DISCUSSION

The aim of this study was to address the issue of antigen presentation and accessory signaling abilities of human T cells, in their role of APCs for *ex vivo* CD4 T cells. Antigen presentation to CD4 T cells involves specialized cells, which apart from expressing MHC class II molecules constitutively, also have accessory signaling capability. Since human T cells express MHC class II antigens upon activation, they are capable of providing the 'first' signal, their accessory signaling capability, however, remains controversial. The question, therefore, is whether a T cell expressing MHC class II molecules can successfully present antigen to CD4 T cells, leading to activation. There are some reports of such T-T interaction leading to proliferation, while others show induction of anergy [19]. However, these reports will have to be viewed taking into account two caveats. Firstly, in most of these studies T cell clones have been used as responders. T cell clones are 'memory' T cell populations and 'memory' T cells have been reported to have enhanced expression of cell surface molecules involved in co-stimulation [255], and hence their requirements of co-stimulatory signals are believed to be less stringent than naive T cells [20,255]. Therefore, using such 'memory' cells as responders may give a biased assessment of the co-stimulatory potential of T cell APCs. Also in most of these studies on T cell antigen presentation, T cell clones have been used as stimulators. It follows, that the co-stimulatory capabilities or lack of it would be very different from that of freshly activated *ex vivo* T cells. Consequently, these studies may not present a complete picture of human T cells, as APCs, for CD4 T cells. In our study, we have

tried to circumvent these limitations by adopting the allorecognition system, which enabled us to use predominantly 'naive' *ex vivo* T cells as responders. We have also used freshly activated *ex vivo* T cells as stimulators in our assays. The results of the study are discussed below.

1. Analysis of antigen presentation by ex *vivo* human T cells expressing MHC class II antigens :

The first question was, whether the allo-ligand presented on activated T cell APCs is able to elicit an alloproliferative response from responder CD4 T cells. When we used activated T cells as APCs, the responder CD4 T cells failed to show a proliferative response to the allo-ligands on activated T cell APCs, while the same responders showed an excellent proliferative response to the same allo-ligands on a professional APCs, like monocytes from PBMCs, in a standard *in vitro* T cell proliferation assay [Fig. 12]. This, suggested that activated T cell APCs are unable to generate a primary proliferative alloresponse from *ex vivo* T cell responders.

The absence of alloresponse with activated T cells as APCs could be due, in part, to the low density of allo-ligands on the activated T cells [Fig. 5]. The other [and not mutually exclusive] possibility, of course, is a lack of accessory signaling by the activated T cell APCs. It is not yet clear what constitutes the mandatory accessory signals required for T cell activation and proliferation. There are reports of accessory signals being provided by 'by-stander'

in sharp contrast to our findings with activated T cells used as APCs. This may be because the tumor cells may have accessory signaling abilities, which are different, as compared to the non-tumor T cells used as APCs.

MHC class II is known to contribute more significantly in an alloresponse than MHC class I [262,263] and hence we tried to enhance the potential of H9 to elicit an alloresponse, by upregulating MHC class II levels using PHA/PMA treatment, as shown in Fig. 8. Surprisingly, there was no significant enhancement in the alloresponse to PHA/PMA treated H9 over the responses against H9 [Fig 10], indicating that there was no correlation between the response elicited and the level of MHC class II allo-ligand expressed in this case. One possible reason for this apparent discrepancy could be that the upregulated MHC class II allo-ligand expressed by PHA/PMA treated H9 may be in some way defective in its ability to elicit an alloresponse. Another possibility was that the alloresponse seen in Fig. 9, was due to the presence of responder syngeneic 'by-stander' cells in the responder PBMCs population, similar to what was seen with the activated T cell APCs [Fig. 13]. It is also possible that the co-stimulating ability of H9 may be different from that observed with PBMCs and that, in itself, may be the crucial factor involved in the T cell-APC interaction. Repertoire of peptides loaded on to MHC class II molecules in normal and PHA/PMA treated H9 may be different resulting in the modification of conformation of MHC class II molecules expressed on H9. Association of MHC class

cells [249,264]. In this context, when we used responder-syngeneic accessory cells as 'by-stander' cells, activated T cell APCs elicited a small, but significant proliferative response from the responder T cells [Fig. 13]. This proliferation was possibly because the activated T cell APCs, used as stimulators, were secreting some growth factors, such as IL-2, in alloresponse to the responder syngeneic accessory cells added. Our experiments addressing this issue, showed no proliferative response, when stimulator-syngeneic accessory cells were added as 'by-stander' cells [Fig. 13]. 'By-stander' help is by definition haplotype independent and, therefore, what we were observing with responder-syngeneic accessory cells cannot be called 'by-stander' help, and is an artifact of the allorecognition system. However, a simple assay to detect the presence of IL-2 [Fig. 14], did not give the answer. It is possible that either very little IL-2 was produced by the activated T cell stimulators, in response to responder-syngeneic accessory cells, or some growth factor other that IL-2 was involved.

We also used a human tumor T cell line, H9, to ask the question whether it can present antigens to responder T cells. Confirming earlier reports [216], we have shown that H9 constitutively expressed low levels of MHC class II [Fig. 6 and 7]. Upon activation the expression of MHC class II on H9 was significantly enhanced [Fig 8]. When used as allostimulators, H9 elicited a weak proliferative alloresponse from the responder T cells [Fig 9]. The ability of H9 cells to elicit a primary alloresponse was

II with Ii during their synthesis is known to alter the final conformation of MHC class II [128,129].

Ii modulates the kind of peptides that bind to MHC class II and thus it has a role to play in allorecognition [49,69,126]. Our attempts to analyze the level of Ii mRNA expression in normal H9 and PHA/PMA treated H9 cells [Fig. 11], showed that the level of Ii expressed in H9 is significantly lower than that expressed in the control B cell line; and that there was no concomitant upregulation in the expression of Ii in the PHA/PMA treated H9 cells, along with the upregulation of MHC class II. This suggests that a bulk of the MHC class II expressed in PHA/PMA treated H9 might not have been associated with Ii, and hence might have a different conformation. The lack of enhancement in the allostimulating potential of PHA/PMA treated H9 may de due to one or more of these possibilities.

2. T-T interaction results in the induction of tolerance in *ex vivo* responder T cells :

The fact that CD4 T cells did not respond to allo-ligands presented by activated T cell APCs, suggested that, either, the MHC class II allo-ligands on T cell were being ignored by the responder T cells, or that the activated T cell APCs were inducing tolerance for the allo-ligand in the responder T cells. The experiments shown in Figs 16-18 demonstrated that the T cell responders exposed to MHC class II allo-ligands on activated T cell APCs were clearly rendered

tolerant, as they showed significantly lowered response [as compared to the control T cells, which have not seen the allo-ligand] to the same allo-ligands presented by professional APCs, in the restimulation assay [Fig 16-18]. This non-responsiveness or tolerance was not due to a non-specific phenomenon, such as cell death, because responder T cells that showed tolerance in the restimulation assay, were capable of responding to a 'third party' allo-ligand and to polyclonal stimulating agents such as PHA [Figs. 16 and 17].

It is possible that the tolerance seen is the result of some suppresser factor secreted by the activated T cell APCs and does not involve the interaction of the TCR-MHC class II allo-ligand. A few lymphokines have been shown to be anti-proliferative, namely IFN-γ [265], and such secreted factors may contribute to the decrease in proliferative response, used as a read-out assay for tolerance induction. Rather than looking for the presence of such cytokines, we addressed the issue by priming with activated T cell APCs of the responder-phenotype, as an additional control. If activated T cells APCs were secreting such factors, because of their activated status, the inhibition of proliferation would manifest in this scenario as well. As shown in Fig. 18, presence of allo-ligand on T cell APCs appeared to be necessary, rather than their activated status *per se,* to induce tolerance.

It has been reported that 'primed' T cells can be activated more

easily than 'naive' T cells [20,257]. This might be because 'primed' or memory T cells are less stringent in their requirements for co-stimulation, as compared to, 'naive' T cells. Since, T cell APCs were found to be incapable of eliciting primary alloresponse from CD4 T cells, we explored the possibility of T cell APCs being capable of eliciting, at least, a recall response from PBMC-primed responders. Our results in Fig. 15 show that T cell APCs could not elicit an alloresponse even from 'primed' responders.

An interesting issue here is the crossreactivity between the allo-ligands expressed by professional APCs and by activated T cell APCs, in light of the possibility that allorecognition can be peptide-specific [10], and that allo-ligands may be tissue- and cell-type-specific [11,114]. Our results indicate that the allo-ligands expressed by activated T cell APCs could prime, practically, all the T cells capable of responding to allo-ligands on professional APCs for tolerance, since the read-out assay we have used for tolerance is the ability to respond to professional APCs. Given the fact, that the haplotype of MHC class II expressed on the activated T cells and professional APCs used in these restimulation experiments is the same [since they are obtained from the same individual, donor 2], there appears to be a substantial cross-reactivity between the self-peptides that contribute to form the allo-ligands. Recent data on the origins of self-peptides contributing to allo-ligands show that many of these come from MHC proteins themselves [40,109,124]. There is evidence for serum albumin to be another source of such peptides

[125]. Most of these proteins are abundantly available to most cells and may therefore be presented by both professional APCs and activated T cell APCs as part of their allo-ligands.

3. Characterization of the mechanism of tolerance induction :

Activated T cell APCs, used in these experiments, express CD4 or CD8 co-receptor molecules on their surfaces along with MHC class II. There is evidence to suggest that the ligation of surface MHC class I molecules in the non-polymorphic region, may induce non-responsiveness in the responder T cells [254]. APCs expressing CD8 molecules may be inducing non-responsiveness by this mechanism [255]. There is also the possibility of CD8 activated T cell APCs killing the responder T cells specific for the allo-ligand.

Our experiments using, both CD4 T cells and CD8 T cells as APCs induced ligand-specific tolerance [Fig 19] suggesting that the ligand-specific tolerance seen in our experimental system is not dependent on the co-receptor expression on the T cell APCs. CD8 expressing activated T cell APC induced a hyper-response to IL-2, in responder T cells, as compared to, the unprimed T cells, indicating the presence of high affinity IL-2R expressing 'anergized' cells. This suggested that responder T cells tolerised by CD8 expressing T cells were not deleted or killed, but were present in the system. Therefore the possibility of CD8 activated T cell APCs killing responder T cell appears unlikely.

T cell tolerance can be mechanistically explained, either by deletion of the concerned T cells, or by the induction of non-responsiveness, i.e., induction of anergy. Our experiments using CD8 expressing T cell APCs indicated that the tolerized T cells were not killed during the induction of tolerance. Physical identification of non-responsive T cells is experimentally impractical in a heterogeneous *ex vivo* bulk population, such as the one we are dealing with. During the induction of anergy, T cell clones have been reported to undergo some, but not all the changes seen during complete activation [202]. Such anergic T cells are, however, unable to produce enough IL-2 to drive clonal expansion [198,203]. However, induction of expression of the high-affinity IL-2R is seen during induction of anergy and these anergized T cells, in fact, respond better to exogenous IL-2 [204] than non-anergic cells [202]. We, therefore, reasoned that if the tolerance we see during T-T interaction is a consequence of a similar mechanism leading to anergy, an increased response of the anergized responder cell population to IL-2 should be detectable, even over the background responses of 'by-stander' T cells. Our data, in Fig. 19 and 20, showed the enhanced response of the tolerized responders to IL-2, supporting the notion of induction of 'anergy' rather that 'deletion', as the form of tolerance.

As reviewed earlier, the induction of anergy in T cell clones is an active process involving protein synthesis [205,206] and the level

of IL-2R mRNA is increased following tolerization [269]. Anergic T cells, thus, appear to be unable to produce IL-2 for their proliferation, while they show enhanced responses to IL-2 as compared to normal T cells due to higher levels of IL-2R. Our data strongly suggest that the tolerised population, that we obtain after a T-T interaction, has similarly anergic T cells, which would account for the enhanced response to IL-2.

In studies with human T cell clones it has been shown that addition of IL-2 can prevent induction of anergy *in vitro* [204]. A similar phenomenon has been demonstrated *in vivo* [206]. We attempted to mimic the situation by providing exogenous IL-2 during the tolerising T-T interaction. Fig. 21 shows that presence of exogenous IL-2 in the T-T priming interaction prevented the induction of anergy and subsequent hyper-responsiveness to IL-2. These data demonstrate that incorporation of exogenous IL-2 during priming with activated T cell APCs prevents the induction of tolerance and the appearance of hyper-responsiveness to IL-2. The anergised T cells with enhanced IL-2R levels may be unable to synthesize IL-2, and provision of external IL-2 may enable them to undergo complete activation. The availability of IL-2 therefore appears to be one of the critical point during the induction of anergy in this system.

What could be the basis for limiting amounts of IL-2 being available? In a T-T interaction, the stimulators are activated T cells

and would therefore have enhanced expression of high-affinity IL-2R on their surface, as compared to, responder T cells, most of which would express quantitatively lesser numbers of IL-2R, and those predominantly of the low-affinity type [268]. Thus, it can be envisaged that the activated T cell APCs could compete for the IL-2 produced by responder T cells in response to the allo-ligand on the APCs. Since these activated T cells are irradiated, they would not proliferate in response to the IL-2, but they would be capable of adsorbing it and thus possibly depriving the responder T cells of IL-2. It has been demonstrated that effectively depriving T cells of IL-2 during activation leads to the induction of anergy [269]. In order to examine if such competition for IL-2 between responder T cells and irradiated activated T cells would cause anergy, we have added activated T cells of the responder phenotype, as 'by-standers', for adsorbing IL-2 during priming with allo-PBMCs, and see if 'by-stander' high-affinity IL-2R expressing cells could convert this normal priming interaction into a tolerising interaction. If this were to happen, it would suggest that the competition for IL-2 between responder T cells and irradiated activated T cells could be the reason for this tolerance. Our experiments described in Fig. 22, showed that the presence of these 'by-stander' activated T cells did not prevent effective priming, suggesting that simple competition for IL-2 is unlikely to be the basis for induction of anergy.

4. Analysis of the co-stimulatory function of activated T cell APCs :

It is still not clear what constitutes the mandatory accessory signals required for T cell activation and proliferation. Recent reports implicate the CD28/CTLA4-B7/BB1 interaction as one of the major accessory signals for T cell activation [187,191]. Ligation of CD28 on responder T cells by the B7 family of molecules on APCs is a major co-stimulatory pathway that has been shown to augment lymphokine secretion by inhibiting the degradation of various lymphokine mRNAs [182]. Allogenic responses to BCL can be blocked with antibodies to B7 [190]. Further, both blocking of CD28-B7 interaction [189,191] and APCs that do not express B7 have been shown to induce anergy in PBMCs [189].

Naive T cells do not express members of the B7 family of ligands, but B7.1 and B7.2 has been shown to be expressed on T cell clones and long-term activated *ex vivo* T cells [170,171]. T cells activated for 7-10 days *in vitro* have been shown to express B7.1 [173], which happens to be the earliest time point for the expression of B7 reported. In order to address the question of the role played by CD28-B7 interaction in our experimental system, it was necessary, either to use a T cell line, which would be expected to express B7 molecule, based on the reports in the literature, and/or to demonstrate the presence or otherwise of B7 on the T cell APCs, that we have been using routinely for our experiments.

In the experiment we did, the 15 day old T cell line failed to elicit an alloresponse from responder T cells [Fig 23], suggesting that, either our protocol of activation failed to express members of B7 family, or though members of B7 family of ligands were expressed, they were not relevant in a T-T interaction of the kind that we are studying.

We had used PHA-stimulation to generate the 15 day old T cell line, used in these experiments. The activation protocol which we followed for the expression of MHC class II on T cell APCs [Table 1] also included PHA as one of the T cell activators. As shown in Fig. 24, 48-h activated T cells do express CTLA-4-binding ligands, suggesting that during T-T interaction CD28 and B7 are available for their potential functions, as accessory molecules. Thus, the induction of anergy following T-T interaction may not be due to the absence of surface expression of B7 on the T cell APCs.

Thus, despite the expression of ligands for CTLA4, activated T cell APCs function as tolerogens. This may be because the ligand/s for CTLA4 expressed on T cells are in some way functionally inadequate [either because their levels are too low to be effective or they are in some way defective for signaling through CD28/CTLA4], or because the CTLA4 molecules that are also expressed on activated T cells sequester them, making them functionally unavailable to the responder T cells. It is also possible that CD28-B7-mediated co-stimulation may not be directly relevant to T-T interactions. We,

therefore, next addressed the effect of CD28 ligation on the induction of anergy by activated T cell APCs.

Antibody against human CD28 has been demonstrated to replace fixation-sensitive co-stimulatory function of APCs [187]. Similar findings have been reported in the murine system, where it has been shown that anti-CD28 can provide a co-stimulatory signal to prevent the induction of anergy in T cell clones [188]. We, therefore, reasoned that adopting such an approach using anti-CD28 antibody we could investigate the relevance of the role of CD28-B7-mediated co-stimulation in a T-T interaction. If anti-CD28 antibody prevented the induction of anergy, it would mean that the CTLA4 ligand/s expressed on the activated T cell APCs are in some way functionally inadequate. On the other hand, if such an antibody mediated co-stimulation did not affect the induction of tolerance, CD28-B7-mediated co-stimulation may not be relevant in a T-T interaction.

Incorporation of antibody to CD28 added into the culture, with whole PBMCs used as responders, did not bring about a primary proliferative response, when activated T cell APCs were used as stimulators [Fig. 27]. PBMCs as APCs, however, elicited a good proliferative response [Fig. 27]. The presence of FcR-bearing cells in the responder PBMCs should be sufficient to provide cross-linking of CD28 on responder T cells in the presence of anti-CD28 antibody, but this did not appear to be sufficient to drive a primary proliferative alloresponse. The next obvious question was if addition of anti-

CD28 antibody could prevent the induction of anergy. In the experiments that we did, presence of anti-CD28 antibody during priming made no difference to the induction of ligand-specific tolerance by activated T cell APCs [Fig. 28].

It therefore appears that CD28-B7 interaction may not be relevant in the context of the T-T interaction, resulting in the induction of anergy. In this connection, it is interesting to note that T cells from CD28-deficient mice show low but significant antigen-specific proliferative responses in the presence of professional APCs, suggesting either that non-CD28-related co-stimulatory pathways exist, or that CD28-B7-mediated co-stimulation is not absolutely essential for T cell priming [270]. It is possible that the activated T cell APCs used here may be deficient in some other molecule essential for complete co-stimulation and therefore CD28-mediated co-stimulation may not be sufficient to prevent the induction of anergy, or that they may express some unique molecules that signal the induction of anergy.

It is also possible that the activated T cell APCs could be providing an active negative signal, rather that not providing a positive signal. If this active negative signal is associated with the membrane of the T cell APCs, rather that being a soluble, secreted factor, there is a possibility of eliminating the signal by fixing the membrane of the APCs with paraformaldehyde. Our results in Fig. 29, show that the answer to the question of active signal is not easy.

A simple approach of abolishing the signal does not give the answer.

An important factor in a T-T interaction is the bi-directionality of the signaling, as compared to, other T cell-aberrant APC interactions. In the T-T interaction, both the stimulators and the responders express co-receptor and accessory molecules such as CD4/8, LFA-3, ICAM-1, CD28 *et cetera*, and this may contribute to the unique outcome of T-T interactions. Yet another unusual pair of ligands expressed by activated T cell APCs is the B7 family and CTLA4. It has been reported that constitutive expression of B7.1 on resting B cells in transgenic mice results in a defect in T-dependent antibody production [195]. There are also differences in the signaling properties of CD28 and CTLA4. Cross-linking of CD28 provides co-stimulatory activity, whereas anti-CD28 Fab fragments inhibit it. On the other hand, cross-linking of CTLA4 inhibits co-stimulatory activity of B7-expressing APCs but anti-CTLA4 Fab fragments enhance it [193]. Therefore, if CTLA4 cross-linking leads to signaling, these data suggest that the outcome of CTLA4-mediated signaling may be inhibition of T cell function. In such a scenario, it is possible that the high expression of CTLA4 and its ligand/s on activated T cell APCs could mediate a negative signal.

Thus, we have shown that the T-T interaction leads to the induction of anergy and unavailability of IL-2 during priming appears to be one of the factors leading to the induction of anergy, but the actual contribution of various accessory molecules which may result

in the apparent deficiency of IL-2, still remains an enigma.