NK cells.

Besides various transcription factors, various adaptor molecules in the signal transduction cascade downstream of cytokine receptors have been implicated in regulating the Th1 or Th2 cytokine response. Signal transducers and activators of transcription (Stat) are critical in transmitting cytokine dependent signals.

IFN-γ, IL-2 and IL-6 activate Stat 1 and therefore, Stat 1 deficient mice are highly susceptible to viral and some bacterial infections, a phenotype similar to that seen in IFN-αR and IFN-γR deficient mice (Nguyen, K. B. et al. 2000.).

Stat 4 is activated by IL-12 and therefore, Stat 4 deficient mice exhibit defective IL-12 responses and Th1 differentiation (Wurster, A. L. et al. 2000.). The phenotype is similar to that seen in IL-12 and IL-12R deficient mice (Wu, C. et al. 2000.).
Stat 6 is activated by IL-4 and mice deficient in Stat 6 fail to develop Th2 immune responses (Wurster, A. L. et al. 2000.). IL-4 dependent proliferation is blocked in Stat 6 deficient mice, have impaired regulation of IL-4 inducible genes and attenuated allergic and asthmatic disease (Miyata, S. et al. 1999.). However, all IL-4 responses are not abrogated as anti-apoptotic effects are preserved in Stat 6 deficient mice. IL-13 also activates Stat 6, so IL-13 responses are also abrogated in Stat 6 deficient mice. Interestingly, mice deficient in both Stat 4 and Stat 6 develop default Th1 responses (Glimcher, L. H. and K. M. Murphy. 2000.).

There have been identified various inhibitory proteins that function to dampen or terminate cytokine signals. One such family is the SOCS (suppressor of cytokine signaling) family which has eight members- SOCS1-7 and CIS (cytokine inducible SH2 domain containing protein). However, the role of SOCS1 cytokine phenotype has been studied extensively. SOCS1 is induced by IL-2, IL-4, IL-6, LIF, IFN-α/β, IFN-γ. SOCS1 deficient mice have activated Stat 1 in the liver, have elevated expression of IFN-γ inducible genes, are hypersensitive to IFN-γ and have elevated levels of IFN-γ (Marine, J. C. et al. 1999.; Alexander, W. S. et al. 1999.).

In addition to the selective expression of the transcription factors in Th1/Th2 cells and signaling proteins, the role of chromatin remodelling at the cytokine loci in regulating the expression of cytokine genes have been analysed (Agarwal, S. and A. Rao. 1998.). DNase I hypersensitivity sites, that reflect local changes in chromatin structure and therefore the gene accessibility for transcription, have been identified in IL-4 locus during Th2 differentiation and not during Th1 differentiation (Agarwal, S. and A. Rao. 1998.). Demethylation of DNA also reflects the change in chromatin accessibility, which are also
seen at IL-4 locus during Th2 development and not Th1 development (Bird, J. J. et al. 1998.). GATA-3 and c-Maf have been reported to induce chromatin remodelling either directly or indirectly by recruiting modifying enzymes. Similar to IL-4 locus, specific changes in IFN-γ locus also occur during Th1 differentiation and DNAse hypersensitive sites have been identified in the IFN-γ gene (Agarwal, S and A. Rao. 1998.).

2.1.4. Flexibility of Th1/Th2 T cell cytokine response

Stability of committed Th lymphocytes is a controversial issue and contradictory reports exist for the reversibility of Th1 and Th2 cells. Th1 polarised cells with flexible cytokine profiles have been identified from lung parenchyma of influenza virus-infected mice suggesting the plasticity of an immune response in an effector site (Doyle, A.G. et al. 1999.). Using in vitro model for the differentiation of IFN-γ producing CD4 Th1 cells, it has been shown that exposure of differentiated Th1 cells to IL-4 during priming converts them into IL-4 producers whereas Th2 cells cannot be reprogrammed to Th1 type (Perez, V. L. et al. 1995.). In a similar system, presence of IL-12 during priming results in the selective increase in the IFN-γ producers but does not affect the IL-4 production in the clones that were primed in the presence or absence of IL-12 (Manetti, R. et al. 1994.). The reversal of Th phenotype has also been reported to be dependent on the number of stimulations. Restimulation of in vitro primed IFN-γ producing Th1 cells with Ag in the presence of IL-4 resulted in some cells stopping the expression of IFN-γ and becoming IL-4 producers (Assenmacher, A. M. et al. 1998.). Long term Th1 and Th2 populations originating from repeated stimulations in the presence of IL-12 or IL-4 respectively failed to show reversibility in their cytokine profile while Th1 or Th2 cells stimulated for a
shorter time period of 1 week could be reprogrammed to become Th2 or Th1 respectively (Murphy, E. et al. 1996.). Recently, the expression of various cytokines have been shown to be correlated with the cell division. The cells can start producing cytokines before they undergo complete cell division (Richter, A. et al. 1999.). However, cells that undergo less than four cell divisions have the ability to express alternate cytokines when restimulated under opposing conditions (Grogan, J. L. et al. 2001.) even in the absence of Stat 4 or Stat 6. The cells, however, could only sustain polarised cytokine expression in the presence of Stat signals. Cytokine genes are positioned away from the heterochromatin in naïve cells suggesting their accessibility while after polarisation most of the silenced cytokine genes are repositioned to the heterochromatin.

Developing Th1 cells selectively acquire IL-12Rβ2 expression (Szabo, S. J. et al. 1997.), which is inhibited in Th2 cells due to the activity of GATA-3 (Ouyang, W. et al. 1998.), although in the presence of forced expression of IL-12Rβ2, IL-4 is still dominant for Th2 development of T cells exposed to both IL-4 and IL-12 in vitro (Nishikomori, R. et al. 2000; Heath, V. L. et al. 2000.). Loss of IL-12Rβ2 expression has been suggested to be responsible for the stability of Th2 response.

The ability to modulate the cytokine profile of an ongoing primed T cell response is potentially useful in the treatment of a range of clinical situations where altering the Th1/Th2 balance is required since many diseases are characterised by either Th1 or Th2 T cell cytokine phenotype, for instance, allergy which is mediated by Th2 cells and its products.

In this study, issues such as factors regulating helper T cell cytokine phenotype and the plasticity of a cytokine profile have been addressed using a previously established system.
Maleylaton of proteins makes them the ligands for a class of receptors, scavenger receptors (SRs). SRs are expressed on the cells of the monocyte lineage and bind to various polyanionic ligands (Pearson, A.M. 1996.). It has been suggested that the physiological role of SRs is to remove or "scavenge" substances such as acetylated-LDL, oxidized-LDL from the blood circulation. They also bind to bacterial components like lipid A, lipotechoic acid, and to intact bacteria, thus helping in uptake and clearance of bacteria or toxic components (such as lipid A) from the system. SRs also mediate the adhesion of macrophages to lymphoid tissue sections which may be responsible for homing of macrophages to these organs. Scavenger receptors have also been implicated in clearance of apoptotic cells. Two types of SRs have been identified and characterised: (a) Class A which are present mainly on monocytes, peritoneal and tissue macrophages. Some dendritic cells, endothelial cells and smooth muscle cells have also been shown to express class A SRs. Three subclasses have been described under this category viz. SR-AI, SR-AII and MARCO and they show minor structural differences from each other. SR-AI and SR-AII exhibit similar binding properties to ligands such as acetylated-LDL, oxidized-LDL, maleyl-BSA and polynucleotides such as PolyI/PolyG. (b) Class B SRs which include CD36 (thrombospondin receptor) and SR-BI. These receptors are expressed on monocytes, capillary endothelial cells and interestingly on B lymphocytes. It has also been reported that the two classes of SRs differ in their affinity for maleylated proteins and distribution levels per cell on different APCs.

Thus by targeting proteins to APCs via SRs, it would be possible to analyse antigen presentation related issues. Reprogramming of a Th response can also be studied since it has been shown that immunisation with native protein results in the generation of Th2
cytokine response while immunisation with its maleyl counterpart generates a Th1 response (Singh, N. et al. 1998.).
Results
2.2 Results

2.2.1 Effect of scavenger receptor ligation on APCs on costimulation

It has been earlier shown in the laboratory that maleylating protein immunogens so that they become SR ligands leads to better presentation to T cells in vitro and to greater immune responses in vivo (Abraham, R. et al. 1995.). There is also a shift in Th1/Th2 balance wherein immunisation with maleyl-protein skews the cytokine response towards Th1 type while immunisation with the native protein results in relatively Th2 type of cytokine response (Singh, N. et al. 1998.). As mentioned in the review, there are several factors regulating Th1/Th2 balance. Therefore, the effect of maleyl-proteins binding to SRs on macrophages on Th polarising cytokines- IL-10, IL-12, IL-1β and TNF-α, various costimulatory molecules- CD80, CD86, CD40, CD24 and adhesion molecules-CD11a/CD18, CD54 was tested.

2.2.1.1 Production of accessory cytokines

Adherent PECs from CBA/J mice were titrated at various doses from $3 \times 10^6$/ml to $3 \times 10^3$/ml and were either unstimulated or stimulated with LPS (10 μg/ml) in the absence or presence of maleyl-BSA (100 μg/ml) for 48 h. At 48 h, the culture supernatants were collected and tested for the presence of IL-10 and IL-12 (Fig 1A and 1B) or IL-1β and TNF-α (Fig 1C and 1D) using ELISA. Fig 1 shows that the ligation of SR on macrophages by maleyl-protein does not induce the production of IL-10, IL-12, IL-1β or TNF-α. LPS was used as a positive control and it induces the production of the cytokines tested. Moreover, binding of maleyl-BSA to SRs on macrophages does not alter the ability
FIG 1: Ligation of SR on macrophages does not induce the production of accessory cytokines
CBA/J-adherent PECs were incubated at various cell concentrations with either medium alone (open circles) or maleyl-BSA (100 μg/ml; filled circles), LPS (10 μg/ml; open triangles) or LPS + maleyl-BSA (filled triangles). Culture supernatants were harvested at 48 h and assayed for the cytokines by ELISA. The experiment shown is a representative of four independent experiments.
of LPS to induce the production of IL-10, IL-12, IL-1β or TNF-α since macrophages stimulated with LPS in the presence of maleyl-BSA produce cytokines equivalent to when stimulated with LPS alone. Thus, maleyl-BSA alone or in conjunction with LPS does not affect the production of IL-10, IL-12, IL-1β or TNF-α by macrophages.

2.2.1.2 Expression of costimulatory molecules

Adherent PECs from BALB/cJ mice were treated with maleyl-BSA (50 μg/ml) for 48 h. At 48 h, the cells were stained for various cell surface costimulatory molecules CD80 (Fig 2A), CD86 (Fig 2B), CD40 (Fig 2C) and CD24 (Fig 2D). There was, however, no significant difference in the expression of CD80, CD86, CD40 and CD24 on macrophages induced by maleyl-BSA. As a control, cells were stimulated with LPS (25 μg/ml) or IFN-γ (0.3 μg/ml) for 48 h. LPS stimulated macrophages showed higher levels of CD24, CD80 and CD86 while IFN-γ stimulated cells showed higher levels of CD86 as compared to unstimulated cells. Thus, the expression of molecules involved in T cell costimulation is not altered upon SR ligation by maleyl-BSA.

2.2.1.3 Expression of adhesion molecules

In an experimental set up described above, the effect of binding of maleyl-BSA to SRs on macrophages on the expression of CD11a/CD18 (Fig 3A) and CD54 (Fig 3B). Expression of CD11b/CD18 (Fig 3E) was used as a control cell surface marker for macrophages. Expression of MHC class I (Fig 3C) and II (Fig 3D) was used as control cell surface markers as the expression of MHC class I is expected to be unaltered while that of MHC class II to be upregulated by IFN-γ. As shown in Fig 3, maleyl-BSA by itself does not
**FIG 2**: Expression of costimulatory molecules on peritoneal macrophages

BALB/cJ-adherent PECs were incubated with either medium (plain thin black) or LPS (25 µg/ml; thick black) or IFN-γ (0.3 µg/ml; thin grey) or maleyl-BSA (500 µg/ml; thick grey) for 48 h. At 48 h, the cells were stained for CD80 (panel A), CD86 (panel B), CD40 (panel C) and CD24 (panel D). This experiment is a representative of three independent experiments.
FIG 3: Expression of adhesion molecules on peritoneal macrophages
BALB/cJ-adherent PECs were incubated with either medium (plain thin black) or LPS (25 µg/ml; thick black) or IFN-γ (0.3 µg/ml; thin grey) or maleyl-BSA (500 µg/ml; thick grey) for 48 h. At 48 h, the cells were stained for CD11a/CD18 (panel A), CD54 (panel B), MHC class I (panel C), MHC class II (panel D) and CD11b/CD18 (panel E). This experiment is a representative of three independent experiments.
modulate the expression of CD11a/CD18, CD54, MHC class I and MHC class II. However, when LPS and IFN-γ were used as positive controls, LPS upregulated the expression of CD54 while IFN-γ upregulated the cell surface levels of MHC class II and CD54. The cell surface expression of CD11b/CD18 remained unaltered. Thus, SR ligation by maleyl-BSA does not modulate the expression of adhesion molecules.

2.2.2 Consequences of scavenger receptor ligation on antigen presentation

The cytokine commitment of T cells has been shown to be regulated by lineage of APC presenting the ligand to T cells as well as the longevity of peptide-MHC complexes on the surface of APC. Maleyl-BSA has been shown to bind to macrophages, B cells and dendritic cells (Singh, N. et al. 1998.). To test these possibilities the kinetics of generation and the decay of peptide-MHC complexes derived from native and maleyl-protein was analysed in an in vitro antigen presentation assay.

2.2.2.1 Generation of peptide-MHC complexes

Peritoneal macrophages, bone marrow derived dendritic cells and splenic B cells were pulsed with maleyl-CA (300 µg/ml) for various time periods at the end of which the antigen was washed off extensively. The cells were then rested for 90 min and fixed. Fixed APCs were then used to stimulate CA specific T cell clone, D10.G4.1, for 24 h. Fig 4A shows the proliferative response of D10.G4.1 against antigen pulsed various APCs as a function of time. Macrophages process and present maleyl-CA more rapidly than bone marrow derived dendritic cells, which, however, become as efficient as macrophages by 60 min. Splenic B cells present maleyl-CA much slowly than both macrophages and bone
FIG 4: Macrophages present maleyl-CA efficiently than splenic B cells and bone marrow derived dendritic cells
Peritoneal macrophages (filled squares), splenic B cells (filled circles) and bone marrow derived dendritic cells (open squares) were pulsed with maleyl-CA (300 µg/ml) for the indicated time periods and then rested for 90 min. The cells were then fixed and used as APCs to stimulate D10.G4.1 for 24 h. At 24 h, response was measured as $^3$H-thymidine incorporation (panel A). The percent maximum response is depicted in panel B. The background proliferation in response to unpulsed APCs was less than 1500 in all the groups. The above experiment is a representative of five independent experiments.
marrow derived dendritic cells. These differences in the processing and presentation abilities of various APC lineages could be due to the type of SRs present on the cell surface, the number of SRs and the endocytosis efficiency of the type of SR present on an APC surface. Therefore, the proliferative response of D10.G4.1 triggered by an APC type was normalised. Fig 4B shows the normalised response for each APC type wherein the response at each time period is calculated as the percent of maximal response shown by an APC type. The data suggest that there exist differences in the kinetics of generation of peptide-MHC complexes derived from maleyl-CA between different APC lineages.

2.2.2.2 Stability of peptide-MHC complexes

Peritoneal macrophages, bone marrow derived dendritic cells and splenic B cells were pulsed with maleyl-CA (300 µg/ml) for 1 h and then washed extensively. The cells were rested for various time periods, then fixed and used to stimulate D10.G4.1 for 24 h. Fig 5A shows the proliferative response of D10.G4.1 against various maleyl-CA pulsed APCs as a function of time. It shows that dendritic cells process and present maleyl-CA much faster and for a longer time than macrophages and B cells. However, when the response is normalised as described above (section 2.2.2.1) as indicated in Fig 5B, the data show that all the APC lineages tested here have a similar decay kinetics and the peptide-MHC complexes persist for equivalent time.

Fig 4 and Fig 5 collectively show that in spite of initial differences in the generation of peptide-MHC complexes by various APCs, the ligand thus generated has an equivalent half-life. Therefore, it is unlikely that the different APCs would play a preferential role in regulating cytokine profile in this system.
**FIG 5: Stability of peptide-MHC complexes derived from maleyl-CA is comparable between different APC lineages**

Peritoneal macrophages (filled squares), splenic B cells (filled circles) and bone marrow derived dendritic cells (open squares) were pulsed with maleyl-CA (300 μg/ml) for 1 h. The antigen was then washed off and the cells were then rested for the indicated time periods following which they were fixed. Fixed cells were then used to stimulate D10.G4.1 for 24 h. At 24 h, the response was measured as \(^{3}\text{H}\)-thymidine incorporation (panel A). The percent maximum response is depicted in panel B. The background proliferation induced by unpulsed APCs was less than 1000 for all the groups. The above experiment is a representative of four independent experiments.
To compare the stability of peptide-MHC complexes between native and maleyl-protein, peritoneal macrophages were pulsed with either CA (100 μg/ml) or maleyl-CA (100 μg/ml) for 1 h. At the end of 1 h, the antigen was washed off and the cells were rested for various time periods following which they were fixed and used to stimulate D10.G4.1 for 24 h. Fig 6A shows that the response generated by maleyl-CA pulsed macrophages is much higher and persists for a longer time than that generated by CA pulsed macrophages. The normalised response in Fig 6B shows that the decay rate of the response generated by maleyl-CA is much lower than that of CA thereby suggesting that peptide-MHC complexes derived from maleyl-CA persist for a longer time than those derived from CA.

2.2.3 T cell priming by different APC lineages

The data so far suggests that the decay rate of the peptide-MHC complexes derived from maleyl-protein is comparable between different APCs but they persist for a longer time on the APC surface as compared to those generated from native protein. To directly address the role of ligand density and its longevity in regulating Th cytokine phenotype, *in vitro* antigen pulsed APCs were used as immunogens. Peritoneal macrophages were pulsed with either CA (1 mg/ml) or various concentrations of maleyl-CA (1 mg/ml and 0.1 mg/ml), thereby generating different ligand densities on the cell surface, for 1 h. The cells were washed extensively and 5 x 10⁵ cells were transferred i.p into syngenic naive recipients. Day 7 post transfer, splenocytes were harvested and restimulated *in vitro* with either CA (Fig 7A) or maleyl-CA (Fig 7B). The response generated by unpulsed macrophages was used as the background proliferation. Both the panels in Fig 7 show that there is no significant *ex vivo* T cell response generated
FIG 6: Peptide-MHC complexes derived from maleyl-CA persist for a longer duration than those derived from native CA

Peritoneal macrophages were pulsed with CA (100 µg/ml, open circles) or maleyl-CA (100 µg/ml, filled circles) for 1 h and rested for various time periods following which they were fixed and used as APCs to stimulate D10.G4.1 for 24 h. At 24 h, response was measured as ³H-thymidine incorporation (panel A). Panel B shows the percent maximum response. The background proliferation in response to unpulsed APCs was less than 1000. The above experiment is a representative of four independent experiments.
Results

by antigen pulsed macrophages, when used as immunogens, as compared to unpulsed macrophages. The data, therefore, suggest that neither native nor maleyl-CA pulsed macrophages can prime T cells in vivo.

Next, dendritic cells were pulsed with various concentrations of OA or maleyl-OA (1, 0.1 and 0.01 mg/ml) for 1 h. The cells were washed extensively and $5 \times 10^5$ dendritic cells were then transferred i.p into naïve syngenic recipients. Day 7 post transfer, the proliferation response of splenocytes against OA (Fig 7C) or maleyl-OA (Fig 7D) as in vitro recall antigens was scored in a restimulation assay. As shown in Fig 7C and 7D, only the maleyl-OA pulsed dendritic cells elicit a T cell response in a dose dependent manner, which is more evident when maleyl-OA is used as a recall antigen (Fig 7D). However, OA pulsed dendritic cells fail to generate an ex vivo proliferation response suggesting that OA pulsed dendritic cells do not efficiently prime T cells in vivo. Thus, maleyl-protein by itself does not induce immunogenic potential on macrophages. Further, increased immunogenicity of maleyl-protein may be due to increased peptide-MHC complex levels.

2.2.4 Regulation of cytokine phenotype by antigen dose

The cytokine commitment of T cells is regulated by the dose of antigen during priming. To analyse this, the culture supernatants from the above restimulation assays were tested for IFN-γ and IL-10. T cells primed by maleyl-OA pulsed dendritic cells produced detectable levels of cytokines. Therefore, relative dominance of Th1 or Th2 cytokine response could be compared between the groups of maleyl-OA pulsed with different concentrations of the antigen (Fig 8). As shown in Fig 8A, the amount of IFN-γ produced decreases as the dose of antigen is decreased to 0.01 mg/ml of maleyl-OA, although there is not a significant
FIG 7: Different APC lineages have different abilities to prime T cells in vivo
Peritoneal macrophages were either unpulsed (cross symbols) or pulsed with CA (1 mg/ml; open circles) or maleyl-CA (1 mg/ml; filled circles) or maleyl-CA (0.1 mg/ml; filled triangles) for 1 h. Bone-marrow derived dendritic cells were either unpulsed (cross symbols) or pulsed with 1 mg/ml (circles), 0.1 mg/ml (squares), 0.01 mg/ml (triangles) of OA (open symbols) or maleyl-OA (filled symbols) for 1 h. The cells were washed extensively and 5 x 10⁵ cells were transferred i.p into syngenic naive recipients. Day 7 post transfer, splenocytes from macrophages primed mice (panels A and B) or dendritic cells primed mice (panels C and D) were restimulated in vitro with various concentrations of CA (panel A), maleyl-CA (panel B), OA (panel C) or maleyl-OA (panel D) for 72 h. The response was measured as ³H-thymidine incorporation at 72 h. The above experiment is a representative of two independent experiments.
difference in the production of IL-10 (Fig 8B) with respect to the priming dose. As seen in
Fig 8C, the IFN-γ/IL-10 ratio decreases as the dose of priming antigen decreases
suggesting that higher dose of antigen favours Th1 response while low dose of priming
antigen favours Th2 response. However, since OA pulsed dendritic cells failed to generate
a T cell response and elicit cytokine production, no comparison could be made between
native and maleyl-OA.

2.2.5 Reprogramming of an established Th1 or Th2 T cell response

It has been already shown in the laboratory that native protein immunisation (non-SR
directed delivery) leads to a Th2 response while maleyl-protein immunisation (SR directed
delivery) results in a Th1 response. Whether the cytokine fate of a T helper cell is fixed or
reversible is still incompletely understood phenomenon. The system of SR or non-SR
mediated delivery of antigen would help to gain information about the flexibility of an
ongoing or established T cell cytokine response since it is possible to generate a Th1 or
Th2 polarised T helper cell population under physiological conditions and follow their fate
in vivo upon re-exposure to the same antigen but delivered by different mode.

2.2.5.1 Modulation of cytokine balance in vivo

Mice were immunised with 300 µg of either CA or maleyl-CA in saline, i.p. Fourteen days
later, primed mice were boosted with 300 µg of either CA or maleyl-CA in saline, i.p.
Seven days after secondary immunisation, splenocytes were harvested and in vitro
restimulation was done. Cytokine analyses by ELISA were done on the culture
supernatants collected at 72 h. Fig 9 shows the amounts of IFN-γ and IL-10 produced
FIG 8: Effect of antigen dose on cytokine profile
Bone-marrow derived dendritic cells were pulsed with different concentrations of maleyl-OA (1, 0.1, 0.01 mg/ml) and injected i.p into naive syngenic recipients. Day 7 post transfer, splenocytes were harvested and restimulated in vitro with maleyl-OA for 72 h. At 72 h, the culture supernatants were collected and tested for the cytokines IFN-γ and IL-10 by ELISA. Panel A shows IFN-γ, panel B shows IL-10 and panel C shows the ratio of IFN-γ to IL-10 for each group. The above experiment is a representative of two independent experiments.
**Results**

upon *in vitro* restimulation. As shown in Fig 9A, there is a significant reduction in the IFN-γ produced by T cells from maleyl-CA primed mice receiving CA during secondary immunisation as compared to the mice receiving maleyl-CA during primary as well as secondary immunisation. On the other hand, there is marked reduction in the level of IL-10 produced by T cells from CA primed mice receiving maleyl-CA as the secondary immunogen as compared to the mice receiving CA as primary and secondary immunogen. There is a concomitant increase in the level of IL-10 produced by T cells from maleyl-CA primed mice receiving CA during secondary immunisation in comparison to the mice receiving maleyl-CA during primary and secondary immunisation. The shift of the cytokine profile becomes more evident in Fig 9C which depicts the ratio of IFN-γ to IL-10. It suggests that there is a switching of the cytokine profile from Th1 to Th2 or vice versa depending on the nature of the secondary immunogen.

IL-5 is another hallmark cytokine of Th2 response. In a similar experiment described in the above paragraph, the amount of IFN-γ and IL-5 was quantitated. There is marked increase in the IFN-γ production when secondary immunisation is done by maleyl-CA while primary immunogen is CA as compared to the situation when both primary and secondary immunisation is done by CA (Fig 10A). Conversely, IFN-γ decreases in case of secondary immunisation with CA even though primary immunisation is by maleyl-CA. Although, there is no significant difference with respect to the IL-5 production amongst various groups and maleyl-CA followed by maleyl-CA shows maximum IL-5 levels (Fig 10B), the relative dominance of Th1 or Th2 phenotype becomes self-evident when the ratio of IFN-γ to IL-5 (Fig 10C) is calculated. Maleyl-CA as a secondary immunogen increases the IFN-γ to IL-5 ratio with CA as the primary immunogen and CA reduces the IFN-γ to IL-5 when
FIG 9: Modulation of IFN-γ to IL-10 ratio in vivo
Mice were immunised with either CA or maleyl-CA (mCA; 300 μg per mouse), i.p. On day 14, CA-immune mice were boosted with either CA or maleyl-CA (300 μg per mouse), i.p. Similarly, maleyl-CA immune mice were boosted with either CA or maleyl-CA, i.p. On day 21, splenocytes were restimulated in vitro with titrating concentrations of CA for 72 h. At 72 h, culture supernatants were collected for estimation of IFN-γ (panel A) and IL-10 (panel B) by ELISA. Panel C shows the ratio of IFN-γ to IL-10. The symbols are CA/CA (open circles), CA/mCA (filled circles), mCA/CA (open triangles) and mCA/mCA (filled triangles). The above experiment is a representative of six independent experiments.
FIG 10: Modulation of IFN-γ to IL-5 ratio in vivo

Mice were immunised with either CA or maleyl-CA (mCA; 300 µg per mouse), i.p. On day 14, CA-immune mice were boosted with either CA or maleyl-CA, i.p. Similarly, maleyl-CA immune mice were boosted with either CA or maleyl-CA, i.p. On day 21, splenocytes were harvested and restimulated in vitro with titrating concentrations of CA for 72 h. At 72 h, culture supernatants were collected for estimation of IFN-γ (panel A) and IL-5 (panel B) by ELISA. Panel C shows the ratio of IFN-γ to IL-5. The symbols are: CA/CA (open circles), CA/mCA (filled circles), mCA/CA (open triangles) and mCA/mCA (filled triangles). The above experiment is a representative of six independent experiments.
Results

used as a secondary immunogen with maleyl-CA as primary immunogen. Thus, Fig 9 and Fig 10 collectively suggest that it is possible to modify an ongoing Th1 or Th2 response in vivo.

Same experimental approach was extended to a situation wherein an immunodominant peptide (Der p 1 p111-139) derived from house dust mite Dermatophagoides pteronyssinus (HDM) type I protein was used. The house dust mite is a major source of aeroallergens causing respiratory symptoms in susceptible individuals. The protein by itself elicits an allergic Th2 response with high IgE, IgG1 antibodies and low IFN-γ. For immunisation, p111-139 was co-adsorbed with either poly-L-lysine (PLL) or maleyl-poly-L-lysine (maleyl-PLL) on alum. C57BL/6 mice were immunised with p111-139-PLL (300 µg p111-139 per mouse) or p111-139-maleyl-PLL. Fourteen days later, secondary immunisation was done with either p111-139-PLL or p111-139-maleyl-PLL in each group. Seven days later, splenocytes were harvested and restimulated in vitro with titrating concentrations of p111-139 for 72 h. The culture supernatants were collected and tested for IFN-γ (Fig 11A), IL-10 (Fig 11C) and IL-5 (Fig 11E). IFN-γ increases when p111-139-mPLL is used during secondary immunisation of p111-130-PLL primed mice in comparison to when p111-130-PLL is used during both primary and secondary immunisation while reverse happens when p111-130-maleyl-PLL is used for secondary immunisation (Fig 11A). Conversely, there is a decrease in IL-10 (Fig 11C) or IL-5 (Fig 11E) when the secondary immunisation is done using SR directed delivery of antigen. Fig 11B and Fig 11D show the ratio of IFN-γ/IL-10 and IFN-γ/IL-5 respectively, for all the groups. There is a significant increase in the IFN-γ/IL-10 and IFN-γ/IL-5 ratio when the peptide is delivered to SR using maleyl-PLL as a carrier to mice that were primed against
FIG 11: Modulation of Th1/Th2 cytokine balance in vivo against an allergic peptide, Der p 1 (p111-139)

Mice were immunised with either p111-139-PLL or p111-139-mPLL (100 μg/ms). Fourteen days later secondary immunisation was done with either p111-139-PLL or p111-139-mPLL in each group. On day 21, splenocytes were harvested and restimulated in vitro with titrating concentrations of p111-139. At 72 h, culture supernatants were collected and assayed for IFN-γ (panel A), IL-10 (panel C) and IL-5 (panel E). Panel B shows ratio of IFN-γ to IL-10 and panel D shows ratio of IFN-γ to IL-5. The symbols are: PLL/PLL (open circles), PLL/mPLL (filled circles), mPLL/PLL (open triangles) and mPLL/mPLL (filled triangles). The above experiment is a representative of six independent experiments.
peptide co-adsorbed with PLL. Conversely, the ratio decreases when the peptide is given with PLL as a carrier to the mice primed against peptide using maleyl-PLL as the carrier. Thus, by delivering the peptide co-adsorbed with a non-immunogenic SR ligand during secondary immunisation is sufficient to shift the cytokine balance from relatively Th2 type to Th1 type and vice versa.

2.2.5.2 Abrogation of allergic manifestations in vivo

Since the above data show that it is possible to shift an established or ongoing Th2 response to Th1 cytokine response, the next question was whether it can lead to any abrogation of allergy in vivo. Eosinophils are a prominent cell type in the allergic inflammatory infiltrate and are major mediators of the symptoms of allergy. Therefore, a model of allergic peritonitis was used (Das, A. M. et al. 1999.), in which allergic challenge causes infiltration with eosinophils and neutrophils. C57BL/6 mice were immunised with p111-139-PLL and one week later they were boosted with either p111-139-PLL or p111-139-maleyl-PLL on alum. As a control for non-allergic response mice were immunised as well as boosted with p111-139-maleyl-PLL. Seven days post secondary immunisation the mice were challenged with p111-139 (10 μg per mouse, i.p) and peritoneal cellular infiltrate was collected 6 h later. The numbers of eosinophils and neutrophils extravasating the peritoneum were counted using Leishman’s stain (described in materials and methods). Fig 12A shows that the numbers of eosinophils and neutrophils extravasating in mice receiving p111-139-maleyl-PLL was much lower than in mice receiving p111-139-PLL wherein priming was done against p111-139-PLL. The mice that were primed and boosted
with p111-139-maleyl-PLL showed minimum numbers of eosinophils and neutrophils recruited into the peritoneum.

In a similar experimental system, skin reactivity test was performed to further investigate the role of SR directed delivery of the immunogen in modulating clinical symptoms in vivo. The immunisation procedure has been described above. However, at the time of challenge, the peptide was delivered i.d mixed with Trypan blue as a marker and the diameter of the skin lesion was measured at various time periods. Fig 12B shows that the diameter of the skin lesion was significantly reduced when the p111-139-PLL primed mice were boosted with p111-139-maleyl-PLL as compared to the mice which were primed and boosted with p111-139-PLL. Mice receiving p111-139-maleyl-PLL as the primary and secondary immunogen showed very small skin lesions. Thus, the above data suggest that it is possible to abrogate pre-established allergic manifestations.
FIG 12: Abrogation of allergic manifestations in vivo

Mice were primed with p111-139-PLL on alum. Seven days later mice were boosted with either p111-139-PLL (panel B, open circles) or p111-139-maleyl-PLL (panel B, filled circles) on alum and a week later were challenged with p111-139 in saline i.p (panel A) or in saline containing Trypan Blue i.d. (panel B). For panel A, peritoneal cells were harvested six hours post challenge and numbers of total cells (black bars), agranulocytes (hatched bars), eosinophils (dotted bars) and neutrophils (plain bars) were counted. For panel B, the diameter of the skin lesion was measured at various time periods post challenge. For both the panels, mice primed and boosted with p111-139-maleyl-PLL (panel B, filled triangles) were used as controls representing a non-allergic phenotype. Three mice were included in each group. The above experiment is a representative of three independent experiments.
Discussion
2.3 Discussion

During a primary immune response, CD4 T cells get committed to either Th1 or Th2 cytokine phenotype and the imprinted profile is recalled upon re-exposure to the antigen against which they are primed. This commitment is subject to stringent regulation by numerous factors, although the phenomenon is still not completely understood. To study the mechanism of cytokine regulation a previously established system was used. It has been earlier shown that immunisation of mice with maleylated protein results in the generation of relatively Th1 cytokine response whereas the T cells from mice that are immunised with native proteins show a Th2 cytokine profile. Maleylated proteins are the ligands for scavenger receptors (SRs) that are present on the macrophages, B cells and dendritic cells, the cells that constitute the class of professional APCs. Thus, the targeted delivery of maleyl antigen shifts the cytokine balance towards Th1 type. What is the basis for this differential commitment of T cells?

Since maleyl proteins bind to SRs and are taken up by APCs via receptor mediated endocytosis, the effect of ligation of scavenger receptors by maleyl proteins on costimulation on APCs was studied. Cytokines such as IL-12 have been shown to play a major role in programming of helper T cells to a Th1 response (Magram, J. et al. 1996.; Trinicheri, G. 1997.) while IL-10 has been shown to favour a Th2 response (Hsieh, C. S. et al. 1992.). Costimulatory molecules such as CD80/CD86 (Kuchroo, V. K. et al. 1995.), CD28 (Lenschow, D. J. et al. 1996.), ICOS (Tafuri, A. et al. 2001.; Dong, C. et al. 2001.) and CD40 (Kamanaka, M. et al. 1996.; Campbell, K. A. et al. 1996.; Soong, L. et al. 1996.) have been implicated in differentially regulating the cytokine commitment of T cells. However, binding of maleyl-BSA to SRs on macrophages does not induce the production of Th polarising cytokines, IL-12, IL-10, TNF-α and IL-1β (Fig 1) and neither does it alter the expression of various costimulatory (CD80, CD86, CD40 and CD24)/adhesive molecules (CD11a/CD18, CD54) (Fig 2 and 3). The data therefore argues against the role of cytokines, such as IL-12, IL-10, TNF-α and IL-1β during priming of T cells in the differential
commitment of T cells in this system. There is also no significant effect on the expression of CD80, CD86 and CD40 upon binding of maleyl-BSA to SRs on macrophages suggesting that the preferential generation of a Th1 response by maleyl-proteins is not a result of differential expression of these costimulatory molecules.

The uptake of maleyl-proteins by SRs via receptor mediated endocytosis would result in degradation of the protein in endolysosomal compartments thereby allowing generation of peptides that can be loaded onto MHC class II molecules and presented on the surface of APCs. APCs from different lineages such as B cells have been reported to prime Th2 T cell clones (Gajewski, T. F. et al. 1991) while macrophages and dendritic cells are required for priming of Th1 T cell clones (Hsieh, C. S. et al 1993.; Macatonia, S. E. et al. 1993). In the system used here, APCs of different lineages differ in their ability to generate peptide-MHC complexes derived from maleyl-CA (Fig 4). During the initial time periods macrophages process and present maleyl-CA faster than dendritic cells which become as efficient as macrophages by 60 min. B cells, on the other hand, process and present maleyl-CA very slowly. Interestingly, the persistence of these peptide-MHC complexes on the surface of these APCs is equivalent between different APCs (Fig 5). This data together suggest that the stability of the peptide-MHC complexes on different APC lineages is comparable although they are generated with different efficiencies. Thus, it is unlikely that differential longevity of peptide-MHC class II complexes on an APC type contributes to this dichotomous commitment of T cells.

The role of ligand density has been critically studied in regulating cytokine phenotypes. In fact, when the stability of peptide-MHC complexes derived from native protein is compared to those derived from maleyl-CA (Fig 6), the peptide-MHC complexes are generated at a higher level from maleyl-CA and persist for a longer time. Although the relative ligand levels have been read out using a T cell clone, D10.G4.1, which is an indirect measurement of peptide-MHC complex levels, the method has been used in other systems (Cella, M. et al. 1997.; Guery, J-C. et al. 1996.). The data therefore suggest that it is the ligand density and/or
duration of the availability of peptide-MHC complexes that determine the cytokine commitment of T cells in this system. It is consistent with the findings using TCR transgene bearing T cells (Kupeic-Weglinski, J. W. et al. 1988.; Constant, S. et al. 1995.) wherein \textit{in vitro} priming of T cells with high doses of peptide results in their differentiation to Th1 cells while cells primed with low doses of antigen get committed to Th2 cytokine phenotype.

The next attempt was to use an approach which would directly address the role of ligand density in regulation of cytokine phenotype. \textit{In vitro} antigen pulsed macrophages were used as immunogens. However, they failed to prime T cells \textit{in vivo} (Fig 7A and B) but when \textit{in vitro} antigen pulsed dendritic cells were used to prime mice, they were able to generate an \textit{ex vivo} T cell response (Fig 7C and D). This proliferative response was significant with maleyl-OA pulsed dendritic cells but was very low with OA pulsed dendritic cells. Dendritic cells pulsed with various concentrations of maleyl-OA showed differences in the cytokine profile of primed T cells. The relative dominance of IFN-\(\gamma\) increased with an increase in the dose of maleyl-antigen (Fig 8). No cytokines were detected when OA pulsed dendritic cells were used as immunogens, hence, it failed to allow any comparison to be made between native and its maleyl counterpart.

Recently, various transcription factors have been identified that are associated with either Th1 (T-bet) (Szabo, S. J. et al. 2000.) or Th2 (c-Maf, GATA-3) (Hodge, M. R. et al. 1996.; Ho, I-C et al. 1996.; Kim, J. et al. 1999.; Zheng, W-P. and R. A. Flavell. 1997.; Ouyang, W. et al. 1998.; Zhang, D. H. et al. 1997.) cytokine profile and chromatin remodelling of various cytokine alleles have been found to be associated with a particular Th phenotype. It would be therefore, now interesting to analyse the relative expression of these factors in the T cells primed against either native or maleyl protein, by the use of a TCR transgenic mouse system wherein it is possible to track antigen specific T cells by the use of anti-clonotypic antibodies.

Given the dichotomy in cytokine phenotype with native versus maleyl protein, it became interesting to know whether this cytokine response is fixed or can it be reversed? Is it
possible to modulate an ongoing or established cytokine response of a T helper cell upon re-exposure to the same antigen under different microenvironment conditions?

Initial work was done using model protein antigens as immunogens. T cells from mice primed with CA when boosted with maleyl-CA shifted their cytokine phenotype towards the Th1 phenotype as seen by an increase in IFN-γ to IL-10 ratio (Fig 9) or IFN-γ to IL-5 ratio (Fig 10). An opposite pattern was seen wherein T cells from mice initially primed with maleyl-CA altered their cytokine profile from IFN-γ dominant Th1 to Th2 when they received CA during secondary immunisation (Fig 9 and 10). Thus, SR directed delivery of the antigen during secondary immunisation could reprogram an established Th2 dominated response towards Th1 end of the cytokine spectrum.

Following this, it was necessary to ask if the immunomodulatory Th1 skewing resulting from SR directed delivery could be seen for antigens that have a prominent Th2 immunogenicity, such as allergens. A dominant epitope of the Der p 1 allergen of HDM was used for this purpose (Higgins, J. A. et al. 1994.). Secondary immunisation with Der p 1 co-adsorbed with maleyl-PLL on alum was able to skew the Th1/Th2 balance towards IFN-γ (Fig 11). Eosinophil infiltration at the site of disease is associated with pathogenesis in allergic inflammation. SR directed delivery of Der p 1 also inhibits the recruitment of eosinophil in vivo (Fig 12A). Skin reactivity test also shows a reduced lesion upon SR directed delivery of the allergen peptide (Fig 12B). Thus it is possible to reprogram the pattern of cytokine production by T cells. This is in fact consistent with a recent report wherein major shrimp allergen, tropomyosin has been used (Rajagopal, D. et al. 2000.) and SR mediated delivery of the allergen skews the otherwise Th2 response to a Th1 type.

It has been reported that the avidity of peptides for a given MHC class II molecule influences the Th1/Th2 outcome of the T cell, with higher ligand density resulting in a preferential Th1 commitment of T cells. SR mediated delivery of antigens to APCs results in the generation of higher density of peptide-MHC class II complexes on the surface of an APC, both in vitro (Fig 6) and ex vivo (Singh, N. et al. 1998.), which persist for a longer
duration of time. It is, therefore, possible that the increase in the ligand density generated by antigen delivery to SRs promotes a Th1 dominant response even in experienced T cell populations, which may account for the reprogramming of ongoing Th2 response, although how a higher ligand density on an APC is translated to a Th1 response in molecular terms is still unclear.

Various models have been put forward to explain the regulation of Th differentiation upon secondary stimulation (Coffman, R. L. and S. L. Reiner. 1999.). Instructive model of cytokine commitment suggests that the cells are fated for a cytokine phenotype through a program of molecular switches and occurs in response to the cytokines present in the microenvironment during priming. It can therefore change depending on the availability of the cytokine type. On the other hand, selective model suggests that the commitment of T cells is stochastic but fixed and cytokines subsequently act by favouring the selective outgrowth of a particular lineage. In a hybrid instructive-selective model, the cytokines are required to decide the fate as well the maintenance of the Th polarisation. In the system used here, it is possible that T cells primed against native protein get committed to Th2 phenotype as a consequence of low ligand density but the Th2 type T cells are selected by the dominant Th2 cytokines. Nonetheless, it might still be a mixed population of dominant Th2 T cells, cells with a Th0 phenotype and a small fraction of surviving cells with Th1 phenotype. When this population of T cells is restimulated with maleyl-protein, the small fraction of Th1 T cells are preferentially selected by Th1 cytokines (as would be predicted by selective model) while at the same time the Th2 cells are being reprogrammed to Th1 type by cytokine dependent signaling (as would be predicted by instructive model). Thus, it may actually follow a hybrid instructive-selective model of Th regulation.

Taken together, the data shows that maleylated proteins generate a relatively greater dominance of Th1 response and that rather than major modulation of costimulatory functions, it is the level/duration of ligand availability that plays a major role in this differential commitment.