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# The activation pathway of CD8 positive suppressor T cells induced by human intestinal epithelial cells

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# The Activation Pathway of CD8 Positive Suppressor T Cells Induced by Human Intestinal Epithelial Cells

by

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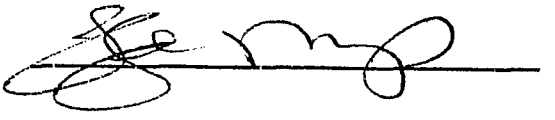

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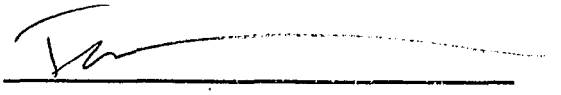

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# Abstract


The Activation of $CD8^+$ Suppressor T Cells
Induced by Human Intestinal Epithelial Cells

by

Yin Li

Advisor: Professor Lloyd Mayer

The immunologic hyporesponsiveness of the human gastrointestinal tract to dietary proteins has been demonstrated to be mediated by the generation of suppressor T cells. Human intestinal epithelial cells are able to function as antigen presenting cells to process and present antigens to T cells and selectively induce the activation of a $CD8^+$ suppressor T cell subset, in a manner distinct from other conventional antigen presenting cells (i.e. macrophages). The activation of $CD8^+$ suppressor T cells appears to be regulated by the binding of a novel CD8 ligand (non class I molecule), expressed on epithelial cells, to CD8 molecules on T cells. Co-culture of normal epithelial cells with peripheral blood T cells activates a CD8 associated src-like protein tyrosine kinase, $p56^{lck}$ and induces cellular substrate phosphorylation, including a unique 97kD protein. The upregulation of $p56^{lck}$ is found to be an early and essential event during T cell activation in this system. The increased

enzymatic activity of $p56^{lck}$ can be measured within 1 minute, maintained for about 5 minutes after stimulation with epithelial cells. The selective binding to CD8 and activation of CD8-associated $p56^{lck}$, but not CD4-associated $p56^{lck}$ is demonstrated by both antibody blocking and selective stimulation of a murine transfectant expressing human CD8. The molecular weight of two putative candidate CD8 ligands expressed on epithelial cells have been identified by both western blot and $^{35}S$ metabolic labelling assay. While binding to and stimulation through CD8 molecules are necessary, cross-linking CD8 alone with monoclonal antibody is not sufficient to drive T cell proliferation. By employing specially constructed bifunctional antibodies, T cells expressing suppressor function can be activated by the bifunctional antibody anti-CD3/CD8. Similar to epithelium mediated stimulation, anti-CD3/CD8 induced T cell activation involves protein tyrosine kinase $p56^{lck}$ activation, which can be inhibited by genestein. In contrast to cross-linking CD3 and CD8, bifunctional antibody anti-CD3/CD28 activates T cells carrying cytotoxic function. These data imply that different combinations of surface stimulation deliver unique signals to T cells and trigger specific T cell subset activation.

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# ========== Introduction ==========

## Part I: The Induction of Immunosuppression in the Gut

The mucosal immune system is unique in its location, juxtaposed to the external environment, and in its requirement to maintain homeostasis. Under the influence of a complex and distinctive array of antigens present in the intestinal lumen, the mucosal immune system is distinguished from the systemic immune system by a number of features. These include 1) secretory IgA, a mucosal immunoglobulin; 2) mucosal T cell populations with mucosa specific regulatory properties or effector capabilities; and 3) mucosal "homing", a mucosa-oriented trafficking system for immunocompetent cells initially primed and activated in the mucosa which migrate through the systemic circulation and back to the various mucosal lymphoid tissues underlying the epithelium. Such "homing" speaks for a unique mucosal immune system.

An important component of the intestinal mucosal barrier the mucosal immune system in the gastrointestinal tract, or gut-associated lymphoid tissue (GALT), plays a critical role in providing for host defense at mucosal surfaces. One feature of GALT is immune exclusion. Upon the entry of mucosal pathogens, the mucosal immune system is activated and secretes a specialized immunoglobulin, sIgA, which mediates immune exclusion by one of two ways. First, transport of secretory IgA from the basolateral

surface of the epithelial cell is mediated by binding to secretory component (SC) and transport to the apical surface results in the ability (IgA) to trap pathogens prior to adherence to mucosal surfaces, and to facilitate their degradation by mucosal surface proteases before they are bound to and subsequently endocytosed by intestinal epithelial cells (Andre et al., 1974: Walker et al., 1977 and Lake et al., 1979). Second, the formation of IgA-pathogen immune complexes can be cleared in the liver via a secretory component mediated transport system (Peppard et al., 1981; Russell et al., 1981 and Socken et al., 1981). There phagocytic cells of the reticulo-endothelial system ingest and neutralize pathogens. Therefore, the mucosal immune system can either prevent the penetration of potentially harmful pathogens into the systemic circulation or promote their clearance from the circulation.

Another important feature of the GALT, immunologic suppression following oral administration of protein antigens, or oral tolerance, has recently received much attention. The major role of this immunosuppression is most likely to maintain homeostasis by protecting the exposure of the systemic immune system to excess enteric foreign antigens which have penetrated through the mucosal surface layer. Orally administered proteins subject to, but escaping enzymatic digestion in the gut, can be absorbed as macromolecular antigens. Normally, up to 2% of dietary proteins may be taken up in the gut in this way (Mowat, 1987). The introduction of such macromolecular antigens to the mucosal immune system, most commonly results in systemic immune unresponsiveness. Oral tolerance is affected by both the humoral

and cellular arms of the immune system (Challacombe, 1983 and Titus, 1981). However, the mechanisms relating to the generation of oral tolerance are not well understood. Active suppression by suppressor T cells generated in the gut is generally thought to play a central role. Successful transfer of oral tolerance by splenic T cells and more specifically $CD8^+$ T cells was reported in 1976 in rats fed bovine serum albumin (Thomas et al., 1976). The generation of suppressor T cells has been attributed either to unique antigen processing by intestinal epithelial cells (Strobel et al., 1983), or more recently to the expression of a unique CD8 binding ligand on the surface of intestinal epithelial cells.

*Oral Tolerance*

Oral tolerance is the state of antigen-specific hyporesponsiveness of humoral or cellular immunity induced by antigen feeding prior to systemic immunization. The reported phenomenon of oral tolerance dates back to 1829 in an anecdotal report by Dakin who described that South American Indians ate leaves of the poison ivy plant to avoid the contact hypersensitivity reaction to urushiol (Dakin et al., 1829). Subsequently, many experiments in a variety of animal models, including guinea pig (Wells, 1911 and Chase, 1946), mice (Andre et al., 1975; Richman et al.,1978 and Challacombe, 1983), rats (Mattingly et al., 1978) and pigs (Newby et al., 1981), have demonstrated the existence of oral tolerance. Accumulated data from these studies have shown that oral tolerance is a complex immunological phenomenon which affects many different aspects of the immune response. Split

tolerance is sometimes induced with normal or upregulated responses in the humoral or cellular arm with hyporesponsiveness in the other. Even within the humoral immune system, suppression may be specific for distinct antibody isotypes (i.e. IgA production is spared with suppression of IgG and IgM).

The mechanisms relating to the generation of oral tolerance have been extensively studied and the activation of suppressor T cells after feeding antigens has been well documented. The suppression of delayed type hypersensitivity (DTH) after intestinal challenge with contact sensitizing agents has been shown to be mediated by suppressor T cells (Asherson et al., 1977 and Miller et al., 1979). These suppressor T cells can be detected in Peyer's patches, mesenteric lymph nodes and spleen in the case of prolonged oral antigen feeding (Asherson et al., 1977 and Mattingly et al., 1978). Some investigators have suggested that active suppression may relate to other suppressive mechanisms such as suppressive immune complexes (Andre et al., 1975; Kagnoff, 1978 and 1980), tolerogenic peptides (Strobel et al., 1983 and Ferguson st al., 1988) and anti-idiotypic antibodies (Jackson et al., 1981). However, most studies demonstrate that the generation of suppressor T cells plays a central role in the active suppression of both humoral and cellular systemic tolerance (Ngan et al., 1978 and Richman et al., 1978).

Further evidence for the cellular nature of suppression comes from the fact that pretreatment of ovalbumin fed mice with cyclophosphamide or 2'-deoxyguanosine, inhibiting suppressor T cells, abrogates the induction of oral tolerance (Mowat et al., 1982;

Dorf et al., 1984 and Mowat et al., 1986). The phenotype of these suppressor T cells have been studied as well. They found that while in vitro lymphocyte proliferation could not be induced using whole lymphocyte preparations from lymph nodes from orally tolerized animals, if cells from these nodes were treated with anti-Ly1 antibody plus complement then significant proliferative responses were observed (Silverman et al., 1982). While Silverman et al., suggested that the suppressor T cells elicited by oral antigen challenge were of the $Ly1^+$ phenotype, most studies support a central role for $CD8^+$ cells within the $Ly1^+$ lymphocyte population.

The involvement of the unique antigen absorption and processing by the intestinal mucosa in the induction of suppressor T cells in oral tolerizied animals has been suggested by Strobel and Ferguson. In experiments designed to examine the role of the gut in the "processing" of fed antigen, Strobel and others have found that when serum is collected from BALB/c mice 1 hr after feeding ovalbumin, and administered intraperitoneally into naive recipients, there is transfer of tolerance for cell mediated immune responses but not for antibody responses (Strobel et al., 1983). Since the modified antigen present in serum after feeding is the result of proteolytic digestion in the gut as well as absorption through the epithelium and subepithelial tissues of the gut, these investigators attempted to mimic the change of physical and chemical appearance of ovalbumin after intestinal processing. However, parenteral injection of mice with either deaggregated or denatured ovalbumin did not produce the same pattern of unresponsiveness as "intestinally processed" Ag (Bruce and

Ferguson, 1986). Furthermore, the transfer of serum from ovalbumin fed pre-irradiated mice into naive recipient mice, did not result in suppression of delayed type hypersensitivity to ovalbumin. These data suggest that the altered antigen processed in the gut alone may not be sufficient to induce the generation of suppressor T cells and oral tolerance. It also implies that the generation of suppressor T cells in oral tolerized animals is a complex interaction among gut epithelial cells, altered antigens and T cells, with some unique surface molecules on gut epithelium which may play an important role in triggering suppressor T cell activation.

*Antigen Absorption and Processing in the Gut*

Since undigested macromolecules in the gut have been found to be able to cross the intestinal mucosa and induce immune hyporesponsiveness, the route of antigen uptake and the mechanism of antigen processing and presentation have received considerable attention. There are three potential pathways for antigen entry into the lamina propria: 1) Membranous epithelium or M cells (the specialized epithelium overlying Peyer's patches); 2) paracellular (through tight junctions between epithelial cells) (Hecht et al., 1988); 3) transcellular (through the absorptive epithelium). M cells are known to be capable of endocytosing large particulate antigens from the gut lumen and translocating them into the subepithelial space. However, the limited number of M cells in the gut seem to be insufficient to handle the abundant dietary antigen load in the gut lumen. The failure of M cells to express class

II antigens and their lack of cytoplasmic lysosomes make it impossible for them to act as true antigen presenting cells (Owen et al., 1986; Bjerke et al., 1988). Paracellular transport is equally inefficient. Antigen transport through tight junctions is quite rare, with only some ions diffusing through in normal conditions. However, under disease conditions or after contact with certain bacterial toxins (Hecht et al., 1988), the tight junctions may be broken and permeability increased. In this setting, presumably the normal pathway of antigen access through mucosa is disturbed. Lastly, recent studies evaluating intestinal villous absorptive epithelium show that this group of cells express surface HLA class II molecules, possess cytoplasmic lysosomes, process and present antigens and mediate T cell proliferation in vitro, making them good candidates as antigen presenting cells. The huge number of enterocytes in the gut suggest that these cells might be the major cell type responsible for antigen transport and presentation.

*Expression of Ia Molecules on the Enterocyte Surface*

Expression of MHC class II (Ia) molecules by enterocytes was first identified in the guinea-pig by Wiman and colleagues in 1978 (Wiman et al., 1978). Subsequently, class II molecules have been identified on intestinal epithelium in the mouse (Parr et al., 1979), rat (Mason et al., 1981), and humans (Hirata et al., 1986). From immunohistochemical studies in human, class II molecule bearing enterocytes exist mainly in the small intestine, predominantly in the ileum, and less so in the colon. The luminal side of the enterocyte appears to express much more class II antigen than the

basolateral side (Mayer et al., 1987). This may be due to the large surface area of the microvillus structures (membrane overlapping). Although intracellular granular staining has been noted both in tissue sections and isolated enterocytes, the specific organelles containing class II molecules have not been defined, and the recycling pathway of these molecules is unknown.

Normal adult enterocytes constitutively express class II molecules on the cell surface at low density. However, the intensity of expression is found to be inducible in various situations. Both in Trichinella spiralis-induced inflammation (Barclay et al., 1982) and graft-versus-host disease (Mason et al., 1981; Barclay et al., 1982), an increase in class II expression is observed. In in vitro experiments, Cerf-Bensussan et al. have shown that incubation of class II negative epithelial cell lines with supernatant from concanavalin A stimulated spleen cells or IEL can induce the expression of class II molecules (Cerf-Bensussan et al., 1984). The soluble inducing factor involved is believed to be IFN-$\gamma$. This hypothesis was confirmed by the studies in our laboratory in which in the presence of recombinant IFN-$\gamma$ in the medium, expression of class II molecules on a human malignant intestinal cell line, DLD-1, was significantly intensified (Mayer et al., 1987). In the gut, enterocytes live in intimate association with intraepithelial cells and lymphocytes in the lamina propria. Some immunological functions of enterocytes are presumably controlled and regulated by lymphocyte-derived factors or by the direct contact with neighboring T cells. However, constitutive expression of a basal level of class II molecules on enterocytes may also be independent

of T cell effects since class II molecules are also expressed on enterocytes from nude rats (Mayrhofer et al., 1983).

From ontogenetic studies, class II molecules are not detectable on the intestinal epithelium during fetal development in the mouse (Natali et al., 1981). Class II molecules do not appear until approximately a week after birth and do not reach adult levels until about one month of age in the rat (Mayrhofer et al., 1983). The developmental expression of class II molecules is directly correlated with the development of oral tolerance. In the rat, tolerance to beta-lactoglobulin cannot be generated at nine days of age, but tolerance is easily induced in adults. Similarly in mice, tolerance to ovalbumin can not be induced until seven days of age (Hanson et al., 1981). Therefore, expression of class II molecules or co-regulated molecules on the intestinal epithelium may be involved in the mechanism of tolerance induction. However, it is also possible that both the expression of class II molecules and the development of oral tolerance are only upregulated following the maturation of mouse gut, and these two events are not necessarily related.

*The Antigen Presenting Function of Enterocyte*

Expression of class II molecules on the cell surface is necessary but not sufficient for a cell to function as an antigen presenting cell. Degradation or a conformational change in macromolecules are required for the binding of peptides to class II molecules and eventual presentation to T cells (Allison et al., 1987). Studies in both rat and human have been undertaken to investigate the

capability and the efficiency of the gut epithelium to present and process antigens. It has been shown in our laboratory that isolated human enterocytes can present the soluble protein antigen, tetanus toxoid, to primed T cells (Mayer et al., 1987). Pretreatment of enterocytes with glutaraldehyde or paraformaldehyde block the T cell proliferative response demonstrating that processing is required. The results comparing T cell proliferative responses to enterocytes or adherent cells in antigen specific assays suggest that the efficiency of antigen presentation by either epithelial or adherent cells is similar. However, using fluorescein labelled tetanus toxoid, enterocytes were found to take up this antigen at a slower rate than monocytes. Human enterocytes also are capable of secreting IL-6 and expressing GM-CSF receptor, ICAM-1 and LFA-3 molecules (Mayer et al., 1990). Taken together, human enterocytes exhibit all functions required for true antigen presenting cells. Similarly, antigen processing and presentation by rat epithelium has been reported. Rat enterocytes can present ovalbumin to primed lymph node T cells (Bland et al., 1986a and 1986b). Like the human system, this presentation can be blocked by pretreatment with paraformaldehyde, chloroquine, ammonium chloride or monensin, but not leupeptin. When evaluating the processing capability of rat enterocytes, the degradation of radiolabelled ovalbumin (antigen processing) is less efficient in enterocytes than in macrophages, or B cells (Bland et al., 1989). For example, ovalbumin processed by enterocytes can not be presented by fixed splenic adherent cells.

Typically, proteins in the gut lumen are first degraded into

single amino acids, biamino- or triamino-peptides, which then can be taken up by absorptive enterocytes. Therefore proteins may be "preprocessed" by intestinal enzymes and the processing requirement by enterocytes may be less critical. The enzymes required for processing of macromolecules in enterocytes needs to be further defined.

*Ligands Involved in the Epithelium-T Cell Interaction*

In addition to stimulation of antigen specific responses, both human and rat enterocytes can act as stimulators in the mixed cell culture system of epithelial cell-T cells, similar to primary allogeneic mixed lymphocyte reactions. These T cell responses can be inhibited by antibodies to CD8 while no inhibition is detected in the presence of anti-class I or anti-CD4 monoclonal antibodies (Bland et al., 1987; Mayer et al., 1987). Intact enterocytes selectively stimulate T cell subpopulations bearing the suppressor/cytotoxic phenotype. This result raises an interesting question regarding the role played by class II molecules in $CD8^+$ T cell proliferation. Many studies have shown that CD4 and CD8 antigens act as accessory molecules on the T cell surface and bind to MHC class II and class I molecules respectively during cell-cell interactions. These interactions serve to strengthen and stabilize the relatively weak binding of the T cell receptor to antigen/MHC complexes. The fact that CD4 binds to class II molecules and CD8 binds to class I molecules is clearly documented in binding studies, in which cell lines transfected with and expressing CD4 or CD8 molecules bind to class II or class I expressing cells respectively.

Direct binding of CD4 to class II and CD8 to class I molecules has also been achieved by using constructed vesicles expressing purified CD4, CD8, class I or class II molecules (Doyle et al., 1987 and Rosenstein et al., 1989). Antibodies against these molecules can prevent the binding. However, class I and class II may be not the only ligands that can bind to CD8 or CD4, respectively. The gp120 envelope protein of HIV has been found to specifically bind to CD4 and result in the infection of T cells and monocytes by these viruses. To date no other potential ligands have been identified.

*A Potential Novel Ligand for CD8*

Since anti-CD8 antibody can block the T cell proliferation where normal enterocytes serve as stimulators, this suggests that the CD8 molecule on the T cell surface may be involved in the interaction between T cells and enterocytes. The addition of anti-class I antibody, the recognized ligand for CD8, into the same reaction does not result in an inhibitory effect. This raises the possibility that an alternative ligand for CD8 exists on the enterocyte surface. As will be described below, CD8 molecules expressed on intraepithelial lymphocyte (IEL) consist of $\alpha\,\alpha$ homodimers, not the $\alpha\beta$ heterodimers expressed on peripheral blood T cell. The CD8 $\alpha\alpha$ homodimer might bind to a novel CD8 ligand instead of class I antigen. Recently, our laboratory has screened a panel of monoclonal antibodies raised by immunizing mice with normal human enterocytes. Four monoclonal antibodies were identified which had the ability to inhibit $CD8^+$ T cell proliferation in MLRs where freshly isolated human enterocytes

served as stimulators. The surface molecules of enterocytes recognized by these four monoclonal antibodies might be potential ligands for CD8 molecules. Two of these monoclonal antibodies have been tested for this hypothesis.

*Suppressor T Cell Induction by Enterocytes*

$CD8^+$ T cell populations can either be suppressor cells or cytotoxic cells. Further functional analysis of enterocyte stimulated T cell populations suggests that these responding T cells act as suppressor cells when added to a variety of T and B cell proliferation assays. In the human system, T cells induced by enterocytes can non-specifically inhibit primary, secondary, and unrelated mixed lymphocyte reactions and show no cytotoxic activity (Mayer et al., 1987). These suppressor T cells can also inhibit mitogen-induced T cell proliferation and B cell differentiation in the presence of T cells and PWM. Results from cell staining indicate that these enterocyte stimulated T cells are stained with an anti-CD8 antibody, but not with the monoclonal antibody 9.3 (anti-CD28), which recognizes the cytotoxic T cell within the $CD8^+$ population. In the rat system, enterocyte stimulated T cells are also suppressor cells (They stain with the monoclonal antibody MRC OX-8, which recognizes suppressor T cells), but in contrast to humans are antigen-specific (Bland et al., 1986b). Thus the suppressor T cells induced in these two systems seem to have different functional capabilities, one mediating antigen non-specific inhibition, the other antigen specific. The factors relating to this dichotomy may include the different

isolation procedures for intestinal epithelial cells, the different species and different cell culture system. Interestingly, isolated epithelial cells from inflamed mucosa, e.g. from patients with inflammatory bowel disease activate mainly $CD4^+$ helper T cells rather than $CD8^+$ suppressor T cells. Therefore, the defect in epithelial cells coupled with an increased class II MHC molecule expression on epithelial cells from IBD patients changes the normal epithelial cell-T cell interaction in the gut and may also change the direction of immune responses.

The preferential activation of $CD8^+$ T cells by normal enterocytes is of particular interest given the predominance of $CD8^+$ T cells in the intraepithelial space. These findings suggest that enterocytes may play an important role in the activation of IEL.

*The Phenotype and Function of IEL*

The intraepithelial lymphocytes (IEL) are a population of cells lying above the basement membrane, amongst the epithelial cells. The unique location of IEL, between epithelial cells which separate the antigen-rich intestinal lumen from the subepithelial lymphoid tissue of the lamina propria, may relate to the finding that this group of cells functions differently from other lymphocytes in the mucosal system.

There are about 9-39 IEL/100 small intestinal epithelial cells under normal conditions and a larger number in some inflammatory states. More than 95% of human IEL are positive for the pan-T-marker CD3 and there is no staining of surface or cytoplasmic immunoglobulin (Selby et al., 1981). Around 80% of

CD3 positive IEL co-express CD8 and are not stained by the H366 monoclonal antibody, which identifies the subset of cytotoxic T cells within the CD8 population (Trejdosiewcz et al., 1987). From a functional point of view, IEL are less activated than T cells in other lymphoid organs. Normal IEL fail to express HLA-DR, Tac antigen (IL-2R), $C_{3b}$ receptor, and transferrin receptor (Hirata et al., 1986 and Selby et al., 1984). There is no staining of IEL with either OKT10 (for "activated" lymphocytes) or Ki67 (for proliferating cells) monoclonal antibodies. IELs are stained with mAb HML-1 which recognizeds an integrin which is not expressed on peripheral blood T cells or LPL unless activated (Cerf-Bensussan et al., 1987 and Schieferdecker et al., 1991). T cell receptors on human IEL are mainly of the $\alpha$–$\beta$ type (TCR2), and not the $\gamma$–$\delta$ type (TCR1) found in murine IEL (Goodman et al., 1988). Both CD4 and CD8 bearing IEL express predominantly the CD45RO marker which is the surface antigen expressed on memory lymphocytes, rather than CD45RA which is expressed on naive lymphocytes (Mowat et al., 1990).

IEL have a low rate of spontaneous proliferation and do not respond to the lectins phytohemagglutinin, concanavalin A, and pokeweed mitogen, even after addition of autologous macrophages or IL-2 (Greenwood et al., 1983). Recently it has been shown that proliferation of human IEL can be induced by stimulation of these cells by the addition of sheep red blood cells, probably via CD2, and IL-2. The mechanism of such activation is as yet unknown, although activation by alternate pathways, not through the T cell receptor is under consideration. The possibility of IEL activation induced by neighboring enterocytes is also being investigated.

However, IELs may have effects on enterocytes as well. IEL can secrete a variety of lymphokines including IL-2, IL-3. IL-4, IL-6, TGF-β, TNF-α and IFN-γ (Madara et al., 1989; Evans et al., 1992 and Sollid et al., 1987). IFN-γ has been shown to directly affect the barrier function of the epithelium (Lowes et al., 1992) as well as enhance the expression of class II MHC molecules (Ferguson, 1972) or secretory component (Mayrhofer, 1980 and Schrader et al., 1983) on enterocytes. Secretory component can be further increased by IL-4 (Mayrhofer, 1980) and TNF-α (Petit et al., 1985). In addition, IFN-γ can manipulate ion secretion by the intestinal epithelium (Lefrancois, 1991). Since IEL can produce a broad range of lymphokines, IEL may be able to regulate local mucosal immunity and epithelial cell growth and function. Studies from Greenwood et al. have been shown that IEL are capable of suppressing immunoglobulin production by peripheral blood mononuclear cells in vitro (Greenwood et al., 1983). Therefore, IEL may possibly have suppressive responses to antigens passing through the villous epithelium. Interestingly, IEL do not express class II histocompatibility antigens (HLA-DR, Ia-like), even in inflammatory states (Cerf-Bensussan et al., 1984; Selby et al., 1981 and 1984).

As discussed above, the majority of IEL express CD8. A unique feature of the CD8 molecule on IELs is that it is often a homodimer of the CD8α chain, whereas in other tissues CD8 is a heterodimer consisting of α and β chains (CD8αβ) (Viney et al., 1989; Mosley et al., 1990; Guy-Grand et al., 1991 and Banadeira et al., 1991). Therefore, recognition of class I molecules promoting IEL activation

signalled by this form of CD8 may be potentially different. It is not known whether the CD8α homodimer can still interact with classical class I MHC or requires signaling by other class I-like molecules on enterocytes for activation. Furthermore, although intestinal epithelial cells predominantly "present antigen" to and activate $CD8^+$ T cells, the stimulation of $CD8^+$ T cells can only be blocked by anti-CD8 but not anti-class I antibodies. Thus, it has been suggested that a novel CD8 ligand is required on intestinal epithelium. Since IEL also have a novel form of CD8, the nature of this interaction may be important for the induction of the unique mucosal immune phenomenon "oral tolerance". In fact, IEL expressing the CD8α chain homodimer appear to be more difficult to activate by mitogens or other conventional stimuli (Sollid et al., 1987 and Bandeira et al., 1991).

Generally, IEL can be divided into two classes, granulated and non-granulated cells. Evidence suggests that the non-granulated population of intraepithelial lymphocytes is thymus-dependent whereas the granulated cell population is not under thymic control (Mayrhofer, 1983 and Mayrhofer et al., 1983). These granulated IEL may develop their repertoire via selection from either classical class I or II, or non-classical MHC/HLA molecules expressed on epithelial cells. Supporting this concept is the finding that IEL have been detected in athymic mice. However, other evidence suggests that intraepithelial lymphocytes arise from precursor cells within the Peyer's patches and migrate via the mesenteric lymph nodes and thoracic duct lymph back to the mucosal epithelium via the systemic circulation (Cahill et al., 1977).

*The Phenotype and Function of LPL*

The lamina propria and Peyer's patches are believed to be important for lymphocyte activation in the gut. The lymphoid tissue within the lamina propria just below the basement membrane of the gastrointestinal tract is composed predominantly of mature plasma cells, T cells, mast cells, macrophages and other less frequent elements such as eosinophils, basophils, and neutrophils. While IEL are almost exclusively T cells, lamina propria lymphocytes (LPL) are about equally divided among B cells and T cells. $CD4^+$ lymphocytes are the predominant T cell subset, twice as numerous as $CD8^+$ lymphocytes, similar to peripheral blood (Selby et al., 1983). By double-labelling flow cytometric analyses, James and coworkers have shown that, when compared with peripheral blood lymphocytes, lamina propria T cells contain a significantly higher percentage of $Leu3^+$, $Leu8^-$ and $Leu3^+$, $2H4^-$ cells (helper-inducer phenotype), and a corresponding decrease in $Leu3^+$, $2H4^+$ cells (suppressor-inducer phenotype). In addition, they contain fewer $Leu2^+$, $Leu15^+$ cells (suppressor-effector phenotype), but similar proportions of $Leu1^+$, $9.3^+$ cells (cytotoxic phenotype) (James et al., 1986). Thus it appears that helper/inducer and cytolytic cells constitute the bulk of T cells within the lamina propria.

In contrast to IEL, lamina propria lymphocytes have undergone prior activation. Freshly isolated LPL T-cells contain IL-2 receptor mRNA, in contrast to T cells from peripheral blood which only show detectable levels of IL-2 receptor mRNA after culture with mitogens or antigens (Zeitz et al., 1988). LPL T-cells have

increased class II MHC and IL-2 receptor expression, and high expression of mRNA for IL-2 and IFN-γ. None of the unstimulated cell populations produce IL-2, but after mitogen-induced activation LPL T-cells produce significantly more IL-2 and express more mRNA for IL-2, than circulating lymphocytes. Titration assays of monoclonal antibodies to both CD2 and CD3 have shown that LPL T-cells are significantly more sensitive to activation compared with peripheral blood T cells (Targan et al., In press). Furthermore, LPL T-cells respond more rapidly (by proliferation) after cross-linking CD2 than after cross-linking CD3.

LPL T-cells seem to possess predominantly helper function in in vitro assays. Addition of unstimulated LPL T-cells to the pokeweed mitogen stimulates peripheral blood B cells in mixing experiments to induce high rate immunoglobulin synthesis (Elson et al., 1985). Therefore, helper function of LPL T-cells is more potent than that of its peripheral blood counterpart. In one recent experiment, after incubation with anti-Leu8 antibody, peripheral blood $CD4^+$ cells exhibited suppressor function in immunoglobulin synthesis assays, while LPL T-cells did not (Kanof et al., 1988). Further studies showed no difference in helper function of isolated $CD4^+$, $Leu8^-$ T cells and the suppressor-inducer function of $CD4^+$, $Leu8^+$ T cells from both the intestine and blood. Probably the vigorous helper function of LPL T-cells comes from quantitative differences in $CD4^+$, $Leu8^+$ cells which are fewer in number in the LPL population. In the same immunoglobulin synthesis assay, lamina propria $CD8^+$ T cells display either suppressor or helper functions (contrasuppressor cells) (Lee et al., 1988). After addition

of $CD4^+$ cells, the helper function of lamina propria $CD8^+$ T cells increases. Thus, it may be that the regulatory function of lamina propria $CD8^+$ T cell function is dependent upon $CD4^+$ T cells. LPL T-cells seem to be a group of activated cells having a preferential helper activity, probably mediated by $CD4^+$ cells.

## Part II: Alternate Signal Pathway Through CD8/p56$^{lck}$ or CD4/p56$^{lck}$

In addition to receiving stimulation through T cell receptor, T cells also acquire additional signals provided by antigen presenting cells through accessory molecules. The T cell membrane glycoproteins CD8 and CD4 are two important accessory molecules for antigen-dependent T cell activation. On the one hand, they stabilize and increase the avidity of the interaction between TCR-CD3 and the nominal antigen-MHC complex by binding to monomorphic determinants on MHC class I or II molecules (Doyle et al., 1987; Dembic et al., 1986 and Rosenstein et al., 1989). On the other hand, CD8 and CD4 may play an even more active role in T cell activation by transducing an independent signal.

### *Activation Signals Transduced Through CD8 and CD4 Surface Molecules*

Recently several groups have shown that the intracytoplasmic tails of CD8 and CD4 molecules are physically complexed with a protein-tyrosine kinase p56lck (Rudd et al., 1988a and 1988b; and Veillette et al., 1988a and 1988b). Cross-linking CD8 or CD4 activates the kinase activity of p56$^{lck}$, which phosphorylates numerous intracytoplasmic substrates. In addition to the initial protein-tyrosine kinase activation, stimulation through CD8 or CD4 can induce an ethyleneglycoltetraacetic acid (EGTA)-resistant increase in $[Ca^{2+}]_i$, resulting from intracellular $Ca^{2+}$ mobilization (Ledbetter et al., 1987). Some studies suggest that CD4 may be

functionally associated with a $Ca^{2+}$ channel (Rosoff et al., 1987). Samstag et al. have reported that the signal delivered through CD8 with antibody cross-linking was very sensitive to 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-8), an inhibitor of protein kinase C and cGMP/cAMP-dependent kinases, implying activation of one (or more) of these kinases by CD8-mediated signaling (Samstag et al., 1988). The increase of IL-2 receptor expression, a marker for T cell activation, has been found in the stimulation of CD4 with the human immunodeficiency virus-1 (HIV-1) envelope glycoprotein, gp120, a ligand binding to the CD4 receptor (Kornfeld et al., 1988). Furthermore, a unique murine anti-CD4 monoclonal antibody, B66, has the capacity to deliver a primary activation signal to human $CD4^+$ peripheral blood T cells, that is, to stimulate, in its cross-linked form, proliferation and IL-2 production in the absence of additional stimuli (Carrel et al., 1988). The stimulation through CD8 with monoclonal antibodies may also provide a positive signal to T cell activation and proliferation. In a superantigen driven system, staphylococcal enterotoxin B (SEB) driven isolated $CD8^+$ T cell proliferation, the presence of anti-CD8 monoclonal antibody did not inhibit but rather enhanced T cell proliferation (Heeg et al., 1991).

Although some studies have shown that the stimulation of T cells with lectin or antibodies directed against the TCR/CD3 complex can be blocked by anti-CD8 or anti-CD4 in the absence of antigen presenting cells bearing the appropriate class I or class II ligands (Bank et al., 1985 and van Seventer et al., 1986), recent evidence suggests that such blocking effects are related to the steric

interference of the TCR-CD8 or -CD4 co-receptor complex (Gallagher et al., 1989). These studies raise the possibility that CD8 and CD4 might play a direct role in signal transduction. In our human intestinal epithelium stimulated T cell activation system, freshly isolated intestinal epithelial cells can selectively trigger $CD8^+$ T cell proliferation from unselected peripheral blood T cells (Mayer et al., 1987). The binding of CD8 molecules on T cells appears to be critical for this event. However, whether cross-linking CD8 alone is sufficient to drive $CD8^+$ T cell proliferation is addressed in this thesis.

The CD8 and CD4 transmembrane protein molecules on T cells were initially identified in rat (Williams et al., 1977 and White et al., 1978), and subsequently in mouse (Ledbetter et al., 1981) and human (Reinherz et al., 1979a and 1979b). The cloning and sequencing of mouse and human CD8 and CD4 have established them as members of the immunoglobulin (Ig) gene superfamily and their amino terminal external domains homologous to the Ig V region.

*The Biology and Function of CD8*

Human CD8 has three different chains. One is the product of the CD8α gene, and the other two forms, $CD8\beta_1$, and $CD8\beta_2$, are differentially spliced products of the CD8β gene and differ only in the sequences of their cytoplasmic domains (Norment and Littman, 1988). Most CD8 molecules expressed on peripheral blood T cells are heterodimers of CD8α and either $CD8\beta_1$ or $CD8\beta_2$ (Norment and Littman, 1988), whereas CD8 αα homodimers are the predominant

form on human IELs (Viney et al., 1989; Mosley et al., 1990; Guy-Grand et al., 1991 and Banadeira et al., 1991).

In the thymus, in addition to CD8 αα homodimers, multimers of human CD8 α chain exist and are disulfide linked to the 46kD CD1 glycoprotein (Ledbetter et al., 1985 and Snow et al., 1985). CD1 is a class I related molecules encoded on a different chromosome (Calabi et al., 1986). While CD1 is typically associated noncovalently with $\beta_2$-microglobulin, no $\beta_2$ microglobulin has been found associated with the CD1 that is linked to CD8 in human thymocytes (Ledbetter et al., 1985 and Snow et al., 1985). Besides thymocytes, human and murine enterocytes also can express CD1 molecules on their surface. This CD1 molecule has been identified as CD1d, one of the CD1 isoforms, by using a tissue immuno-staining technique. It is not clear whether CD1 expressed on enterocytes can bind and present small dietary peptides to T cells or associate with the CD8α chain. Recently, a noncovalent association between CD8 and class I MHC proteins has been described on the surface of human T cell clones (Bushkin et al., 1988). The physiological significance of such an association is not known.

The human CD8α chain is a single, glycosylated 34-kD polypeptide (Snow et al., 1983). The carbohydrates on CD8α chain are all O linked rather than N linked (Snow et al., 1983). The external portion of the protein consists of a 96-amino acid V-like domain and a membrane-proximal, 65-amino acid hingelike region or connecting peptide. This is followed by a 24 amino acid hydrophobic transmembrane segment and a highly basic 29-amino acid cytoplasmic tail (Littman et al., 1985). Two cysteine residues

each in the hinge, transmembrane region and cytoplasmic tail are used by CD8 α chains to form dimers or multimers (Tykocinski et al., 1988).

Neither CD8α nor CD8β chains contain a kinase domain. The signaling role of CD8 has been linked to the finding that a protein tyrosine kinase, $p56^{lck}$, is physically associated with the CD8α chain in $CD8^+$ T lymphocytes (Rudd et al., 1988b and Veillette et al., 1988a). Cross-linking CD8 with monoclonal antibodies induces $p56^{lck}$ activation. Cotransfection of CD8α and $p56^{lck}$ cDNAs into HeLa cells results in stable CD8/$p56^{lck}$ interactions as measured by immune coprecipitation (Shaw et al., 1990). Other proteins expressed exclusively in lymphoid cells, including $CD8\beta_1$ and $CD8\beta_2$ are therefore apparently not required for the stable interaction between $p56^{lck}$ and CD8α. In addition, binding of $p56^{lck}$ to the cytoplasmic tail of either $CD8\beta_1$ or $CD8\beta_2$ was not found when these chains were expressed as chimeric transmembrane proteins (Shaw et al., 1990). These results suggest that $p56^{lck}$ binds to various dimeric forms of CD8 only through the cytoplasmic tail of CD8α.

The interactive domains of the CD8/$p56^{lck}$ complex have been defined as amino acid residues 15-23 of $p56^{lck}$ and 191-197 of CD8α. Indeed, this six-amino acid sequence from the cytoplasmic domain of CD8α, VCKCPR, when appended to the cytoplasmic domain of vesicular stomatitis virus (VSV) glycoprotein (G), allows the chimeric protein to stably co-associate with $p56^{lck}$ upon cotransfection into HeLa cells (Shaw et al., 1990). The myristylation and cell surface membrane association is not required for the complex formation of CD8/$p56^{lck}$. Although the cytoplasmic portion

of the CD8α chain contains two cysteine residues, there is no disulfide bond formation between them. However, the mutation of these cysteine residues will reduce the binding avidity between CD8 and $p56^{lck}$. This finding suggests that free cysteine residues might employ a metal ion to stabilize the formation of the protein complex as suggested in the formation of the human immunodeficiency virus Tat protein dimer (Frankel et al., 1988).

Since p56lck only binds to CD8α, distinct heteromeric and multimeric forms of CD8 might play a role in modulating signal transduction. For example, three heterodimeric human CD8 molecules could be generated by CD8α, $CD8\beta_1$ and $CD8\beta_2$. In the case of the gut, the CD8 molecules expressed on IEL are αα homodimers which means that each CD8 molecule can associate with one $p56^{lck}$, and that the signal transduction through CD8 might be unique. This unique signal might be important in triggering $CD8^+$ suppressor T cell activation induced by enterocytes. Since the presence of an associated molecule might influence the binding of $p56^{lck}$ or its kinase activity, it would be important to address how the different forms of dimeric CD8 in particular T cells could play a role in T cell differentiation and activation.

*The Biology and Function of CD4*

In contrast to CD8, the human CD4 molecule is a single transmembrane polypeptide chain with a molecular weight 55kD (Terhorst et al., 1980 and Dialynas et al., 1983). The extracellular portion of CD4 consists of 374 amino acids. This is followed by a hydrophobic transmembrane segment containing 26 amino acids

and a highly basic cytoplasmic tail containing 38 amino acids (Maddon et al., 1985). The CD4 molecule is an N-linked glycoprotein. The external portion of the CD4 polypeptide has six cysteine residues which form three intrachain disulfide loops by connecting adjacent cysteines (Classon et al., 1986). There are six additional cysteines in human CD4, two in the transmembrane region and four in the cytoplasmic tail (Maddon et al., 1985). However, these cysteine residues do not form intrachain disulfide bonds.

CD4 also belongs to the Ig gene superfamily, like CD8, although less homologous to true V regions (Maddon et al., 1985 and Tourvieille et al., 1986). The first 100 amino acids of the amino terminus of CD4 contains two cysteine residues which form an intrachain loop. This part of the protein is most closely related to Ig V regions (Maddon et al., 1985, Tourvieille et al., 1986 and Littman et al., 1987). Following this V-like region is a short sequence that is similar to Ig J segments. In contrast to the structure of the CD8 polypeptides, CD4 contains a second V-like domain (V') just downstream of the J-like segment (Tourvieille et al., 1986 and Clark et al., 1987). However, this V' domain turns out to be severely truncated containing only a sequence related to the carboxy terminal half of an Ig V region. The V' domain of CD4 is also followed by a J-like sequence (J') (Tourvieille et al., 1986). The carboxy-terminal half of the external domain approximate to membrane of CD4 has been referred to as a connecting peptide which contains two degenerate V-like domains. The intracytoplasmic tail of CD4 contains only 38 amino acids and has

no kinase activity.

A signaling function for the CD4 receptor has been associated with the finding that the intracytoplasmic tail of CD4 is physically associated with the protein tyrosine kinase $p56^{lck}$ (Rudd et al., 1988a and Veillette et al., 1988a). This association does not require lymphoid specific factors to maintain a stable interaction, myristylation, or cell membrane association. In the genetic reconstitution of CD4/$p56^{lck}$ by cotransfection of CD4 and lck cDNAs into HeLa cells by Shaw et al., it was shown that the CD4/$p56^{lck}$ complex is formed before transport to the Golgi apparatus (Shaw et al., 1989). Further evidence from genetic mapping in the same system has demonstrated that amino acids 15-23 of $p56^{lck}$ and the 30 membrane-proximal residues of CD4 are involved in the formation of the CD4/$p56^{lck}$ complex. The involvement of cysteine residues at positions 419 and 421 in the CD4 molecule and position 20 and 23 in $p56^{lck}$ are critical for complex formation. Since these cysteines are not disulfide linked, it suggests that a metal ion may be required for the CD4/$p56^{lck}$ interaction similar to the human immunodeficiency virus Tat protein dimer.

The cysteine-rich amino-terminal domain of $p56^{lck}$ required for both CD8 and CD4 binding is not shared by any other member of the src family. Thus, the physical association of CD4/$p56^{lck}$ and CD8/$p56^{lck}$ may be unique among the binding of a transmembrane polypeptide with a non-receptor tyrosine kinase in the src family (Shaw et al., 1990).

CD8 and CD4 are functionally as well as structurally coupled to $p56^{lck}$. Stimulation through CD8 and CD4 with antibody mediated

cell-surface cross-linking leads to a rapid increase in the enzymatic activities of the associated $p56^{lck}$ (Rudd et al. 1989 and Veillette et al., 1989). Recently Thompson et al. have stated that antigen presentation to antigen-specific, MHC-restricted human cytotoxic T lymphocyte (CTL) clones as well as heteroconjugate-mediated coengagement of CD4 with the CD3/TCR complex results in the enzymatic activation of CD4-associated $p56^{lck}$ (June et al., 1990 and Ledbetter et al., 1990). Cross-linking CD8 or CD4 with antibody induces rapid (within 1 minute), but transient activation of $p56^{lck}$, with a return to baseline activity level noted within 5 minutes.

*$p56^{lck}$, a src-like Protein Tyrosine Kinase*

The protein-tyrosine kinase $p56^{lck}$ belongs to the src gene family and is found to be expressed almost exclusively in cells of the lymphoid lineage (Marth et al., 1985 and Veillette et al., 1987), mostly in T lymphocytes and NK cells (Einspahr et al., 1990). The lck gene is located on human chromosome 1 p32-p35. lck encodes a 509 amino acid polypeptide with a migration at approximately 56kD on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Marth et al., 1985 and Voronova et al., 1986). The functional domains of $p56^{lck}$ can be classified into five regions (Veillette et al., 1991): 1) an amino-terminal glycine residue important for myristylation and membrane association; 2) a segment of 60 amino acids distinct from the other members of the src family; 3) the presumed substrate interactive domain containing src homology domain 3 (SH3) and 2 (SH2), which span residues 66-122, 123-234 respectively; 4) the catalytic or kinase domain (SH1

domain), which extends from residue 235-494 and contain sites for ATP-binding at Lys 273 and autophosphorylation (tyrosine 394); and 5) a carboxy-terminal regulatory domain spanning amino acids 495-509, containing a important regulation site (tyrosine 505).

It has been suggested that the association of $p56^{lck}$ within T cells consists of three parts: binding of the N-terminal of $p56^{lck}$ to a specific internal membrane myristyl-lck receptor similar to $p60^{src}$ (Resh et al., 1990), the noncovalent linking of $p56^{lck}$ to CD4 or CD8 antigens (Shaw et al., 1989 and Turner et al., 1990), and finally the interaction of SH3 to the cytoskeleton (Pawson, 1988). The SH2 domain has been shown to be the major region for substrate binding.

The carboxy terminus encoded by QYQPQP of $p56^{lck}$ is an important regulatory domain as is the case for other members of the src family. Within this carboxy domain, the tyrosine residue 505 remains phosphorylated in the resting state (Veillette et al., 1988b). During the activation of $p56^{lck}$, Tyr 505 is dephosphorylated by a tyrosine phosphatase shown to be the leucocyte-common antigen, CD45 (Ostergaard et al., 1989 and Mustelin et al., 1989). Mutation of Tyr 505 to a phenylalanine (which can not be phosphorylated) renders $p56^{lck}$ constitutively activated and capable of oncogenic transformation in rodent fibroblasts (Marth et al., 1988; Amrein et al., 1988 and Abraham et al., 1990). Therefore, Tyr 505 is a negative regulatory site for $p56^{lck}$ by phosphorylation. The tyrosine kinase responsible for Tyr 505 phosphorylation is not $p56^{lck}$ itself (autophosphorylation) but rather a human tyrosine kinase $p50^{csk}$ (Bergman et al., 1992).

The positive regulatory site on lck is tyrosine 394 which is usually not phosphorylated in resting T lymphocytes (Abraham et al., 1990 and Casenellie et al., 1982). Antibody-mediated aggregation of CD4 molecules on T cells as well as mutation of Tyr 505 to phenylalanine on $p56^{lck}$ activates $p56^{lck}$ and this activated form of $p56^{lck}$ is extensively phosphorylated on Tyr 394. The mutation of Tyr 394, the major site of autophosphorylation of $p56^{lck}$, to phenylalanine abolishes the ability to activate $p56^{lck}$ by either CD4 cross-linking or Tyr 505 mutation (Marth et al., 1988; Amrein et al., 1988 and Abraham et al., 1990). Thus Tyr 394 is involved in the positive regulation of protein tyrosine kinase $p56^{lck}$. Although Tyr 394 is critical and required for the activation of $p56^{lck}$, myristylation and membrane association of $p56^{lck}$ facilitate the kinase activity as well (Caron et al., 1992).

*Protein Tyrosine Phosphorylation Following $p56^{lck}$ Activation*

In addition to autophosphorylation, activated $p56^{lck}$ induced by antibody cross-linking, phosphorylates abundant cellular protein substrates. The first identified substrate for $p56^{lck}$ is the $\zeta$ subunit of the CD3/TCR complex (Veillette et al., 1989). The $\zeta$ subunit has been demonstrated to transduce the major signal for TCR stimulation (Nakayama et al., 1989). By using a chimeric transmembrane peptide in which the intracytoplasmic tail of $\zeta$ chain is connected to a truncated CD8$\alpha$ chain, one is able to activate T cells by cross-linking this chimeric molecule with antibody (Irving et al., 1991). Upon the occupation of TCR/CD3 complex with antigen, the $\zeta$ chain is tyrosine phosphorylated. The tyrosine kinase

responsible for this is $p56^{lck}$, since the active form of $p56^{lck}$ following CD4 cross-linking can be coprecipitated with the ζ chain (Burgess et al., 1992). However, the importance of ζ chain phosphorylation in T cell activation through the TCR receptor is unclear. Another physically associated protein which is functionally phosphorylated by $p56^{lck}$ is Raf-1 (Thompson et al., 1991). Raf-1 can be immune coprecipitated with activated $p56^{lck}$ suggesting that it is a physiological substrate for $p56^{lck}$. Other substrates for $p56^{lck}$ include phospholipase C (PLC)-γ1 (Kanner et al., 1992), phosphatidylinositol-3 kinase (PI 3 kinase) (Ullrich et al., 1990), the 42kD mitogen-activated protein (MAP) kinase ($p42^{mapk}$) (Ettehadieh et al., 1992), the 32kD GTP-binding protein (Telfer et al., 1991) and ras GTPase-activating protein (GAP) (Amrein et al., 1992). The regulation of GAP by $p56^{lck}$ links the signal transduction by a tyrosine kinase in the src family to $p21^{ras}$ protein which is an important oncogene encoded cytoplasmic protein responsible for cell transformation (Julian et al., 1990).

<u>$p56^{lck}$ Is Associated with the IL-2 Receptor</u>

Recently, it has been shown that $p56^{lck}$ is also associated with the IL-2 receptor β chain and occupation of IL-2 receptor by IL-2 induces the activation of $p56^{lck}$ in primed T cells (Hatakeyama et al., 1991 and Horak et al., 1991). However, there is no obligate relation between the expression of $p56^{lck}$ and CD8 or CD4 coreceptor molecules, suggesting that $p56^{lck}$ may have additional functions in different signal transduction systems.

*Two Signals Required for T Cell Proliferation*

As described above, signals through CD8/p56$^{lck}$ and CD4/p56$^{lck}$ can induce an ordered cascade of numerous biochemical changes necessary for T cell activation and proliferation. Available evidence suggests that stimulation through the CD8 or CD4 molecule on the T cell is able to activate T cells but not sufficient to drive resting T cell proliferation (Kornfeld et al., 1988). A second signal is required. The combination of two signals on T cells may be also important in the stimulation of specific T cell subsets, (CD8 molecule being expressed on suppressor and cytotoxic T cells and CD4 on helper T cells). In our intestinal epithelium stimulated T cell proliferation system, we have suggested that a novel CD8 ligand expressed on epithelial cells binds to and delivers a signal to CD8$^+$ T cells. This interaction is critical for CD8$^+$ suppressor T cell proliferation. One major focus of this thesis relates to identifying the second signal provided by the intestinal epithelium to CD8$^+$ suppressor T cells.

Recent studies have shown that CD8 and CD4 can each physically associate with the TCR on T cells to some degree in the absence of antigen stimulation (Gallagher et al., 1989). Cross-linking CD4 can induce co-capping of CD4 with the TCR and co-capping of TCR with CD4 can also be seen upon cross-linking CD3 (Anderson et al., 1988). The association of CD4-TCR can be upregulated following T cell activation (Rojo et al., 1989). CD4-TCR as well as CD8-TCR have been recently referred to as the co-receptor complex, and TCR, CD4 and CD8 each play a role in signal transduction (Emmrich et al., 1987; Anderson et al., 1987 and Ledbetter et al., 1988).

In gene transfer studies, it was found that overexpression of CD8 or CD4 facilitates antigen-induced IL-2 production. This capacity depends upon whether or not these molecules act in concert with the TCR binding to the same MHC molecule. Binding to an MHC molecule not involved in TCR antigen presentation does not result in a positive signal (Gabert et al., 1987; Miceli et al., 1991 and Ballhausen et al., 1988). This augmentation of T cell activation by CD8 or CD4 formation of a coreceptor with TCR can be abrogated by truncating the cytoplasmic tail of CD8 or CD4. Zamoyska et al. have demonstrated that the truncated "tailless" CD8 molecules were not able to interact with the protein tyrosine kinase $p56^{lck}$ and have a decreased ability to restore immune responses (Zamoyska et al., 1989).

The TCR associates with a variety of transmembrane proteins including CD8, CD4, CD2 (Brown et al., 1989), ζ (Weissman et al., 1986), η (Baniyash et al., 1988)), the γ chain of the Fc receptor (Orloff et al., 1990), etc. Since tyrosine phosphorylation is an initial and essential event in T cell activation prior to PKC activation (Kanner et al., 1992), the control of this pathway by co-aggregation and inclusion of different T cell proteins within the TCR complex is a primary mechanism by which TCR engagement can send a variety of signals which direct T cell activation and differentiation. The evidence for this is the observation that the formation of a coreceptor between TCR and CD8 or CD4 is able to move the TCR ζ chain close to CD8 and CD4 associated p56lck resulting in tyrosine phosphorylation (Burgess et al., 1992). The components included in the extended TCR complex during T cell activation may be dictated

by the arrangements of the ligands on antigen presenting cells, which binds to this extended TCR complex.

In addition, overlapping substrates in the T cell may account for signaling pathways common to all T cells, whereas subtle differences in kinase activity and substrate specificity may be involved in signaling unique selection, differentiation and activation events.

To investigate the minimal signals required for induction of $CD8^+$ suppressor T cells by intestinal epithelial cells, we have analyzed T cell activation and differentiation induced by the bifunctional antibody anti-CD3/CD8 as well as anti-CD3/CD28. Such a approach has helped us understand the mechanism of T cell activation.

*The Role of CD28 in the T Cell Activation*

Cross-linking CD3 and CD28 by heteroconjugate antibodies induces the proliferation of cytotoxic T cells (June et al., 1987b). In fact, CD28 is not expressed on the surface of suppressor T cells (Li et al., 1990). The T cell surface antigen CD28 is a heavily glycosylated disulfide-linked homodimer with a molecular weight of 44kD on SDS-PAGE (Hara et al., 1985). Each peptide of CD28 contains 202 amino acids and a 41 amino acid residue cytoplasmic domain. CD28 is expressed on 95% of $CD4^+$ T cells and 50% $CD8^+$ T cells and the level of the expression can be enhanced by T cell activation (Damle et al., 1983).

The CD28 molecule was initially defined by the mouse mAb 9.3, however, stimulation of CD28 by soluble bivalent mAb 9.3

alone did not provide sufficient signals for T cell proliferation and IL-2 production (Martin et al., 1986; Hara et al., 1985 and June et al., 1987b). Only with suboptimal stimulation by cross-linking TCR or PHA did CD28 stimulation can cause marked augmentation of T cell proliferation and IL-2 production. Cross-linking CD28 as well as CD3 can also trigger T cell proliferation. In contrast to CD4 and CD8, CD28 does not form complexes with TCR/CD3.

CD28 initiates or regulates a signal transduction pathway that is distinct from those stimulated by the TCR complex (June et al., 1987b). T cell activation by CD28 cross-linking is resistant to immunosuppressants, such as cyclosporin A, and is inhibited by the src protein tyrosine kinase inhibitor, herbimycin. Indeed, ligation of CD28 by a mAb or by its natural ligand B7/BB1, induces independent protein tyrosine phosphorylation in primed T cells, distinct from TCR-induced tyrosine phosphorylation (Vandenberghe et al., 1992). This tyrosine phosphorylation can be inhibited by cross-linking of CD28 and CD45. CD28 stimulation, acting via stabilization of IL-2 mRNA, facilitates activation and proliferation of T cells, which are suboptimally stimulated through TCR. The ability to induce the activation of cytotoxic T cells by CD3/CD28 bifunctional antibodies has helped us to dissect the mechanisms of cytotoxic T cell subset activation.

# Methods

*Cell Culture Medium*

Cell culture medium (CM) consisted of RPMI-1640 with 2mM glutamine (Gibco, Grand Island, NY), supplemented with 50 ug/ml penicillin (Gibco), 50 ug/ml streptomycin (Gibco) and 10% fetal calf serum (Gibco) Interleukin 2 was purchased from Boeringer Mannheim (Indianapolis, IN) and used at dilution of 100 U/ml.

*Cells*

HT-29 and DLD-1 were colon carcinoma cell lines obtained from American Type Culture Collection (ATCC). P815 was murine mastocytoma transfected with human $Fc\gamma$ receptor obtained from ATCC. The murine transfectant 3G4 and 3G8 expressing human CD4 and CD8 antigens respectively were kind gifts from Dr. S.J. Burakoff (Dana-Faber, Boston, MA) The construction of these hybridoma transfectants have been previously described. For construction of the 3G4 cell line (Sleckman et al., 1987), the murine T cell hybridoma BY155.16 was infected with the defective retrovirus, MNST4 carrying human CD4 cDNA. The 3G4 murine transfectant expressing human CD4 antigen was selected by sorting. The establishment of the 3G8 cell line was similar, transfecting BY155.16 with the retroviral expression vector MNCT8 carrying human CD8 cDNA (Ratnofsky et al., 1987).

*Isolation of Peripheral Blood T and Adherent Cells*

1) Rosetting technique: Heparinized venous blood was collected from normal donors. Blood was diluted 1:3 with sterile PBS (5.4mM KCl, 1.5mM $KH_2PO_4$, 140mM NaCl, 8mM $Na_2HPO_4$, pH 7.4), then layered on a Ficoll-Hypaque density gradient, and centrifuged for 30 min at 500g (Mayer et al., 1985). The mononuclear cells were collected from the interface and washed three times with PBS. Cells were resuspended in RPMI 1640 and cell density was adjusted to $5X10^6$ cells/ml. T cells were then separated using a rosetting technique. Mononuclear cells were mixed with neuraminidase treated sheep red blood cells. The rosetting occured overnight at 4°C. The total cell mixture was then applied to Ficoll-Hypaque density gradient centrifugation for 30 minutes at 500g. Rosetted T cells were in the pellet and non-T cells remained in the interface. Non-T cells from the interface were collected, washed three times with PBS and resuspended in CM. Adherent cells were obtained by incubating non-T cells in tissue culture dishes (25 million cells per dish in 10ml CM) for 45 min at 37°C in a humidified 5% $CO_2$ incubator. Then, B cell enriched non-adherent cells were aspirated and dishes were washed vigorously three times with PBS. Adherent cells were harvested by scraping cells off using a rubber policeman. Rosetted T cells were treated with 0.75% ammonium chloride on ice for 5-10 min to lyse sheep red blood cells. The cell lysis was stopped by addition of PBS buffer. The T cell suspension was then washed three times with PBS and finally resuspended in CM medium. Small amounts of contaminating adherent cells in the T cell suspension were eliminated by using the same adherence procedure described above. The staining of T cell preparations

showed >95% $CD3^+$, <1% $CD14^+$ and <1% $sIgG^+$.

2) Nylon wool purification technique: Heparinized venous blood was collected from normal donors and processed as described above. The mononuclear cells were collected and washed three times with PBS. Cells were resuspended in culture medium (RPMI-1640 with 10% fetal calf serum, Gibco, Grand Island, NY) at a concentration of 75 million cells/ml. 150 million cells were applied to a nylon wool column, loosely packed wih 1.2g scrubbed nylon wool fiber (Polysciences Inc., Warrington, PA). The nylon wool column was prewashed in 1% Duponal RA (Witco, New York, NY) at 65°C for one hour, and rinsed thoroughly with tap water and finally with distilled water for 30 minutes. The column was stored at 4°C following autoclaving and balanced with 20ml warm CM (37°C) before use. The cells applied to the column were incubated at 37°C for 45 minutes. Unbound cells, mostly T cells, were slowly eluted with 20ml warm CM and collected in a sterile tube. Cells were pelleted and adjusted to $10^6$/ml in CM. Flow cytometric analysis of nylon wool purified T cells revealed >97% $CD3^+$, <0.1% $CD20^+$, <0.1% $CD14^+$ and the absence of proliferation in response to PHA stimulation.

*Preparation of Human Intestinal Epithelial Cells*

The procedure used for the isolation of intestinal epithelial cells is a modification of the method used by Bull and Bookman for the isolation of lamina propria and intraepithelial lymphocytes (9). Resected tissue specimens removed from patients with colon cancer at least 10 cm away from the carcinoma (histologically normal)

were washed extensively with HBSS (Gibco) containing 50 ug/ml penicillin, 50 ug/ml streptomycin, 50 ug/ml gentamicin (Sigma, St. Louis, MO) and 2.5 ug/ml amphotericin B (Flow Laboratory, Inc., McLean, VA). The surface mucosa was stripped off from the underlying submucosa and minced into tiny pieces. These were placed in calcium/magnesium free HBSS (CMF-HBSS) containing antibiotics and 1mM dithiothreitol (DTT) (Sigma) and incubated in a 37°C water bath for 5 minutes to remove adherent mucus. The tissue pieces were washed again in CMF-HBSS and incubated in CMF-HBSS containing 3mg/ml dispase (Boehringer Mannheim, Indianapolis, IN) in a 37°C water bath for 30 minutes, vortexing every 5 minutes. During this treatment, epithelial cells and intraepithelial lymphocytes were released from the tissue. Cells which remained loosely adherent to the tissue were recovered using a rubber-policeman. Dispase treatment was repeated once. Isolated epithelial cells were washed twice with CMF-HBSS and then applied to Ficoll-Hypaque and Percoll density gradients to improve the viability and purity of the preparation. The purified epithelial cells were >95% viable, <0.1% esterase-positive, <0.1% $OKM1^+$, <0.1% $OKT6^+$, <1% $sIg^+$, and 1-2% $CD3^+$. Cells were usually irradiated 3000 rad and kept in RPMI-1640 containing 5% human agammaglobulinemic serum.

*Antibodies*

Monoclonal antibody (mAb) OKT8 (anti-CD8) and W6/32 (anti-HLA Class I) were obtained from ATCC. VG2 (anti-DR monomorphic determinant) was kind gift of Dr. Shu Man Fu (Univ. of Virginia,

Charlottesville, VA). FFB 2.3 (Anti-CD4) was a gift from Dr. David Posnett (New York Hospital, New York, NY) and mAb 446 (anti-CD3) was defined in this laboratory (Stohl et al., 1987). Monoclonal antibody (mAb) B9 and L12 against human intestinal epithelial cell membrane antigens were generated in our laboratory as previously described (Mayer et al., 1990). These two mAbs were chosen for their ability to specifically inhibit IEC driven $CD8^+$ T cell proliferation. mAb 9.3 (anti-CD28) was a gift from Dr. John Hansen.

Bifunctional antibodies anti-CD3/CD8 and anti-CD3/CD28 were provided by Dr. Alison Tutt in the Tenovus Research Laboratory (Southampton, UK). The method for generation the bifunctional antibodies anti-CD3/CD8 was as follows: murine mAb OKT3 (anti-CD3) and OKT8 (anti-CD8) both from the ATCC were digested with pepsin (Sigma, St. Louis, MO) at pH 4.2 in 0.1mM sodium acetate for 1 hour at 37°C. The $F(ab')_2$ fragments were isolated from the digest mixture by gel filtration on an Ultrogel AcA44 column (LKB Produkter, Bromma, Sweden). Bifunctional antibody containing mouse $F(ab)'_2$ from OKT3 and OKT8 were linked by connecting half-cysteine residues via thioester bonds using the bifunctional cross-linking agent, *o*-phenylenedimaleimide (*o*-PDM, Sigma, St. Louis, MO). Each $F(ab)'_2$ (10mg/ml) was first reduced by 2-mercaptoethanol (2-ME) for 30 minutes at 30°C to obtain Fab fragments then cooled down and kept at 4°C. A half volume of 12mM *o*-PDM dissolved in dimethylformamide was then added to one of the two murine Fab solutions and incubated at 4°C for 30 minutes. The maleimidated Fab was separated from solutes in the reaction mixture by passage through a Sephadex G-25 column. The

isolated maleimidated Fab was incubated with Fab(SH) antibody component of the heterodimer for 18 hours at 4°C. Then bifunctional $F(ab)'_2$ antibodies were separated from other products and residue reagents by passage through an Ultrogel AcA44 column. The composition of final bifunctional $F(ab)'_2$ products were examined by double immunodiffusion, SDS-PAGE and radioactive labelling analysis (Glennie et al., 1987).

*Metabolic Radiolabelling with Translabel $^{35}S$ Methionine/Cysteine*

The HT-29 cell line was cultured in the presence of IFN-γ (500 U/ml) for 24 hours, washed three times with PBS, starved for four hours in methionine/cysteine free RPMI 1640 medium containing 10% FCS (dialysed against PBS to remove methionine and cysteine), and then cultured for another six hours in the presence of Translabel $^{35}S$ methionine/cysteine (20 million cells/mCi$^{35}S$). After radiolabelling, cells were washed three times with PBS and gently scraped using a rubber-policeman. The cell pellet was collected and lysed in lysis buffer [3% NP-40, 0.5% SDS, 150mM NaCl, 20mM Tris-HCl, 1mM PMSF, 5mM Iodoacetamide, 20ug/ml leupeptin, 20ug/ml aprotinin, pH 7.5, (Sigma)] for 30 minutes on ice. The cell free lysate was precleared twice with 25ul 50% protein A sepharose (PAS) and 5ul affinity purified rabbit anti-mouse IgG (RAM) antibody for 60 minutes at room temperature. During this time, mAb coated beads were prepared for immunoprecipitation using affinity purified antibodies from ascites. mAb B9, L12 and W6/32 (anti-class I antigen) as well as an anti-DNP antibody of matched isotype (IgG1) were used in this process. The mixture of mAb, RAM and PAS beads

were rotated for two hours at room temperature. Coated beads were washed and used for immunoprecipitation. After pre-clearing, the lysate was equally aliquoted into individual mAb, RAM and PAS beads and rotated overnight at 4°C. Immunoprecipitates were washed six times with washing buffer (0.1% Triton X-100, 10mM Tris-HCl, 140mM NaCl, pH 8.0), and beads were resuspended in loading buffer and run in 7.5% SDS-PAGE over four hours. Specific bands were detected by autoradiography.

*Western Blotting*

All of the protein samples were run on reducing SDS-PAGE. The slab gel (with the stacking gel removed) was pre-equilibrated in 50ml degassed transfer buffer (25mM Tris, 192mM glycine, 20% methanol, 0.015% SDS, pH 8.3) for 10 min. Then, a "sandwich" was prepared (Towbin et al., 1979) with the following successive layers: i) a porous polyethylene foam sheet; ii) three thickness filter paper; iii) the SDS-PAGE slab gel; iv) a nitrocellulose sheet (0.2um) cut to the size of the gel; v) three more thicknesses of filter paper; and finally vi) another sheet of porous polyethylene foam. All components in contact with the gel were prewetted in the transfer buffer. The sandwich set was secured in a plastic frame and inserted into the transphor electrophoresis unit with the nitrocellulose sheet at the anode. The chamber was filled with transfer buffer and connected to a cooling system. The electrophoretic transfer was accomplished at 25V/cm (with respect to electrode separation) for six hours. Immediately following transfer, the nitrocellulose sheet was stained with Ponceau S

solution (2% Ponceau S, 6% TCA in $dH_2O$, all from Sigma) and rinsed with $dH_2O$ to develop the quantity of protein transblotted onto the paper. The paper was then immersed in the blocking solution (5% milk or 2% gelatin in PBS) and incubated at 37°C for one hour. The sheet was washed five times with washing buffer (0.1% Tween-20 in PBS) for five minutes each and sealed in a plastic bag containing 5ug/ml mouse monoclonal antibody in 0.5% milk-PBS buffer. Incubation took two hours at R.T. or overnight at 4°C. The sheet was washed five times with washing buffer for one minute each and incubated with biotinylated goat anti-mouse IgG diluted 1:500 in 0.5% milk-PBS buffer for 1-2 hours at room temperature. The sheet was again washed five times with washing buffer for five minutes each and incubated with alkaline phosphatase conjugated avidin solution (prepared 30 min before use) for 30 min at room temperature. The sheet was washed five times with washing buffer for one minute each and developed in substrate buffer (0.1mM Tris, pH 9.5) for 10-30 min until bands became clear. Developing was terminated by rinsing the nitrocellulose paper in distilled water. The sheet was stored in the dark. All of the incubations mentioned above were performed on a rocking platform.

*Production of Ascitic Fluid*

Balb/c mice (Jackson Laboratory) were pristane primed and injected with murine hybridoma cells (5 million cells/mouse) suspended in PBS. After approximately 5-10 days, ascites was tapped off and centrifuged for 10 minutes at 500g to remove any cell components. Clarified ascites was incubated at 56°C for 45

minutes to inactivate complement and then stored at -20°C. Ascites could be tapped off from each mouse several times at 1-2 days intervals. The isotype subclass of the monoclonal antibodies was determined by Ouchterlony double immunodiffusion and protein concentration was determined by measuring O.D.

*Affinity Purification of Monoclonal Antibodies*

This method was adopted from Harlow et al. (Harlow et al, 1988). Recombinant protein G (with its albumin domain deleted) sepharose beads were packed into a glass syringe. The column was washed with 200ml washing buffer (PBS plus 0.5M NaCl). Then 10 ml ascites dialysed against 10% 1M Tris, pH 8.0 was passed through the affinity column for two hours at room temperature. After absorption, the column was extensively washed with 300ml washing buffer. Then the monoclonal antibody was eluted with 30ml 0.1M glycine, pH 2.7. Eluates were collected in ten fractions and neutralized immediately in 1/10 volume of 1M Tris, pH 8.0. The protein concentration was determined by spectrophotometry. Fractions containing monoclonal antibodies were pooled, dialysed against distilled water for two days (three changes) and stored at -20°C. The yield of affinity purification could be calculated by comparing immunoglobulin concentration in purified form with one in ascites by ELISA. The column was rebalanced with 50ml washing buffer.

*Flow Cytometric Analysis*

Treated or untreated T cells were washed with PBS and placed

in V-bottomed microtitre cell plates (Becton Dickinson Labwear, Lincoln Park, NJ) ($2X10^5$ cells per well) in a volume of 100ul and stained with FITC-conjugated anti-IL-2 receptor (Becton Dickinson Labware, Lincoln Park, NJ) for 45 minutes on ice. Isotype matched mAbs were used in each experiment with no significant background staining. Cells were then washed with PBS three times and fixed with PBS-paraformaldehyde 1% solution (Fisher Scientific Company, Fair Lawn, NJ). The staining was analysed on a Coulter Profile II (Coulter, Hialeah, FL).

For staining of HT-29 cells with mAb B9, L12, B7/BB1, etc., enterocytes were washed in V-bottomed microtitre cell plates and stained with unconjugated mAb B9, L12, B7/BB1, etc. for 45 minutes on ice. The plates were washed with PBS three times and incubated with FITC conjugated $F(ab)'_2$ goat anti mouse IgG (TAGO, Burlingame, CA) (1:40) for 45 minutes on ice. The plate was washed three times with PBS. Cells were then resuspended in 200ul 1% formaldehyde in PBS and analysed by flow cytometry.

*Radioimmunoprecipitation and In Vitro Tyrosine Kinase Assay*

1) ($\gamma$-$^{32}$P)ATP labeling: Isolated peripheral blood T cells were incubated in an eppendorf tube either with normal intestinal epithelial cells, peripheral adherent cells (10 million T cells : 10 million stimulator cells) or with antibodies cross-linking CD4 or CD8 in a 37°C water bath for varying time periods. At the specific time point, the tube was transferred to ice and 2X NP-40 lysis buffer [2% NP-40, 40mM Tris base (pH 8.0), 300mM NaCl, EDTA 400uM, sodium pyrophosphate 20mM, sodium fluoride 200mM, 1mM PMSF,

5mM iodoacetamide, 20ug/ml aprotinin, 20ug/ml leupeptin and 200uM $Na_3VO_4$] (Sigma) was added. for 30 minutes, vortexing every 5 minutes. The lysate was centrifuged at 14,000 rpm for 10 minutes in a microfuge to remove cell debris. Lysate was then precleared with protein A sepharose (Pharmacia, Piscataway, NJ) (30ul 50% PAS per sample) for 30 minutes at 4°C. The clarified lysate was then incubated with 4G10 (mouse mAb anti-phosphotyrosine) (UBI, Lake Placid, NY) (5ug/ml) or anti-lck sera (gift of Dr. C.E. Rudd) for one hour at 4°C, added to PAS (50ul 50% PAS per sample) and incubated for 2 hours at 4°C. The immunoprecipitates were washed once with PBS, two times with 0.5M LiCl (Sigma) in 20mM Tris (pH 8.0) and once with kinase buffer (10mM $MnCl_2$, 50mM Tris, pH 7.4). The immunoprecipitates were finally resuspended in kinase buffer (30ul per sample), and mixed with ($\gamma$-$^{32}$P)ATP (Amersham, Arlington Heights, IL) (10uCi per sample). After an incubation of 30 minutes at 25°C, the reaction mixture was subjected to 10% SDS-PAGE and autoradiography.

For mAb blocking studies, we incubated T cells with OKT8 (10 ug/ml), or normal intestinal epithelial cells with B9 (10 ug/ml) or L12 (10 ug/ml) respectively at 4°C for 30 minutes. Unbound antibodies were removed by washing. Treated or untreated T cells were then incubated with treated, or untreated normal epithelial cells at 37°C for 2 minutes. Cells was lysed and p56lck enzyme activity were measured as described above.

2) Metabolic $^{32}$Pi labelling: T cells were washed with 0.9% NaCl twice and starved in phosphorus free RPMI 1640 plus 10% dialysed FCS for one hour at 37°C and then $^{32}$Phosphoros (DuPout NEN,

Boston, MA) was added for four hours at 37°C (1mCi $^{32}Pi$ per 10 million cells). The subsequent allogeneic and antibody cross-linking treatment and immunoprecipitation were the same as describeded above. The precipitations were applied to 7.5% SDS-PAGE and developed by autoradiography.

*Inhibition of T cell Proliferation in the Presence of Genestein*

$10^5$ adherent cell depleted T cells used as responder cells were cultured with $10^5$ irradiated (3000 rad) human intestinal epithelial cells or adherent cells as stimulators in 200ul culture medium, in the presence or absence of 200uM genestein (Calbiochem, La Jolla, CA) added at time 0 or 2 hours after the onset of culture. In all experiments one set of T cells was pretreated with genestein (200uM) for 2 hours followed by removal of genestein by washing and then added to cultures containing irradiated IECs or monocytes. All cultures were performed in triplicate in 96-well round-bottomed microtitre well plates (Becton Dickinson Labware, Lincoln Park, NJ) for 120 hours at 37°C in a 5% $CO_2$ humidified incubator. 1uCi[$^3$H] thymidine (ICN, Irvine, CA) was added during the last 18 hours of culture and cells were harvested onto glass fiber filter mats (Skatron, Sterling, VA) for counting. Counts were recorded by a scintillation counter (Model LS3801, Beckman Instruments, Somerset, NJ).

*Suppressor Assay*

The suppressor effect of bifunctional antibody stimulated T cells were assayed by co-culture of peripheral blood T cells ($10^5$

per well) in U-bottom microtitre plates containing PHA (1 ug/ml) (Gibco) in the presence of equal numbers of bifunctional antibody, anti-CD3 mAb (446) or medium as negative control activated T cells (irradiated 3000 rad) for 48 hours at 37°C. Cells were pulsed with 1 uCi [$^3$H] 18 hours before harvesting and $^3$H thymidine incorporation was then measured.

*Cytotoxicity Assay*

Murine P815 cells (from ATCC) transfected with human $Fc_\gamma$ receptors were used as targets in a chromium release assay. Cells were labelled with 100 uCi $^{51}$Cr (sodium chromate, Amersham, Arlington Heights, IL) per $10^6$ cells for 40 minutes and then washed and resuspended at $5X10^3$ cells/well in V-bottomed plates containing effectors (bifunctional antibodies, anti-CD3 mAb or medium as negative control activated T cells) at varying ratios in the presence of absence of mAb OKT3 (2 ug/ml). The plates were centrifuged briefly (200gX4 minutes) before incubation for 4 hours at 37°C. The culture supernatants were collected and $^{51}$Cr release was measured (Haywand et al., 1986). The spontaneous $^{51}$Cr release was less than 10%. Specific lysis was measured as

$$\% \text{ Cytotoxicity} = \frac{\text{Test release - Spontaneous release}}{\text{Maximum release - Spontaneous release}} \text{ X100}$$

The maximum release was determined by detergent NP-40 (0.1%) lysis of target cells.

# Human Intestinal Epithelial Cell Induced CD8+ T Cell Activation Is Mediated Though CD8 and the Activationof CD8-Associated p56lck

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Running Title: IEC Activation of CD8+ T Cells

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**Abbreviations:** IEC, intestinal epithelial cell; mAb, monoclonal antibody; PBT, peripheral blood T cell; TCR, T cell receptor; APC, antigen presenting cell.

Abstract

The activation of $CD8^+$ suppressor T cells by normal intestinal epithelial cells (IEC) in antigen-specific or allogeneic mixed cell culture systems has significant implications for the regulation of mucosal immune responses. In this study, we found that the capacity of epithelial cells to induce $CD8^+$ suppressor T cell activation appeared to be linked to the binding of CD8 molecules on the T cell surface. This appears to be mediated by a non-class I molecule expressed on the epithelial cell surface, which results in the activation of the CD8-associated src-like tyrosine kinase, $p56^{lck}$. Epithelial cell stimulated $p56^{lck}$ activation is an early event (in contrast to monocytes) and is essential for T cell activation since proliferation could be completely abrogated by pretreatment of T cells with genestein, a protein tyrosine kinase inhibitor. Pretreatment of T cells with anti-CD8 or IEC with an anti-epithelial cell mAb B9 inhibited $p56^{lck}$ activation and further confirmed that CD8 on T cell and a CD8 ligand on the epithelial cell were involved in this T cell activation event. The specificity of this reaction was confirmed in experiments where murine transfectants 3G4 and 3G8 were used, we showed that epithelial cells and monocytes both activated $p56^{lck}$ during T cell activation but they stimulated different surface molecules (CD4 or CD8) on T cell, and the time course of $p56^{lck}$ upregulation was also distinct. Although stimulation through CD8 and CD8-associated $p56^{lck}$ was important for epithelial cell induced T cell activation, we were not able to trigger T cell proliferation by cross-linking CD8 alone with monoclonal antibody anti-CD8. These data suggest that a second

signal is required for epithelial cell driven T cell proliferation.

Introduction

Over the past several years it has become increasingly clear that the rules that govern mucosal immune responses differ from those of the peripheral immune system. For one thing, antigen priming via the oral route most frequently results in the development of immunologic suppression or oral tolerance, not an active immune response. Secondly, the cell types in the gastrointestinal tract are not typical of what one would find systemically. Intraepithelial lymphocytes are predominantly $CD8^+$ T cells which by and large are anergic despite exposure to inordinate numbers of foreign antigens in the gut lumen (1-3). In contrast, lamina propria T cells are activated memory cells with limited-proliferative capacity to specific antigen, but a rich source of cytokines (4, 5). Understanding of the underlying mechanisms responsible for these phenomena has been slow to evolve. This is partly due to our limited knowledge of how the primary signal for an immune response, antigen, is handled by the GI mucosa. Our laboratory as well as others have focused on the role of the intestinal epithelial cell (IEC) as an antigen presenting cell (APC) in this system. We and others have demonstrated that IEC can take up, process, and present soluble antigens to primed T cells (6, 7). However, contrary to conventional APCs, normal IEC appear to selectively activate $CD8^+$ T cells. These activated $CD8^+$ cells express suppressor function without any evident cytolytic activity. In this study we define a mechanism whereby normal IEC can selectively activate $CD8^+$ T cells. By binding and crosslinking CD8 on T cells by a non class I ligand expressed on normal IEC, CD8 $\alpha$ chain associated

$p56^{lck}$ is activated. This activation is necessary, but not sufficient for $CD8^+$ T cell proliferation.

Methods

*Isolation of peripheral blood T cells and adherent cells*.

Heparinized venous blood was collected from normal donors, diluted 1:3 with sterile PBS, layered on a Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient, and centrifuged for 30 min at 500g (8). The mononuclear cells were collected from the interface and washed three times with PBS. Cells were resuspended in RPMI 1640 (Gibco, Grand Island, NY) and the cell density adjusted to $5X10^6$ cells/ml. T cells were separated using a rosetting technique. Using neuraminidase treated sheep red blood cells and Ficoll-Hypaque density gradient centrifugation, non-T cells from the interface were collected, washed three times with PBS and resuspended in culture medium, i.e. RPMI-1640 containing 10% fetal calf serum (Gibco), 50 ug/ml penicillin (Gibco), 50 ug/ml streptomycin (Gibco) and 2mM glutamine (Gibco). Adherent cells were obtained by incubating non-T cells in tissue culture dishes (25 million cells per dish in 10ml culture medium for 45 min at 37°C in a humidified 5% $CO_2$ incubator). Then, B cell enriched non-adherent cells were aspirated and dishes were washed vigorously three times with PBS. Adherent cells were harvested by scraping cells off using a rubber policeman. Rosetted T cells were treated with 0.75% ammonium chloride on ice for 5-10 minutes to lyse sheep red blood cells. The T cell suspension was then washed three times with PBS and finally resuspended in culture medium. Small numbers of contaminating adherent cells in the T cell suspension were removed by the adherence procedure described above. The purity of isolated T cell preparations was assessed by staining with resulting

$CD3^+$>95%. $CD14^+$<1% and $sIg^+$<1%.

*Preparation of human intestinal epithelial cells*

The procedure used for the isolation of intestinal epithelial cells is a modification of the method used by Bull and Bookman for the isolation of lamina propria and intraepithelial lymphocytes (9). Resected tissue specimens removed from patients with colon cancer at least 10 cm away from the carcinoma (histologically normal) were washed extensively with HBSS (Gibco) containing 50 ug/ml penicillin, 50 ug/ml streptomycin, 50 ug/ml gentamicin (Sigma, St. Louis, MO) and 2.5 ug/ml amphotericin B (Flow Laboratory, Inc., McLean, VA). The surface mucosa was stripped off from the underlying submucosa and minced into tiny pieces. These were placed in calcium/magnesium free HBSS (CMF-HBSS) containing antibiotics and 1mM dithiothreitol (DTT) (Sigma) and incubated in a 37°C water bath for 5 minutes to remove adherent mucus. The tissue pieces were washed again in CMF-HBSS and incubated in CMF-HBSS containing 3mg/ml dispase (Boehringer Mannheim, Indianapolis, IN) in a 37°C water bath for 30 minutes, vortexing every 5 minutes. During this treatment, epithelial cells and intraepithelial lymphocytes were released from the tissue. Cells which remained loosely adherent to the tissue were recovered using a rubber-policeman. Dispase treatment was repeated once. Isolated epithelial cells were washed twice with CMF-HBSS and then applied to Ficoll-Hypaque and Percoll density gradients to improve the viability and purity of the preparation. The purified epithelial cells were >95% viable, <0.1% esterase-positive, <0.1%

OKM1$^+$, <0.1% OKT6$^+$, <1% sIg$^+$, and 1-2% CD3$^+$. Cells were usually irradiated 3000 rad and kept in RPMI-1640 containing 5% human agammaglobulinemic serum.

*Murine transfectants 3G4 and 3G8*

The murine transfectants 3G4 and 3G8 expressing human CD4 and CD8 antigens respectively were kind gifts from Dr. S.J. Burakoff (Dana-Farber, Boston, MA) The construction of these hybridoma transfectants have been previously described. For construction of 3G4 cell line (Sleckman et al., 1987), the T cell hybridoma BY155.16 was infected with the defective retrovirus, MNST4 carrying human CD4 cDNA and transfectants expressing human CD4 antigen were selected by sorting. The establishment of 3G8 cell line was similar, transfecting the T cell hybridoma BY155.16 with the retroviral expression vector MNCT8 carrying human CD8 cDNA (10).

*Monoclonal antibodies*

OKT8 (anti-CD8) and W6/32 (anti-HLA Class I) were obtained from American Type Culture Collection (ATCC). VG2 was kind gift of Dr. Shu Man Fu (Univ. of Virginia, Charlottesville, VA). FFB 2.3 (Anti-CD4) was a gift from Dr. David Posnett (New York Hospital, New York, NY) and mAb 446 (anti-CD3) has been previously defined in this laboratory (23). Monoclonal antibody (mAb) B9 and L12 against human intestinal epithelial cell membrane antigens were generated in our laboratory as previously described (24). These two mAbs were chosen for their ability to specifically inhibit IEC driven CD8$^+$ T cell proliferation.

*Immunoprecipitation and In vitro tyrosine kinase assay*

Isolated peripheral blood T cell were incubated in an eppendorf tube either with normal intestinal epithelial cells, peripheral adherent cells (10 million T cells:10 million stimulator cells) or with antibodies cross-linking CD4 or CD8 in a 37°C water bath for varying time periods. At specific time point, tube was transferred to ice and cold 2X NP-40 lysis buffer [2% NP-40, 40mM Tris base (pH 8.0), 300mM NaCl, EDTA 400uM, sodium pyrophosphate 20mM, sodium fluoride 200mM, 1mM PMSF, 5mM iodoacetamide, 20 ug/ml aprotinin, 20 ug/ml leupeptin and 200uM $Na_3VO_4$] (Sigma) was added for 30 minutes, vortexing every 5 minutes. The lysate was centrifuged at 14,000 rpm for 10 minutes in a microfuge to remove cell debris. Lysate was then precleared with protein A sepharose (Pharmacia, Piscataway, NJ) (30ul 50% PAS per sample) for 30 minutes at 4°C. The clarified lysate was incubated with 4G10 (mouse mAb anti-phosphotyrosine) (UBI, Lake Placid, NY) (5ug per sample) or anti-lck sera (gift of Dr. C.E. Rudd) for one hour at 4°C, added to PAS (50ul 50% PAS per sample) and incubated for 2 hours at 4°C. The immunoprecipitates were washed once with PBS, two times with 0.5M LiCl (Sigma) in 20mM Tris (pH 8.0) and once with kinase buffer (10mM $MnCl_2$, 50mM Tris, pH 7.4). The immunoprecipitates were finally resuspended in kinase buffer (30ul per sample), and mixed with ($\gamma$-$^{32}$P)ATP (Amersham, Arlington Heights, IL) (10uCi per sample). After an incubation of 30 minutes at 25°C, the reaction mixture was subjected to 10% SDS-PAGE and autoradiography.

For mAb blocking studies, we incubated T cells with OKT8

(10ug/ml), or normal intestinal epithelial cells with B9 (10ug/ml) or L12 (10ug/ml) respectively at 4°C for 30 minutes. Unbound antibodies were removed by washing. Treated or untreated T cells were then incubated with treated, or untreated normal epithelial cells at 37°C for 2 minutes. Cells were lysed and p56lck enzyme activity were measured as described above.

Mixed cell culture responses in the presence of blocking mAbs

Allogeneic mixed cell culture reaction was performed as previously described (6) using $10^5$ irradiated intestinal epithelial cells as stimulators and $10^5$ allogeneic isolated T cells in the presence of mAb anti-CD4, -CD8, class I, class II or an unrelevant isotype matched Ab control (25 ug/ml for each mAb) in CM. All culture were performed in triplicate in 96 well round-bottomed microwell plates for 120 hours at 37°C in a 5% $CO_2$ humidified incubator. During the last 18 hours of culture, 1 uCi[$^3$H] thymidine (ICN, Irving, CA) were added and cells were harvested onto glass fiber filter mats for counting. Counts were obtained and averaged by a scintillation counter (model LS3801; Beckman Instruments, Somerset, NJ).

*Inhibition of T cell proliferation in the presence of genestein*

$10^5$ adherent cell depleted T cells used as responder cells were cultured with $10^5$ irradiated (3000 rad) human intestinal epithelial cells or adherent cells as stimulators in 200ul culture medium, in the presence or absence of 200uM genestein (Calbiochem, La Jolla, CA) added at time 0 or 2 hours after the onset. In all experiments

one set of T cells was pretreated with genestein (200uM) for 2 hours followed by removal of genestein washing and then added to cultures containing irradiated IECs or monocytes. All cultures were performed in triplicate in 96-well round-bottomed microtitre well plates (Becton Dickinson Labware, Lincoln Park, NJ) for 120 hours at 37°C in a 5% $CO_2$ humidified incubator. 1uCi[$^3$H] thymidine (ICN, Irvine, CA) was added during the last 18 hours of culture and cells were harvested onto glass fiber filter mats (Skatron, Sterling, VA) for counting. Counts were recorded by a scintillation counter (Model LS3801, Beckman Instruments, Somerset, NJ).

*Results*

1. *Binding to CD8 on T cells is required for normal intestinal epithelial cell induced CD8+ suppressor T cell proliferation.* We have previously demonstrated that freshly isolated normal human intestinal epithelial cells were able to trigger $CD8^+$ suppressor T cell proliferation in a mixed cell culture system (6). However, the mechanism of the generation of these antigen non-specific $CD8^+$ suppressor T cells was not addressed. To examine the role of specific surface molecules involved in this T cell activation event, we cultured T cells with varying concentrations of either mAb anti-CD4, anti-CD8, anti-class I MHC or anti-class II MHC. As well documented, mAbs against CD4 have the capacity to inhibit $CD4^+$ T cell activation and proliferation (25). Thus by employing anti-CD4, we would be able to highlight any T-T interaction. As seen in figure 1, antibodies to CD4 failed to inhibit $CD8^+$ T cell proliferation induced by intestinal epithelial cells at any concentration. However, $CD8^+$ T cell proliferation could be inhibited by anti-CD8 suggesting that the CD8 molecule itself may be involved directly in this process. Of note was the finding that the same anti-CD8 mAb failed to inhibit the proliferation of isolated $CD8^+$ T cells to PHA (data not shown) suggesting that the effects seen with this mAb had a direct effect on the interaction of epithelial cells and T cells and was not due to nonspecific suppression of $CD8^+$ T cells.

Interestingly, mAbs to class I, the conventional ligand for CD8, failed to inhibit T cell proliferation. In fact the pattern of proliferation was comparable to that seen in anti-CD4. In contrast, anti-class II mAb VG2 was capable of inhibiting T cell proliferation

comparable to anti-CD8.

Since T cell activation can be inhibited by anti-class II Abs present in the culture wells, we pretreated the epithelial cells with the anti-class II mAb VG2 for 30 minutes at 4°C, washed them free of unbound antibody, and used them as stimulators in MLR cultures of isolated $CD4^+$ or $CD8^+$ T cells as responders. Pretreatment with class II mAbs resulted in the inhibition of $CD4^+$ but not $CD8^+$ T cell proliferation. These data suggest that our initial findings relating to inhibition by a polyclonal anti-class II antibody were due to inhibition of T cell activation by this antibody. Furthermore, the finding of $CD8^+$ T cell proliferation in the presence of anti-CD4, anti-class I and anti-class II mAbs provides support for an alternate pathway, potentially a novel ligand for CD8, which may be activating these cells. The increase in $^3H$ incorporation seen in the anti-CD4 and anti-class I mAb treated cultures was reproducible and may represent enhanced access of CD8 to a novel ligand.

2. *Activation of $p56^{lck}$ induced by normal intestinal epithelial cells and monocytes.* Recent findings indicate that CD4 and CD8 molecules serve not only to enhance the weaker binding of the T cell receptor to the MHC/Ag complex on antigen presenting cells but also to transduce signals themselves to T cells through binding to their respective ligands. Although CD4 and CD8 intrinsically do not possess kinase activity, their intracytoplasmic tails are physically associated with the src-like protein tyrosine kinase, $p56^{lck}$ (11, 12). Cross-linking CD4 or CD8 by monoclonal antibodies has been shown to induce $p56^{lck}$ activation. In the experiment shown in figure 2,

we investigated the activation of $p56^{lck}$ protein tyrosine kinase activity after culturing T cells with normal intestinal epithelial cells or monocytes for 2 minutes. Under reducing conditions, the unstimulated T cell lane shows the basal level of $p56^{lck}$ activity. Cross-linking CD4 with anti-CD4 mAb, culture with normal intestinal epithelial cells, or monocytes results in a marked increase in $p56^{lck}$ enzyme activity. No detectable $p56^{lck}$ activity was seen in the stimulator cells alone, i.e. normal epithelial cells or monocytes. These studies imply that during co-culture of T cells with normal epithelial cells or monocytes, $p56^{lck}$ is triggered and activated as an early event.

3. *The pathway of activation of $p56^{lck}$ induced by normal epithelial cells and monocytes is through CD4 and CD8 molecules respectively.* Although stimulation through either CD4 or CD8 molecules can activate $p56^{lck}$, other surface molecules (e.g. CD2) have been reported to have the capacity to activate $p56^{lck}$. To assess the specific molecules involved in a $p56^{lck}$ activation by normal epithelial cells and monocytes, we tested two transfected murine T cell hybridomas expressing either human CD4 (3G4) or CD8 (3G8) molecules. 3G4 and 3G8 cell lines were cocultured with normal epithelial cells or monocytes and $p56^{lck}$ enzyme activity was measured. From figure 3A, one can clearly see that normal epithelial cells activate $p56^{lck}$ in the 3G8 cell line, but not in the 3G4 cell line. In contrast, monocytes activate $p56^{lck}$ in both 3G4 and 3G8 cell lines (figure 3b), although stimulation is much greater in the CD4 expressing line. Which 3G8 appears to be more vigorous

on its response to any stimulators (mAb or IEC) probably due to more expression of CD8 on 3G8 than expression of CD4 on 3G4, the difference in monocytes vs IEC is clear. In the case of epithelial cell stimulation, no detectable upregulation of $p56^{lck}$ enzyme activity in 3G4 cells in comparison with unstimulated background whereas more than 10 times increased $p56^{lck}$ enzyme antivity was shown in 3G8 cells. In the case of monocyte stimulation, besides marked enhanced $p56^{lck}$ enzyme activity in 3G4 cells we only measured about 4 times increase of p56lck in comparison with unstimulated background (the background was higher in this experiment, however, the maximum activation of p56lck was still able to be reached by cross-linking CD8 with antibodies). Since the only human surface molecules expressed on these transfectants were either CD4 or CD8, there data support the concept that IEC express a molecule (non-class I) which binds to CD8 to activate $p56^{lck}$.

4. *Activation of $p56^{lck}$ occurs early and is critical for $CD8^+$ T cell proliferation.* In order to determine the kinetics of $p56^{lck}$ activators, normal intestinal epithelial cells were incubated with T cells for variable time periods, lysed and $p56^{lck}$ activity measured. In figure 4A, epithelial cell stimulated $p56^{lck}$ activation can be seen maximally within 1 minute, persists for 5 minutes and declines at 10 minutes. After 30 minutes, the enzyme activity of $p56^{lck}$ is nearly base line. In contrast to epithelial cells, monocyte stimulated T cell associated $p56^{lck}$ activity increases gradually reaching a maximum at about 30 minutes and maintaining the activated state for up to 2 hours (figure 4B). This differences in kinetics of $p56^{lck}$

enzyme activity induced by normal intestinal epithelial cells versus monocytes may reflect the different functional requirements of $p56^{lck}$ in the activation of different T cell populations.

5. *Genestein, a tyrosine kinase inhibitor, inhibits both normal epithelial cell and monocyte stimulated peripheral blood T cell proliferation.* In order to test whether $p56^{lck}$ activation is critical for $CD8^+$ T cell proliferation, we cultured T cells with normal enterocytes or monocytes in the presence of varying concentration of genestein, a tyrosine kinase inhibitor. Since the kinetics of $p56^{lck}$ activation was different depending upon the stimulator cells, we added genestein early (2 hours prior to culture), at the onset of culture, or 2 days after culture. Preincubation of T cells with genestein for 2 hours resulted in complete inhibition of T cell proliferation induced by enterocytes (figure 5A), while genestein added at the onset of culture of T cells and epithelial cells, only resulted in 50% inhibition. No inhibition of T cell proliferation was seen when genestein was added 2 days after the onset of culture suggesting that inhibition of an early event was critical (data not shown) and genestein's effects were not mediated via non-specific toxicity. In contrast, monocyte stimulation of T cells was inhibited regardless of when genestein was added, consistent with the kinetic data (figure 5B). Preincubation of T cells with genestein allows for the complete inhibition of intracytoplasmic tyrosine kinases, so that the block of an early response to epithelial cell stimulation indicates that the initial tyrosine kinase activation is critical for normal epithelial cell driven T cell proliferation. Activation of tyrosine

kinases is also important in monocyte driven T cell proliferation, but appears to require both early and ongoing events. Therefore, the pathways promoting T cell proliferation used by normal epithelium and monocytes appear to be different.

6. *The activation of CD8/p56$^{lck}$ is necessary but not sufficient for normal intestinal epithelial cell driven T cell proliferation.* Given the findings described above, we wanted to determine whether stimulation through CD8 alone was sufficient to drive T cell proliferation. In this experiment, we cultured PBMC with mAb OKT8, 446 (anti-CD3), L12 (IgG1 negative control) or PHA in the presence or absence of IL-2 for two days. Monoclonal antibody 446 (anti-CD3) was able to stimulate T cell proliferation in the absence of IL-2 (figure 6). In contrast, mAb OKT8 (anti-CD8) could not stimulate T cell proliferation even in the presence of IL-2 despite the fact that p56$^{lck}$ activation was seen (figure 2). These results indicate that signals through CD8 alone are not sufficient to drive T cell proliferation and raises the possibility of the requirement for a second signal.

7. *The activation of p56$^{lck}$ induced by normal epithelial cells is blocked by monoclonal antibody anti-CD8 and B9.* Since mAb anti-CD8 could block epithelial cell stimulated CD8$^+$ T cell proliferation whereas mAb anti-class I antigen, the conventional ligand for CD8, could not block, we employed anti-epithelial cell mAbs to define the ligand for CD8 expressed on epithelial cells. mAbs B9 and L12 were selected for their ability to inhibit normal epithelial cell

induced $CD8^+$ T cell proliferation when present in the culture. B9 stains intestinal epithelium from all sites with equivalent crypt and villus epithelial staining, with the exception of the esophagus, and fails to stain T cells, B cells or monocytes. L12 stains intestinal epithelium more intensively than B9 and also fails to stain PBMC. To determine whether the activation of $p56^{lck}$ in $CD8^+$ T cells induced by normal epithelial cells is through the binding of a novel CD8 ligand on epithelial cells to CD8 on T cells. we used mAb OKT8, and anti-epithelial cell mAbs B9 and L12 to block $p56^{lck}$ activation. Figure 7 shows that OKT8 and B9 can inhibit $p56^{lck}$ activation, while L12 can not. These findings suggest that mAb B9 might recognize a ligand for CD8 on epithelial cell surface or at least be situated near such a ligand.

Discussion

The fact that oral tolerance is mediated via the generation of T suppressor cells is well established (13, 14). The mechanism of activation of such cells is still not defined. In this study we demonstrate that $CD8^+$ suppressor T cells activated by normal IEC require binding to the CD8 molecule itself. In this setting, crosslinking CD8 activates the src-like tyrosine kinase, $p56^{lck}$. This, in turn, may phosphorylate a number of substrates eventuating in nuclear signalling for cell proliferation. Positive activation of cells through CD8 is in contrast to studies by van Seventer et al. (15) who reported that anti-CD8 mAbs could inhibit cytotoxicity of CTL clones. Although we have not found anti-CD8 mAbs to be inhibitory to $CD8^+$ T cell proliferation induced by mitogens, the results from van Seventer et al are not incongruent with ours. In the first case, we are analyzing a distinct phenomenon, suppressor T cell activation. Secondly, we are dealing with a ligand for CD8 expressed on IEC which is not class I, since anti-class I mAb treatment of IEC failed to inhibit $CD8^+$ T cell proliferation. Third, recent studies suggest that the inhibitory effect mediated by soluble anti-CD4 or anti-CD8 interferes with co-receptor formation with the TCR/CD3 complex (16, 17, 18). Furthermore, we are not dealing with a T cell:T cell interaction (CD4/CD8) since antibodies to CD4 also fail to inhibit $CD8^+$ T cell proliferation. In fact, an interesting consistent observation seen is the finding of enhancement of $CD8^+$ T cell proliferation in the presence of anti-CD4 and anti-Class I mAbs. This may relate to an enhanced ability of CD8 molecules to interact with their non-class I ligand (in the case of anti-class I) promoting

a greater response in the $CD8^+$ T cells. In the setting of anti-CD4 treatment, there may be an increase in the ability of $CD8^+$ rather than $CD4^+$ T cells to interact with IEC. Regardless, the end result is a positive growth signal transmitted to $CD8^+$ cells.

The nature of the positive signal to $CD8^+$ T cells appears to be the activation of $p56^{lck}$. Several laboratories have described a src-like tyrosine kinase, $p56^{lck}$, associated with the intracytoplasmic tail of CD4 and the CD8 $\alpha$ chain (11, 12). Crosslinking of these molecules with antibody results in induction of kinase activity with autophosphorylation as well as phosphorylation of a number of associated substrates which also regulate signals from TCR stimulation (21, 22). Conceivably, different binding to CD4 or CD8 may alter the activation and substrate phosphorylation seen. In addition, the kinetics of $p56^{lck}$ activation is critical, since as the multiple substrates move closer to $p56^{lck}$ at varying times, patterns of phosphorylation and signalling may induce specific cellular activation. In the studies presented here, $p56^{lck}$ is activated within 1' of T cell:IEC co-culture, much more rapid than the induction of $p56^{lck}$ by a more conventional APC, monocytes. Interestingly, this activation is inhibited by antibodies to CD8 providing further evidence that the CD8 molecule itself plays a critical role in this process. Lastly, the activation of this tyrosine kinase is critical for the subsequent $CD8^+$ T cell proliferation since treatment of the cells with a tyrosine kinase inhibitor, pre-co-culture, inhibits their ability to proliferate.

It is especially of interest with regard to IEL. Several groups have reported that the CD8 expressed on a major proportion of IEL

is of the αα homodimeric form as opposed to the αβ heterodimeric form seen in peripheral $CD8^+$ T cells (19, 20). Since $p56^{lck}$ is associated with the CD8α chain, it is intriguing to postulate that a double dose of $p56^{lck}$ in cells living adjacent to intestinal epithelial cells (putatively bearing an activating ligand for such CD8 molecules), may account for the activated yet relatively anergic state of IELs described.

However, crosslinking CD8 and activation of $p56^{lck}$ alone is not sufficient for $CD8^+$ T cell proliferation. In the absence of IEC, anti-CD8 mAbs, in the presence or absence of cytokines, failed to promote cell proliferation. Therefore, it appears that a second signal, possibly through the TCR (since our initial studies related to either antigen specific or alloreactive responses) must be required. Our recent studies using bifunctional mAbs have addressed this issue.

Perhaps the most compelling data underscoring the dichotomy between the peripheral and mucosal immune systems comes from the studies utilizing the murine T cell hybridomas transfected with either genes encoding human CD4 or CD8 α. Monocytes stimulate $p56^{lck}$ activation in CD4 transfectants whereas IEC stimulate $p56^{lck}$ in CD8 transfectants. These findings suggest that although IEC express class II molecules, they are either expressed at such a low level to prevent sufficient CD4 binding or, alternatively, as has been suggested in the mouse, are aberrantly expressed (i.e. abnormal form) incapable of binding to CD4 effectively. In either case, such a scenario compromises the interaction of IEC with $CD4^+$ T cells and promotes the interaction of IEC with $CD8^+$ T cells.

The existence of suppressor T cells has been controversial. Direct activation of such cells has been fraught with difficulties. Perhaps given the nature of the mucosal immune system, the gut is the only environment where suppression can be reproducibly induced.

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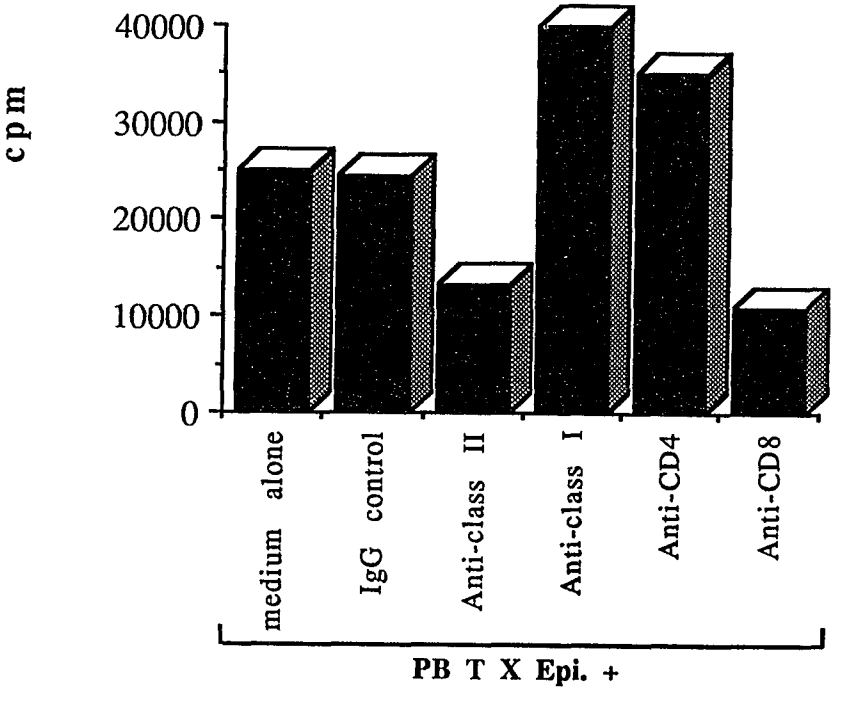



**Figure 1. Inhibition of epithelial cell stimulation of PBT proliferation by specific monoclonal antibodies.** Freshly isolated intestinal epithelial cells were cultured with peripheral blood T cells at ratio 1:1 in triplicate cultures in 96 well U-bottomed plates in the presence of either mAb anti-CD4, anti-CD8, anti-class I, anti-class II or a isotype matched control mAb at 25 ug/ml for 120 hours. $^{3}H$ labeled thymidine was added 18 hours before harvest and thymidine incorporation was measured.

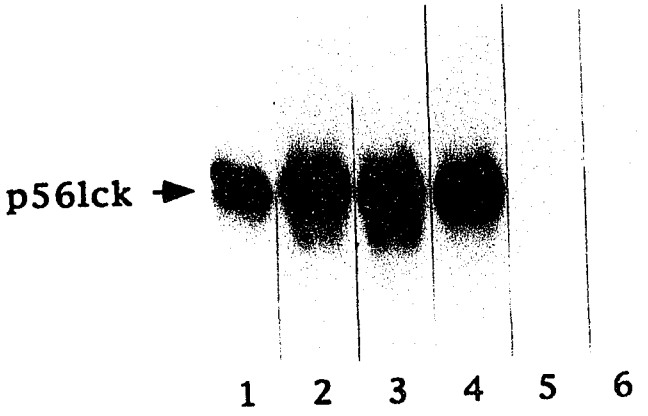
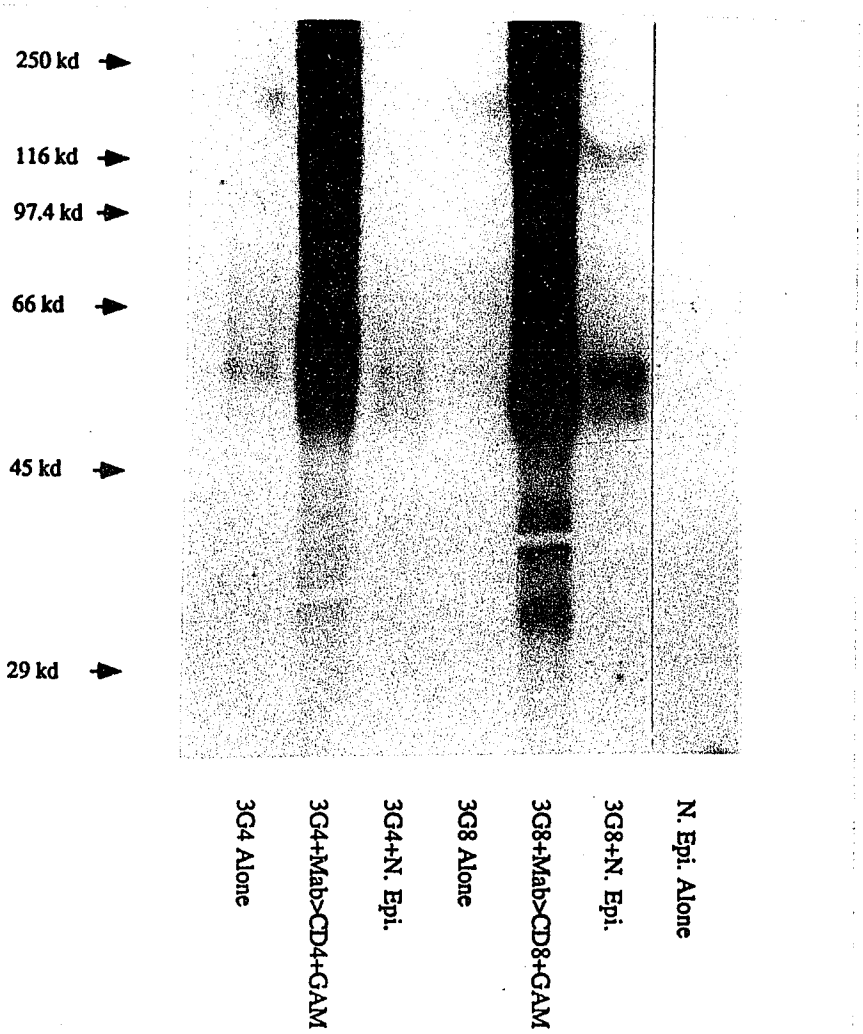
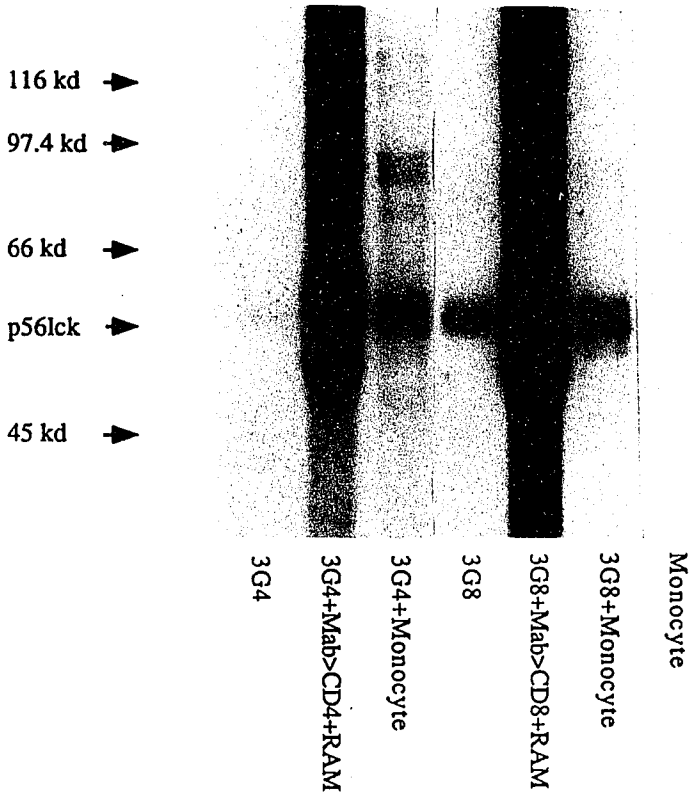



**Figure 2. Determination of $p56^{lck}$ enzymatic activity in activated peripheral blood T cells using an in vitro kinase assay.** Peripheral blood T cells (10 million cells each) stimulated with cross-linked anti-CD4 mAb (for 1 minute), normal human intestinal epithelial cells or monocytes (for 2 minutes) were lysed, immunoprecipitated with anti-lck antibody, and autophosphorylated in the presence of ($\gamma$-$^{32}$P)ATP. Total reactants were analyzed in 10% SDS-PAGE and exposed on film. Lane 1, unstimulated T cells; Lane 2, cross-linked anti-CD4 activated T cells; Lane 3, enterocyte activated T cells; Lane 4, monocyte activated T cells; Lane 5, enterocytes only; Lane 6, monocytes only.

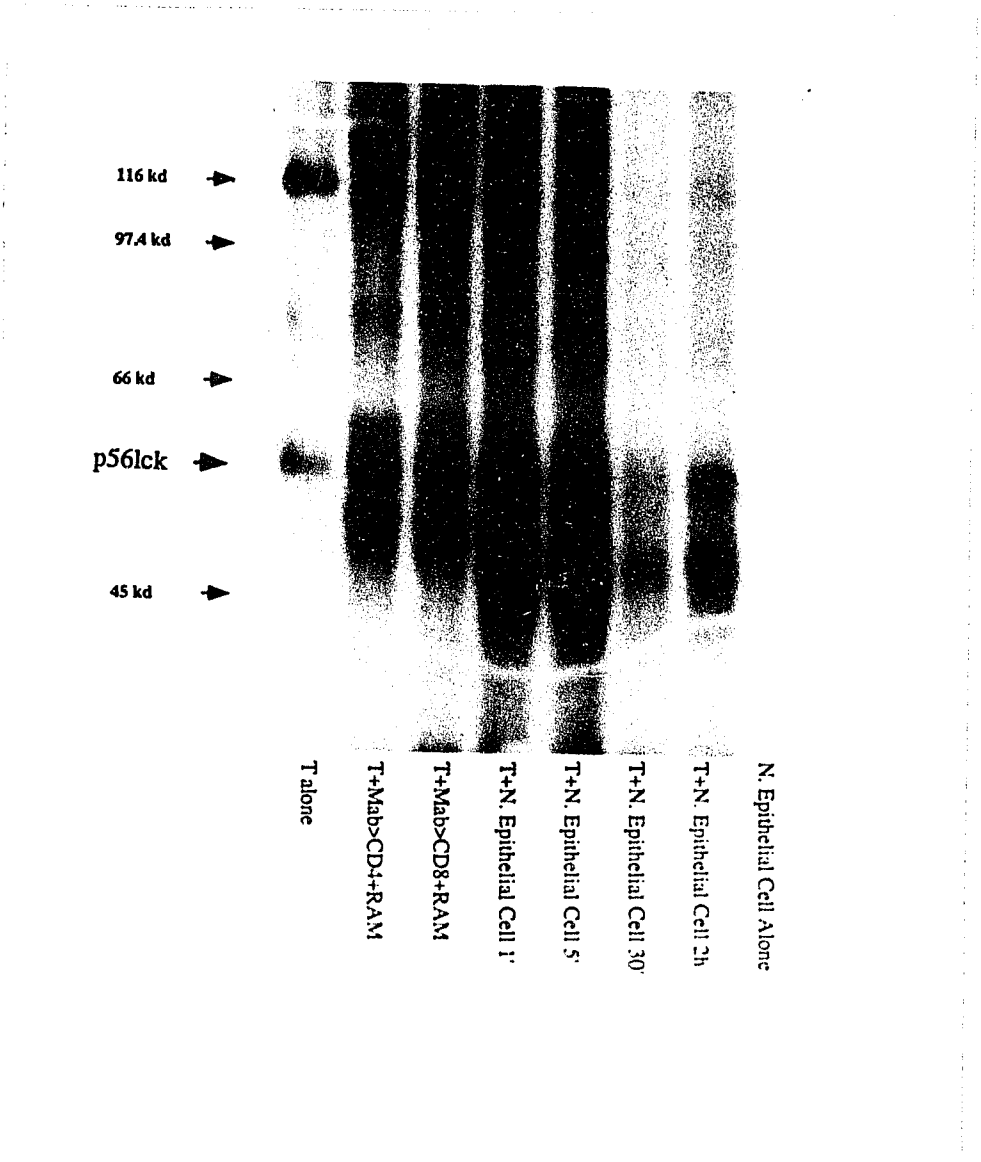



3(A)

**Figure 3. Analysis of T cell surface molecules responsible for $p56^{lck}$ activation induced by intestinal epithelial cells.** (A) Murine T cell hybridomas transfected with human CD4 or CD8 cDNA (3G4 and 3G8) respectively were used as responder cells in co-culture with human intestinal epithelial cells or with anti-CD4 or anti-CD8 mAb as positive control. The level of $p56^{lck}$ enzymatic activity was measured in an in vitro protein tyrosine kinase assay. The position of $p56^{lck}$ is depicted by the arrow. (B) Murine transfectants 3G4 and 3G8 were stimulated by monocytes or with an appropriate mAb (anti-CD4 or anti-CD8 respectively) as positive control and the activation of $p56^{lck}$ was determined.

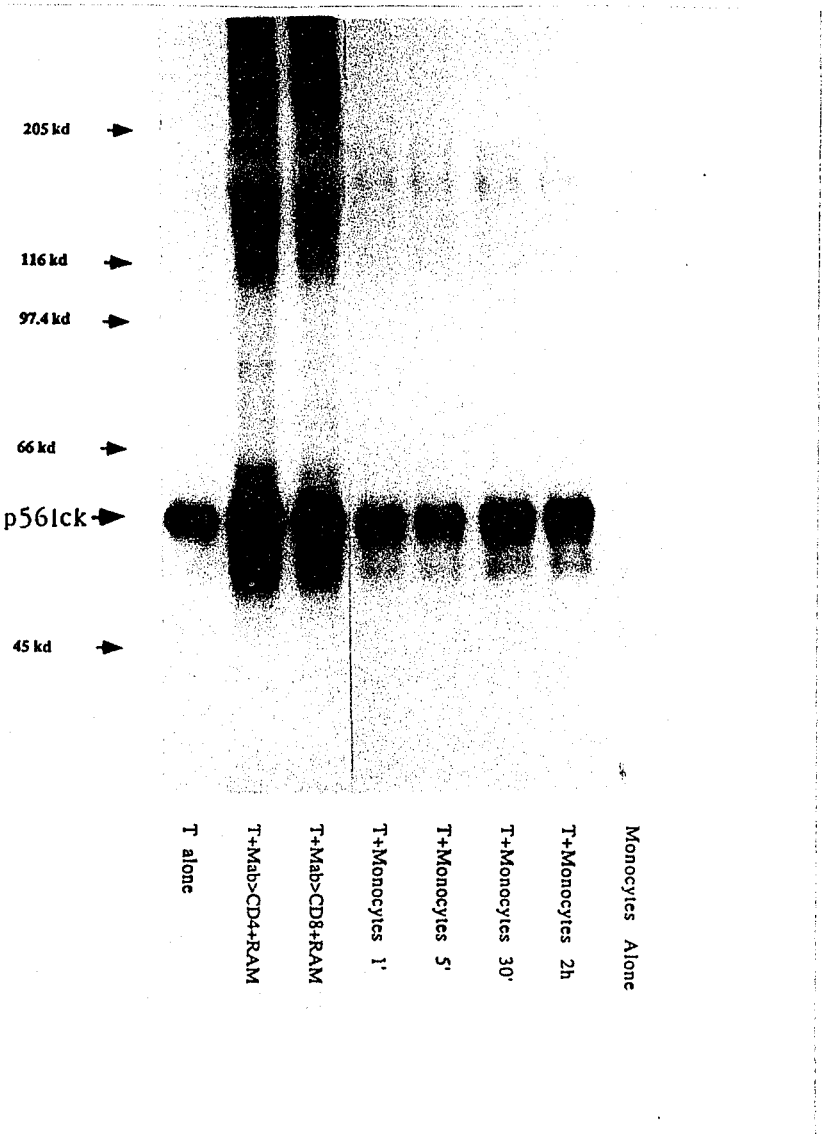

116 kd
97.4 kd
66 kd
p56lck
45 kd
3G4
3G4+Mab>CD4+RAM
3G4+Monocyte
3G8
3G8+Mab>CD8+RAM
3G8+Monocyte
Monocyte

**3(B)**

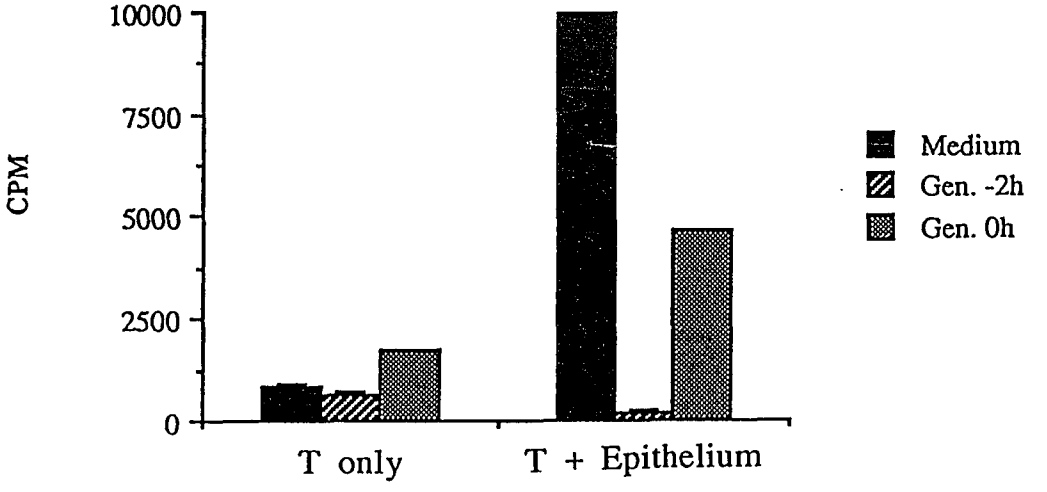



4(A)

**Figure 4. Time course of tyrosine autophosphorylation of $p56^{lck}$ activated by intestinal epithelial cells (A) and monocytes (B).** Peripheral blood T cells were co-cultured with epithelial cells (A) or monocytes (B) for varying time periods. The reaction was stopped by adding cold 2X lysis buffer, lysate was immunoprecipitated with anti-lck antibody, and then $p56^{lck}$ tyrosine kinase activity was measured in an in vitro tyrosine kinase assay. Cross-linking with anti-CD4 or anti-CD8 served as positive controls. The arrow depicts $p56^{lck}$.

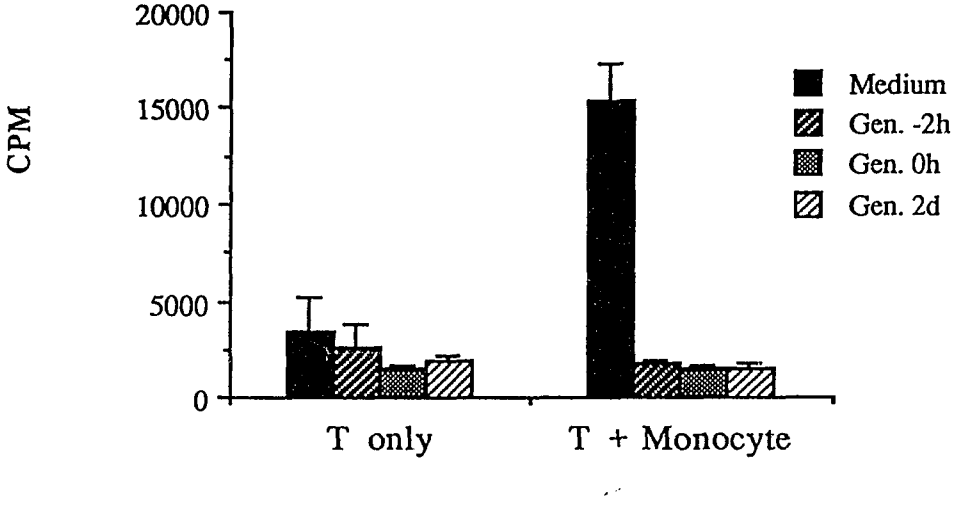

205 kd
116 kd
97.4 kd
66 kd
p56lck
45 kd
T alone
T+Mab>CD4+RAM
T+Mab>CD8+RAM
T+Monocytes 1'
T+Monocytes 5'
T+Monocytes 30'
T+Monocytes 2h
Monocytes Alone

**4(B)**




5(A)

**Figure 5. Inhibition of intestinal epithelial cell stimulated T cell proliferation in the presence of a protein tyrosine kinase inhibitor, genestein.** (A) T cells treated or untreated with genestein (200uM) 2 hours prior to or at the onset of culture were incubated with irradiated intestinal epithelial cells for 5 days. $^{3}H$ thymidine was added 18 hour prior to cell harvesting and thymidine incorporation was measured in a scintillation counter. (B) T cells treated or untreated with genestein (200uM) 2 hours prior to, at time 0, or 2 days after culture were incubated with irradiated monocytes for 5 days and $^{3}H$ thymidine incorporation was measured.


CPM
20000
15000
10000
5000
0
T only
T + Monocyte
Medium
Gen. -2h
Gen. 0h
Gen. 2d

**5 (B)**

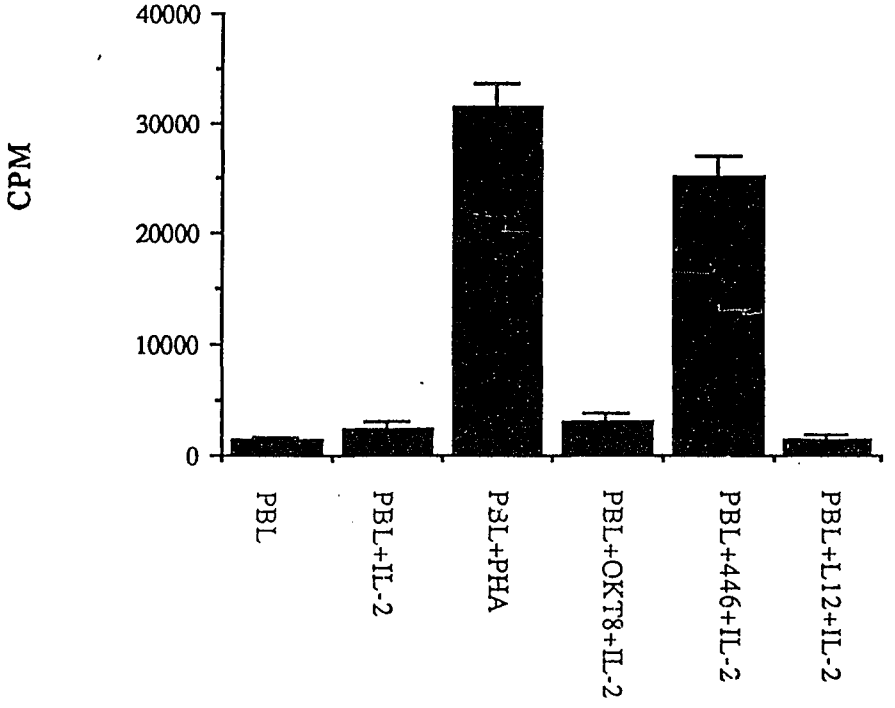



**Figure 6. T cell proliferation in response to monoclonal antibody stimulation.** PBMC containing T cells, B cells, and monocytes were cultured with either monoclonal antibody anti-CD8 (1 ug/ml), 446 (anti-CD3, 1 ug/ml), IgG1 (mAb L12) (1 ug/ml) as a negative control or 1 ug/ml PHA in the presence or absence of IL-2. The cell proliferation was measured by $^{3}H$ thymidine incorporation on day 3.

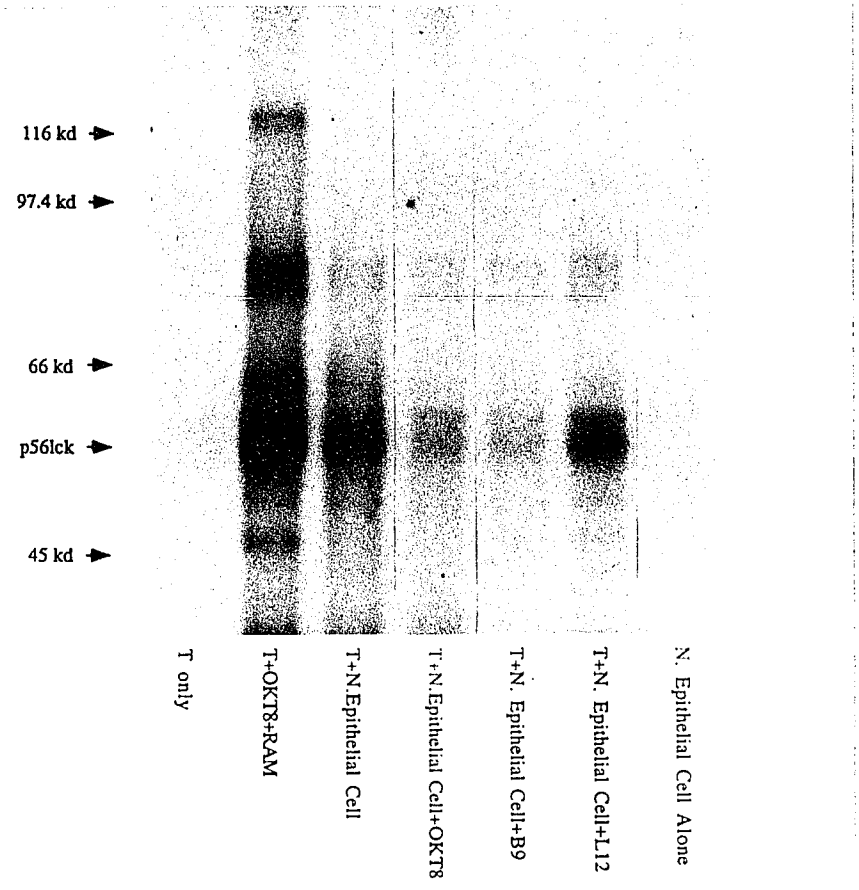



**Figure 7. Inhibition of intestinal epithelial cell induced p56lck activation in peripheral blood T cells by monoclonal antibodies.** Intestinal epithelial cells were treated with monoclonal antibody B9 or L12, or T cells were treated with monoclonal antibody OKT8 (anti-CD8), respectively, for 30 minutes at 4°C, and unbound antibodies were removed by washing. Treated or untreated epithelial cells were co-cultured with treated or untreated T cells, and the induction of p56lck enzymatic activity immunoprecipitated by anti-lck antibody was measured in an in vitro tyrosine kinase assay.

# Complementary Results

1. *Detection of mAbs B9 and L12 recognized molecules by radiolabelled immunoprecipitation.* Since mAb B9 and L12 generated in our laboratory have the capacity to block human intestinal epithelial cell driven peripheral blood T cell proliferation, mAb B9 and L12 recognized surface components on epithelial cells might be involved in epithelial cell:T cell interactions. In order to identify these surface molecules, we labelled HT-29 cells with Translabel $^{35}S$ methionine/cysteine (20 million cells/mCi$^{35}S$) for six hours. Cells were then harvested, lysed and immunoprecipitated with either mAb B9, L12, W6/32 (anti-class I) and an irrelevant mAb (IgG1) as a negative control. Precipitates were resolved on 7.5% SDS-PAGE and bands were determined by autoradiography. From figure 1, the band (arrow b) precipitated by mAb L12 had a molecule weight around 43kd, which was slightly lower than 44kd class I antigen precipitated by mAb W6/32. mAb B9 precipitated two bands. The upper darker band had molecular weight around 55kd and the lower lighter band 53kd. There were two explanations for these results, first was that the upper darker band was recognized by mAb B9 and the lower and lighter was coprecipitated (associated) with the upper band or second was that mAb recognized components had two chains and under reducing condition these two chains were dissociated.

2. *Western blot analysis of mAbs B9 and L12 recognized epithelial components.* HT-29 cells were lysed in 3% NP-40 plus

0.5% SDS lysis buffer and run on 10% SDS-PAGE, and then transblotted onto nitrocellulose membrane. Bands were detected by incubation with either mAb B9, L12 or an irrelevant mAb (IgG1), followed by an alkaline phosphatase conjugated second antibody and developed in the presence of substrate. In the L12 lane (figure 2), the band at 43kd (arrow) was only precipitated by mAb L12 and the band was not seen on other two lanes. This mAb L12 recognized band had the similar molecular weight with the band precipitated from metabolically labelled HT-29 cells shown in figure 1. No specific band was found on mAb B9 lane. This failure to detect a mAb B9 recognized protein might be due to the relatively low resolution of the western blot, or the fact that the mAb only recognizes its antigen in its native form.

3. *Regulation of surface molecule expression on HT-29 cells by IFN-γ.* From results described earlier, the two epithelial cell molecules recognized by mAbs B9 and L12 were candidates for a CD8 ligand. To determine the regulation of expression of these two molecules by IFN-γ, we cultured HT-29 cells with recombinant IFN-γ (500 u/ml) for varying time periods. Cells were then stained and analysed by flow cytometry. After 24 hours of treatment with IFN-γ, we could clearly see the upregulation of class II antigen expression on HT-29 cells. However, no significant change of B9 and L12 expression were seen. The density of mAb B9 and L12 staining was much lower than mAb W6/32 (anti-class I) and VG2 (anti-class II). This supported the notion that mAb B9 and L12 recognized two molecules which were distinct from HLA class I and II antigens.

4. *Detection of protein phosphorylation induced by intestinal epithelial cells.* Results described in the adjoining papers have shown that although both epithelial cells and monocytes activate $p56^{lck}$, the stimulation pathway (through CD8 or CD4 respectively) as well as the kinetics of upregulation of $p56^{lck}$ enzyme activity is different. To identify the intracellular substrates phosphorylated following $p56^{lck}$ activation induced by different stimuli, peripheral blood, T cells were labelled with inorganic $^{32}P$ for 4 hours and then cultured with either epithelial cells, monocytes, or cross-linked mAb anti-CD8. Cells were then lysed immediately in 2X NP-40 lysis buffer and immunoprecipitated with mAb anti-phosphotyrosine (4G10). The precipitates were resolved on 10% SDS-PAGE and bands were detected by autoradiography. As seen in figure 4, p56lck was upregulated in all stimulated T cells. Among many phosphorylated proteins, one band around 97kd (arrow) was present in both cross-linked CD8 and epithelial cell stimulated T cells, but not in control or monocyte activated T cells. Since epithelial cell stimulated $CD8^+$ T cell proliferation is mediated through CD8 and CD8 associated $p56^{lck}$, this phosphorylated 97kd protein supports the concept that epithelial cell induced T cell activation uses a CD8 signal transduction pathway.

5. *Detection of substrate phosphorylation pattern in intestinal epithelial cell stimulated T cells.* Purified peripheral blood T cells were incubated with either cross-linked mAbs (anti-CD4 and anti-CD8), epithelial cells or monocytes for 1-2 minutes. Cells were then lysed and immunoprecipitated with mAb anti-phosphotyrosine

(4G10). ($\gamma$-$^{32}$p)ATP was added into precipitates to initiate a kinase reaction. The total reactants were run on 10% SDS-PAGE and bands were detected by autoradiography. All stimulated T cells showed upregulation of $p56^{lck}$ enzyme activity and no $p56^{lck}$ enzyme activity was detected in epithelial cells or monocytes alone. A 97kd phosphorylated band was seen in epithelial cell but not in monocyte activated T cell cultures. Thus the 97kd phosphoprotein can be detected by two approaches and may be important for epithelial cell stimulation of $CD8^+$ T cells.

During the immunoprecipitation procedure, the anti-phosphotyrosine mAb (4G10) precipitates both kinases and other proteins containing phosphorylated tyrosine residues from either T cells or epithelial cells. Thus conceivably, the novel 97kd tyrosine phosphorylated protein may derive from the epithelial cells. However, since the 97kd phosphoprotein was also seen in $^{32}$P metabolically labelled T cells stimulated by epithelial cells, the participation of substrates from epithelial cells were excluded. Thus far, we have focused on activation of $p56^{lck}$ in epithelial cell stimulated T cell cultures. This does not allow us to conclude that the novel 97kd band is $p56^{lck}$ specific as other kinases may be activated during cell:cell interaction which have not been defined yet.

6. *Staining of monocytes, HT-29 and DLD-1 cells with anti-B7/BB1.* Suppressor cells fail to express CD28 surface marker which is an important costimulatory molecule for monocytes to induce cytotoxic T cells. In this experiment, we decided to examine

whether epithelial cells could express the CD28 ligand, B7/BB1, since intestinal epithelial cells stimulated $CD8^+$ suppressor T cells. IFN-γ (500 u/ml) pretreated or untreated monocytes, HT-29 or DLD-1 cells were incubated with FITC conjugated anti-B7/BB1 antibody. The expression of B7/BB1 was only detected on IFN-γ induced monocytes, not on epithelial cells HT-29 and DLD-1 (figure 6).

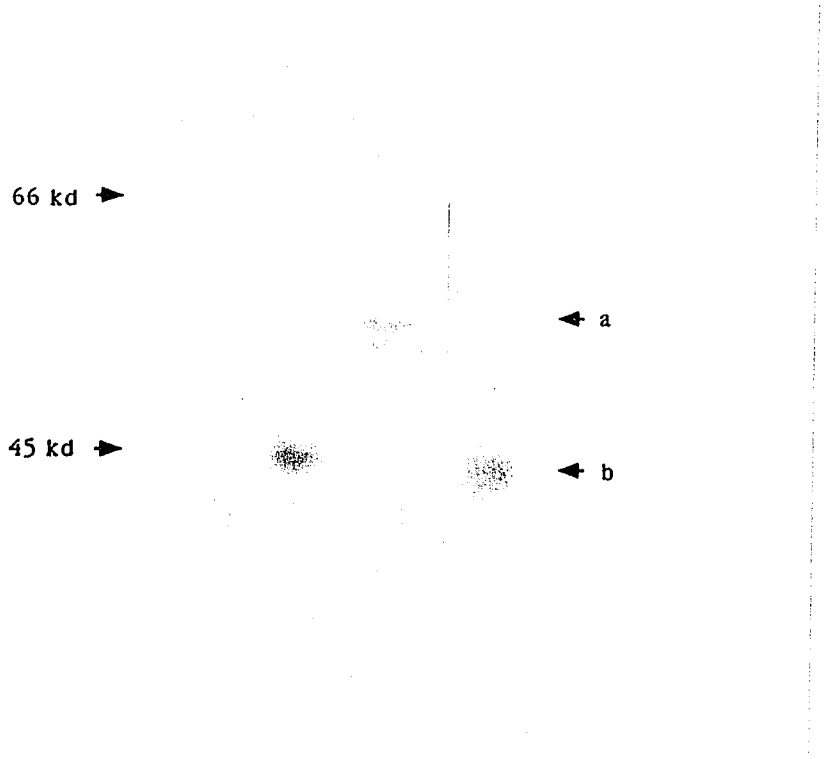



**Figure 1. Precipitation of antigens recognized by monoclonal antibody B9 and L12 in $^{35}S$ labelled HT-29 cells.** HT-29 cells were cultured with IFN-γ (500 U/ml) for 24 hours and then labeled with Translabel $^{35}S$ methionine/cysteine (20 million cells/mCi $^{35}S$) for six hours. Cells were then harvested, lysed and immunoprecipitated with monoclonal antibody B9, L12, W6/32 (anti-class I) or an irrelevant mAb (IgG1) as the negative control. Precipitates were resolved on 7.5% SDS-PAGE and bands were determined by autoradiography.

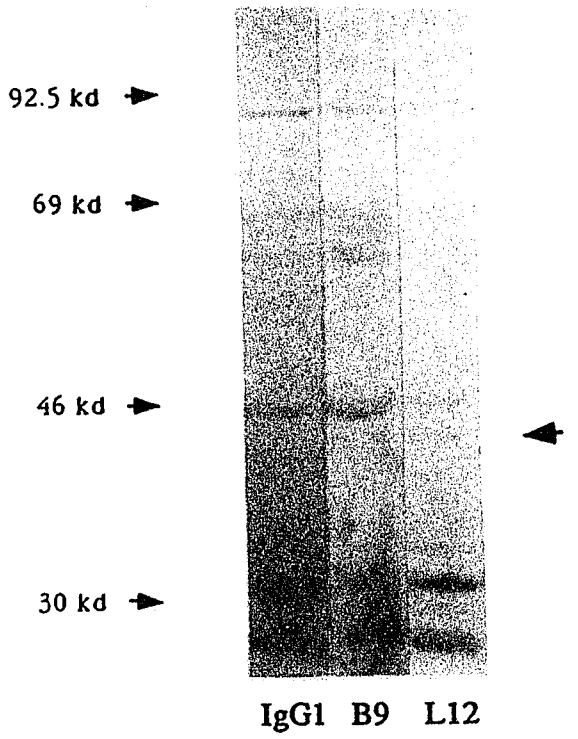



**Figure 2. Western blot analysis of monoclonal antibody B9 and L12 recognized surface components on HT-29 cells.** HT-29 cells pretreated with IFN-γ for 24 hours were lysed in 3% NP-40 containing 0.5% SDS. Lysates were applied to 10% SDS-PAGE and transblotted onto nitrocellulose membranes. Bands were detected by incubating with either monoclonal antibody B9, L12 or an isotype control antibody (IgG1), followed by incubation with alkaline phosphatase conjugated goat anti-mouse IgG and substrate.

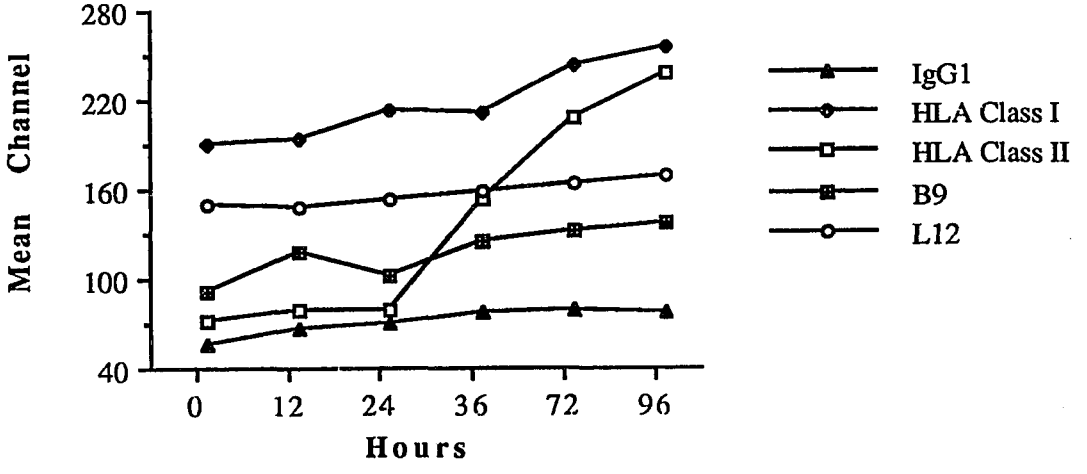



**Figure 3. Regulation of surface antigen expression on HT-29 cells by IFN-γ.** HT-29 cells were pretreated with recombinant IFN-γ (500 U/ml) for varying time in cell culture medium. After each treatment, cells were washed, stained with monoclonal antibodies against class I, class II, B9, L12 or an irrelevant mAb (IgG1), followed by FITC-conjugated goat anti-mouse IgG antibody. Staining was analyzed by flow cytometry.

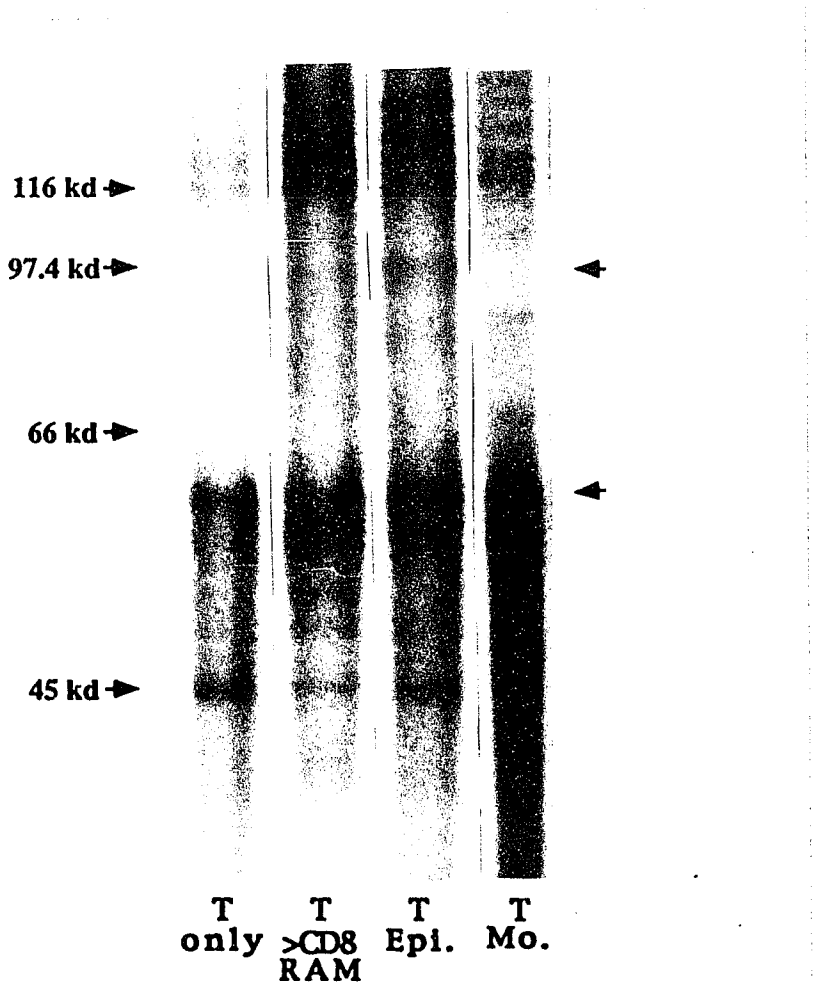



**Figure 4. Detection of a unique p56$^{lck}$ substrate induced by enterocytes in inorganic $^{32}$P metabolically labelled T cells.** Isolated peripheral blood T cells were loaded with inorganic $^{32}$P (10 mCi/10 million cells) in phosphorus depleted medium for 4 hours, then mixed with either epithelial cells ($10X10^6$), monocytes ($10X10^6$) or anti-CD8 mAb plus RAM as stimulators for 2 minutes. Cells were lysed in 2X NP-40 lysis buffer and immunoprecipitated with the anti-phosphotyrosine monoclonal antibody 4G10. The precipitates were resolved on 10% SDS-PAGE and bands were detected by autoradiography.

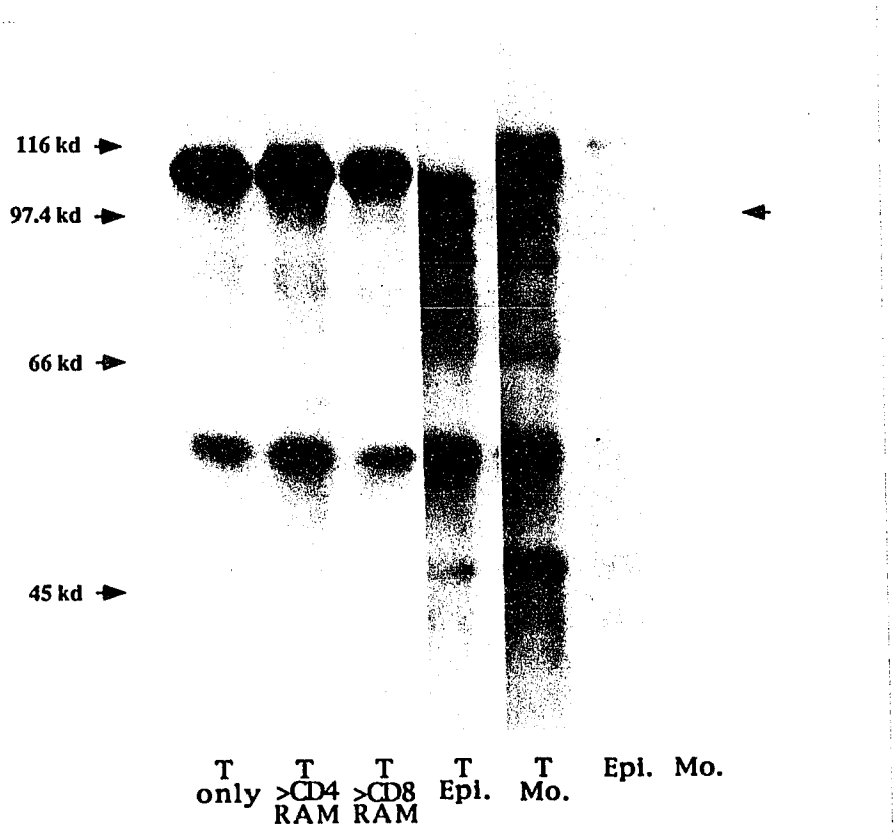



**Figure 5. Pattern of protein phosphorylation in T cells stimulated by epithelial cells versus monocytes.** 10 million T cells were incubated with either normal epithelial cells ($10X10^6$), monocytes ($10X10^6$), anti-CD4 plus RAM or anti-CD8 plus RAM at 37°C for 2 minutes. Cells were lysed in cold 2X NP-40 lysis buffer and immunoprecipitated with the anti-phosphotyrosine monoclonal antibody 4G10 and protein A sepharose beads. The precipitates were washed, resuspended in kinase buffer and mixed with ($\gamma$-$^{32}$P)ATP (10uCi per sample). The reaction mixture was resolved by 10% SDS-PAGE and protein phosphorylation was determined by autoradiography.

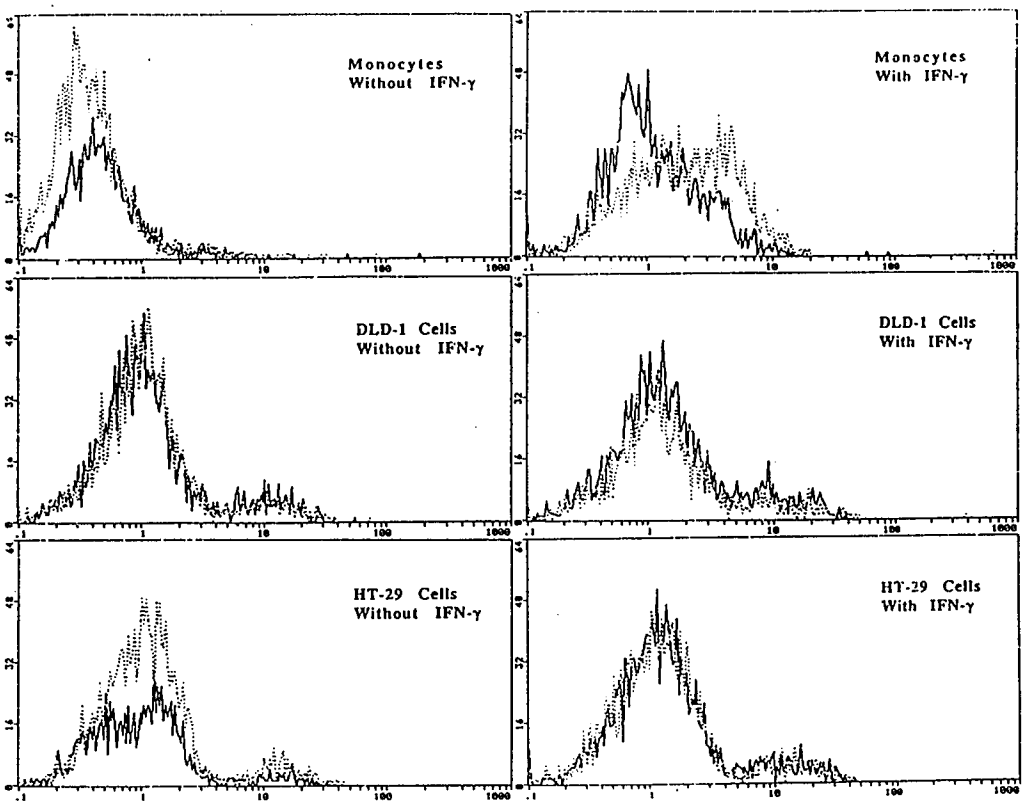



**Figure 6. Measurement of IFN-γ induced expression of B7/BB1 on HT-29, DLD-1 and monocytes.** HT-29 cells, DLD-1 Cells and freshly isolated peripheral monocytes were incubated with recombinant IFN-γ (500 U/ml) at 37°C for 24 hours. After induction, cells were washed and stained with commercial FITC-conjugated anti-B7/BB1 antibody (Becton Dickinson) for 30 minutes at 4°C. The results of staining were analyzed by flow cytometry. The Y axis indicates the cell number and the X axis indicates the staining intensity. The dashed line indicates the cell staining with an irrelevant antibody (L12) and dotted line indicates the cell staining with the anti-B7/BB1 antibody.

# Suppressor T Cells Can Be Induced By Bifunctional Antibody anti-CD3/CD8

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**Abbreviations:** mAb, monoclonal antibody; IL-2, interleukin-2; Ab, antibody; TCR, T cell receptor; CTL, cytotoxic T lymphocyte; CM, culture medium; PBS, phosphate buffered saline; PHA, phytohemagglutinin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Abstract

Using $F(ab)'_2$ CD3/CD8 bifunctional mAbs generated by chemically crosslinking CD3 Fab with CD8 Fab, we were able to induce proliferation of peripheral blood T cells in the presence of IL-2. This capacity to induce proliferation did not require monocytes (although monocytes could substitute for IL-2) or further crosslinking with an anti-mouse antibody. In contrast CD3/CD28 bifunctional mAbs stimulated greater proliferation even in the absence of IL-2 and was a potent stimulus inducing high density IL-2 receptor expression. The interesting findings, however, related to the functional phenotype of cells activated with the two different bifunctional Abs. CD3/CD28 activated T cells exhibited potent cell mediated lympholysis whereas CD3/CD8 activated T cells failed to kill, and, in fact, suppressed baseline killing. This suppressor activity was detected in a number of assay systems demonstrating that the CD3/CD8 mAb stimulated T cells were suppressor cells. Therefore, in the presence of crosslinking of CD8 to CD3, in the absence of a CD28 signal, the predominant cell proliferating is an antigen nonspecific suppressor T cell. Binding to CD28 alters the functional phenotype and possibly the activation requirements.

Introduction

In addition to signalling through the T cell receptor, several T cell surface glycoproteins, CD4, CD8 and CD28 have been demonstrated to be important accessory molecules for enhancing antigen-directed T cell responses by both increasing the avidity of the interaction between the TCR and the antigen-MHC complex and transducing an independent signal (1, 2). Cross-linking CD4 or CD8 activates the kinase activity of $p56^{lck}$ which phosphorylates numerous cellular substrates including CD3 $\zeta$ chain and PLC-$\gamma$1 (3-6). Such cross-linking may enhance or alter the T cell activation process. For example, cross-linking CD8 with monoclonal antibodies (mAb) provides a positive signal to T cell activation. Isolated $CD8^+$ T cell proliferation induced by superantigen staphylococcal enterotoxin (SEB) demonstrated enhanced T cell proliferation in the presence of anti-CD8 mAbs (7). Our laboratory has demonstrated that human intestinal epithelial cells, acting as antigen presenting cells, can selectively activate and expand $CD8^+$ suppressor T cells (8). This T cell activation event is based on signal transduction through the CD8 molecule on T cells and CD8-associated $p56^{lck}$ activation (9). Blocking with an anti-CD8 mAb or the presence of a protein tyrosine kinase inhibitor, genestein, can completely abrogate epithelial cell induced $CD8^+$ T cell activation. However, data from our laboratory have indicated that stimulation through CD8 alone is not sufficient to stimulate T cell proliferation thus suggesting that a second signal is required.

Although some studies have shown that the stimulation of T cells with lectin or antibodies can be blocked by anti-CD4 or anti-

CD8 in the presence of antigen presenting cells bearing the appropriate class I or class II molecules (10, 11), recent results suggest that such blocking effects are related to the steric interference of the TCR-CD4 or -CD8 co-receptor complex (12). It has been demonstrated that to some extent CD4 and CD8 each can physically associate with TCR on T cells in the absence of antigen stimulation and this association can be upregulated following T cell activation (12, 13).

Since the intracytoplasmic tails of both CD4 and CD8 are associated with the src-like protein tyrosine kinase, $p56^{lck}$, which is activated after stimulation (3, 4), the formation of this TCR-CD4, or -CD8 co-receptor would bring kinase and substrate together that would be critical for T cell activation. Although overlapping substrates in the T cell may account for signaling pathways common to all T cells, subtle differences in kinase activity and substrate specificity may be involved in signaling unique selection, differentiation and activation events.

Although CD28, is not associated with the TCR, the binding of CD28 during T cell activation significantly enhances IL-2 production and T cell proliferation (14, 15). Stimulation of CD28 also induces protein tyrosine phosphorylation which is required for the cellular effects elicited by CD28.

To investigate the signals required for proliferation of $CD8^+$ suppressor T cells induced by intestinal epithelial cells, we measured T cell activation and differentiation induced by the bifunctional antibody anti-CD3/CD8 as well as anti-CD3/CD28. CD3/CD8 stimulation results in the activation of suppressor T cells

while CD3/CD28 stimulation activates cytotoxic T cells.

## Methods

### *Culture medium*

Cell culture medium (CM) consisted of RPMI-1640 with 2mM glutamine (Gibco, Grand Island, NY), supplemented with 50ug/ml penicillin (Gibco), 50ug/ml streptomycin (Gibco) and 10% fetal calf serum (Gibco, Grand Island, NY). Interleukin 2 (IL-2) was purchased from Boeringer Mannheim (Indianapolis, IN) and used at dilution of 100 U/ml.

### *Cell preparation and separation by nylon wool*

Heparinized venous blood was collected from normal donors. Blood was diluted 1:3 with sterile PBS and layered on to a Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient, and centrifuged for 30 minutes at 500g. The mononuclear cells were collected from the interface and washed three times with PBS. Cells were resuspended in culture medium (RPMI-1640 with 10% fetal calf serum, Gibco, Grand Island, NY) at a concentration of 75 million cells/ml. 150 million cells were applied to a nylon wool column, loosely packed wih 1.2g scrubbed nylon wool fiber (Polysciences Inc., Warrington, PA). The nylon wool column was prewashed in 1% Duponal RA (Witco, New York, NY) at 65°C for one hour, and rinsed thoroughly with tap water and finally with distilled water for 30 minutes. The column was stored at 4°C following autoclaving and balanced with 20ml warm CM (37°C) before use. The cells applied to the column were incubated at 37°C for 45 minutes. Unbound cells, mostly T cells, were slowly eluted with 20ml warm CM and collected in a sterile tube. Cells were pelleted and adjusted to

$10^6$/ml in CM. Flow cytometric analysis of nylon wool purified T cells revealed >97% $CD3^+$, <0.1% $CD20^+$, <0.1% $CD14^+$ and absence of proliferation in response to PHA stimulation.

*Antibodies*

Anti-CD3 monoclonal antibody (mAb) 446 has been previously defined in our laboratory (20). mAb 9.3 (anti-CD28) was a gift from Dr. John Hansen and mAb OKT8 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Anti-CD4 mAb (FFB2.3) was a gift from Dr. David Posnett (New York Hospital, New York, NY).

Bifunctional antibodies anti-CD3/CD8 and anti-CD3/CD28 were provided by Dr. Alison Tutt in the Tenovus Research Laboratory (Southampton, UK). The method for generation the bifunctional antibodies anti-CD3/CD8 was as follows: murine mAb OKT3 (anti-CD3) and OKT8 (anti-CD8) both from the ATCC were digested with pepsin (Sigma, St. Louis, MO) at pH 4.2 in 0.1mM sodium acetate for 1 hour at 37°C. The $F(ab')_2$ fragments were isolated from the digest mixture by gel filtration on an Ultrogel AcA44 column (LKB Produkter, Bromma, Sweden). Bifunctional antibody containing mouse $F(ab)'_2$ from OKT3 and OKT8 were linked by connecting half-cysteine residues via thioester bonds using the bifunctional cross-linking agent, *o*-phenylenedimaleimide (*o*-PDM, Sigma, St. Louis, MO). Each $F(ab)'_2$ (10mg/ml) was first reduced by 2-mercaptoethanol (2-ME) for 30 minutes at 30°C then cooled down and kept at 4°C. A half volume of 12mM *o*-PDM dissolved in dimethylformamide was then added to one of the two murine Fab

solutions and incubated at 4°C for 30 minutes. The maleimidated Fab was separated from solutes in the reaction mixture by passage through a Sephadex G-25 column. The isolated Fab was incubated for 18 hours at 4°C. Then bifunctional $F(ab)'_2$ antibodies were separated from other products and residue reagents by passage through an Ultrogel AcA44 column. The composition of final bifunctional $F(ab)'_2$ products were examined by double immunodiffusion, SDS-PAGE and radioactive labelling analysis. and residue reagents by passage through and Ultrogel AcA44 column. The composition of final bifunctional $F(ab)'_2$ were examined by double immunodiffusion, SDS-PAGE and radioactive labelling analysis (21).

*T cell proliferation in response to antibody stimulation*

Nylon wool purified T cells were cultured in triplicate in U-bottomed microtitre plates (Becton Dickinson Labware, Lincoln Park, NJ) ($10^5$ per well) in CM with 100 U/ml IL-2 in the presence or absence of antibodies as stimulators. In some experiments, rabbit anti-mouse IgG (Cappel, West Chester, PA) (1 ug/ml) was added as a cross-linking reagent. All cultures were incubated at 37°C, in 5% $CO_2$ humidified incubator for 72 hours. 1uCi[$^3$H] thymidine (ICN, Irvine, CA) was added during the last 18 hours of culture and cells were harvested onto glass fiber filter mats (Skatron, Sterling, VA) for counting. Thymidine incorporation was measured by liquid scintillation counting (Model LS3801, Beckman Instruments, Somerset, NJ) and results were expressed as mean cpm$\pm$SEM for the triplicate cultures. In some experiments, genestein

(25uM) was incubated with T cells for 2 hours prior to co-culture with antibodies.

*Flow cytometric analysis*

After 72 hour culture in the presence or absence of antibodies, cells were washed with PBS and placed in V-bottomed microtitre cell plates (Becton Dickinson Labware, Lincoln Park, NJ) ($2X10^5$ per well) in a volume of 100ul and stained with FITC-conjugated anti-IL-2 receptor (Becton Dickinson Labware, Lincoln Park, NJ) for 45 minutes on ice. Isotype matched mAbs were used in each experiment with no significant background staining. Cells were then washed with PBS three times and fixed with PBS-paraformaldehyde 1% solution (Fisher Scientific Company, Fair Lawn, NJ). The staining was analysed on a Coulter Profile II (Coulter, Hialeah, FL).

*Immunoprecipitation and In vitro tyrosine kinase assay*

Nylon wool purified peripheral blood T cell were stimulated with either anti-CD8 mAb plus rabbit anti-mouse IgG (RAM) in the presence of absence of IL-2, anti-CD3/CD8 in the presence of absence of IL-2 +/- RAM, or anti-CD3/CD28 in a 37°C water bath for 1 minute. Cells were then lysed in cold 2X NP-40 lysis buffer [2% NP-40, 40mM Tris base (pH 8.0), 300mM NaCl, EDTA 400uM, sodium pyrophosphate 20mM, sodium fluoride 200mM, 1mM PMSF, 5mM iodoacetamide, 20 ug/ml aprotinin, 20 ug/ml leupeptin and 200uM $Na_3VO_4$] (Sigma) for 30 minutes, vortexing every 5 minutes. The lysate was centrifuged at 14,000 rpm for 10 minutes in a microfuge to remove cell debris, and then precleared with protein

A sepharose (Pharmacia, Piscataway, NJ) (30ul 50% PAS per sample) for 30 minutes at 4°C. The clarified lysate was incubated with anti-lck sera (gift of Dr. C.E. Rudd) for one hour at 4°C, added to PAS (50ul 50% PAS per sample) and incubated for 2 hours at 4°C. The immunoprecipitates were washed once with PBS, two times with 0.5M LiCl (Sigma) in 20mM Tris (pH 8.0) and once with kinase buffer (10mM $MnCl_2$, 50mM Tris, pH 7.4). The immunoprecipitates were finally resuspended in kinase buffer (30ul per sample), and mixed with ($\gamma$-$^{32}$P)ATP (Amersham, Arlington Heights, IL) (10uCi per sample). After an incubation of 30 minutes at 25°C, the reaction mixture was subjected to 10% SDS-PAGE and autoradiography.

*Suppressor assay*

The suppressor effect of bifunctional antibody stimulated T cells were assayed by co-culture of peripheral blood T cells ($10^5$ per well) in U-bottom microtitre plates containing PHA (1 ug/ml) (Gibco) in the presence of equal numbers of bifunctional antibody, anti-CD3 mAb (446) or medium as negative control activated T cells (irradiated 3000 rad) for 48 hours at 37°C. Cells were pulsed with 1 uCi [$^3$H] 18 hours before harvesting and $^3$H thymidine incorporation was then measured.

*Cytotoxicity Assay*

Murine mastocytoma cell line, P815 cells (from ATCC) transfected with human Fc$\gamma$ receptors were used as targets in a chromium release assay. Cells were labelled with 100 uCi $^{51}$Cr (sodium chromate, Amersham, Arlington Heights, IL) per $10^6$ cells

for 40 minutes and then washed and resuspended at $5X10^3$ cells/well in V-bottomed plates containing effectors (bifunctional antibodies, anti-CD3 mAb or medium as negative control activated T cells) at varying ratios in the presence of absence of mAb OKT3 (2 ug/ml). The plates were centrifuged briefly (200gX4 minutes) before incubation for 4 hours at 37°C. The culture supernatant were collected and $^{51}Cr$ release was measured (Haywand et al., 1986). The spontaneous $^{51}Cr$ release was less than 10%. Specific lysis was measured as

$$\% \text{ Cytotoxicity} = \frac{\text{Test release - Spontaneous release}}{\text{Maximum release - Spontaneous release}} X100$$

The maximum release was determined by detergent NP-40 (0.1%) lysis of target cells.

RESULTS

1. *Peripheral blood T cell activation and proliferation can be induced by bifunctional antibodies anti-CD3/CD8 and anti-CD3/CD28.* Nylon wool purified resting T cells were co-cultured with either bifunctional antibodies made from the chemical cross-linking of two Fab fragments, anti-CD3/CD8 (410 ng/ml), CD3/CD28 (350 ng/ml), or intact mAb anti-CD3 (500 ng/ml), intact anti-CD8 (500 ng/ml) or intact anti-CD28 (500 ng/ml) in the presence or absence of IL-2 (100U/ml). These concentrations were determined to be optimal for stimulation of T cell proliferation. The extent of depletion of adherent cells in the purified T cells was confirmed by their lack of proliferation in response to PHA.

As seen in figure 1A, soluble $F(ab)'_2$ bifunctional antibody anti-CD3/CD8 was capable of triggering T cell proliferation in the presence of interleukin-2 whereas neither soluble anti-CD3 (mAb 446), anti-CD4 (mAb FFB2.3) or anti-CD8 (mAb OKT8) alone or in combination (figure 2A) is stimulatory. T cells in this setting were dependent upon the presence of IL-2 and this dependence could not be bypassed by cross-linking of the bifunctional mAb with a rabbit anti-mouse Ig antibody (figure 1B). However, exogenous IL-2 was not required to drive CD3/CD28 antibody stimulated T cell proliferation (figure 1A), and in fact, proliferation was greater in these cultures. Stimulation with soluble anti-CD28 failed to stimulate T cell proliferation even in the presence of IL-2 (figure 1A).

2. *The effect of bifunctional antibodies on T cells could be*

*blocked by soluble antibodies.* We next examined the effect of independently cross-linking CD3 and CD8 or CD3 and CD28 on T cells. The requirement for physical cross-linking of either CD3 to CD8 or CD3 to CD28 was confirmed by the finding that addition of soluble monoclonal antibody 446 (anti-CD3) or OKT8 to cultures of T cells stimulated with the CD3/CD8 bifunctional antibody greatly reduced (33% and 66% respectively) the T cell response to the bifunctional antibody (figure 2A). Similarly, anti-CD3 (soluble) depressed (50%) the T cell response to CD3/CD28 (figure 2B). Neither the combination of mAb 446 (anti-CD3) and mAb 9.3 (anti-CD28) nor mAb 446 and OKT8 could induce responses similar to those seen with the bifunctional antibodies in our experiments. These data indicate that the physical cross-linking of CD3 to either CD28 or CD8 provides the activation signals to T cells.

3. *Measurement of IL-2 receptor expression on bifunctional antibodies activated T cells.* The findings of induced T cell proliferation correlated with the expression IL-2 receptor on these cells. Stimulation with CD3/CD8 resulted in a marked increase 70% IL-2 receptor expression (figure 3). Exogenous IL-2 needed for the induction of T cell proliferation was not required for IL-2 receptor expression (data not shown). In contrast, CD3/CD28 stimulation of the same T cell preparation resulted in similar IL-2 receptor expression, however the exogenous IL-2 was not required for T cell proliferation induced by this stimulus. (figure 3).

The requirement for interleukin-2 to drive CD3/CD8 antibody activated T cell proliferation could be obviated by the addition of

monocytes (data not shown). Given the nature of the bifunctional antibody $(F(ab)'_2$, monocytes could only be providing either a source of accessory cytokines (IL-1, IL-6?) or secondary cell-cell adhesion events.

4. *Combined signals generated by bifunctional antibody anti-CD3/CD8 activate a suppressor T cell subpopulation.* Thus far, we have demonstrated that by using bifunctional antibodies to cross-link CD3 with other accessory molecules on the T cell surface in nylon wool purified T cells, we more effectively activate T cells than by cross-linking CD3 alone. However, whereas while anti-CD3 mAb is a polyclonal activation stimulus (in cross-linked form), cross-linking CD3 with specific accessory molecules appeared to selectively activate distinct T cell subsets. To examine the function of bifunctional antibody activated T cells, cells were cultured in the presence or absence of antibodies and IL-2 for three days. After harvesting and washing, the suppressor activity of these activated T cells was assayed in a PHA driven T cell proliferation system. As seen in figure 4, nonactivated T cells as well as 446 activated T cells had no effect on PHA stimulated T cell proliferation. However, bifunctional antibody anti-CD3/CD8 activated T cells significantly inhibited T cell proliferation. This effect was dose-dependent and antigen non-specific manner (data not shown). Similarly, bifunctional antibody anti-CD3/CD28 activated T cells were also capable of mediating suppression of the PHA response.

5. *Bifunctional antibody anti-CD3/CD28 activated T cells*

*exhibited cytotoxic function.* The cytotoxic potential of bifunctional antibodies anti-CD3/CD28 and CD3/CD8 activated T cells were then assessed in a $^{51}Cr$-release assay using a human $Fc\gamma$ receptor transfected murine mastocytoma cell line, P815, as a target for NK, LAK or anti-CD3 redirected CTL activity. T cells cultured with IL-2 alone showed high baseline cytotoxic activity which relates to NK or LAK cells activated in IL-2 supplemented cultures (table 1). However, the most marked cytotoxic activity was mediated by anti-CD3 and CD3/CD28 antibody stimulated T cells in the presence of mAb OKT3 (anti-CD3) in a redirected lysis assay.

The most interesting finding was the fact that T cells activated by the bifunctional antibody anti-CD3/CD8 showed no enhanced cytotoxic function and a actually suppressed even basal levels of cytotoxic activity seen in IL-2 cultured T cells. Since antibody activated T cells had proliferated and were washed extensively with PBS, minimal mAb remained on these activated T cells as determined by flow cytometric analysis. Therefore, suppression of CTL activity mediated by CD3/CD8 antibody activated T cells does not appear to relate to blocking of CD3 or CD8 by residual antibodies. Furthermore, human CD8 does not participate in redirected lysis of murine cells. Thus the suppressive activity of CD3/CD8 activated T cells in PHA and cytotoxicity experiments demonstrated above appears to relate to true suppression while the suppression by CD3/CD28 activated T cells (PHA stimulation only) may relate to cytotoxicity. The findings with CD3/CD8 antibody contrast to results observed with other antibody activated T cell systems where cross-linking CD3 and CD8 by solid phase in $CD8^+$

human T cells resulted in the induction of clearly cytotoxic activity (19).

6. *Measurement of $p56^{lck}$ upregulation in bifunctional antibody activated T cells.* Activation of $p56^{lck}$ has been found to be important in the phosphorylation of PLC-γ1 which initiates the $PIP_2$ signal transduction pathway in conventional antigen stimulated T cell activation. Previous studies have demonstrated an association of the src-like tyrosine kinase, $p56^{lck}$, to the α chain of the CD8 molecule (4). Cross-linking CD8 with mAb results in the activation of $p56^{lck}$ and autophosphorylation. In addition, occupying IL-2 receptor by IL-2 also induces $p56^{lck}$ activation and autophosphorylation (16, 17). To determine the level of $p56^{lck}$ activation in bifunctional antibody stimulated T cells, we cultured T cells with antibody in the presence or absence of IL-2 for 1 minute. Cells were immediately lysed in cold lysis buffer. $p56^{lck}$ was immunoprecipitated with an anti-p56lck antibody and was autophosphorylated in the presence of $(\gamma\text{-}^{32}P)ATP$. The total immunoprecipitates were analyzed on SDS-PAGE. As seen in figure 5, cross-linking CD8 remarkably enhanced $p56^{lck}$ activation. Addition of IL-2 to CD8 cross-linked T cells reduced the level of $p56^{lck}$ autophosphorylation at 1 minute (such treatment may alter the kinetics of the phosphorylation). Bifunctional antibody anti-CD3/CD8 was capable of upregulating $p56^{lck}$ enzyme activity, although this effect was weaker than conventional anti-CD8 mAb cross-linking. In this setting, addition of IL-2 to the bifunctional antibody anti-CD3/CD8 stimulated cultures slightly enhanced

$p56^{lck}$ activity while further cross-linking of the bifunctional antibody by RAM had no effect. In contrast, no $p56^{lck}$ activation was seen in T cells cultured in the presence of the bifunctional antibody anti-CD3/CD28.

7. *Genestein, a protein tyrosine kinase specific inhibitor, can block T cell proliferation stimulated by bifunctional antibodies.* Tyrosine kinase activation and sequential protein tyrosine phosphorylation are initial events for T cells activated following engagement of the TCR/CD3 complex. In order to determine whether the activation of $p56^{lck}$ was critical to the stimulation of T cells in our system, we used genestein, a tyrosine kinase inhibitor, to test its effect on T cell proliferation triggered by bifunctional antibodies anti-CD3/CD28 and anti-CD3/CD8. As shown in figure 6, genestein (25uM) inhibited the proliferative capacity of both bifunctional antibodies, although the effect was greater in CD3/CD8 antibody stimulated T cells. In contrast, PHA responses were not inhibited by genestein at this concentration suggesting that the effects seen with bifunctional antibodies were not related to cellular toxicity (data not shown). The inhibition of CD3/CD28 induced T cell proliferation in the absence of $p56^{lck}$ activation suggests that another tyrosine kinase may be involved in this system.

Discussion

Our laboratory has previously defined a system whereby unique antigen presenting cells derived from the gastrointestinal tract (intestinal epithelial cells) can selectively activate $CD8^+$ suppressor T cells (8). These studies implicated the CD8 molecule itself in this process with concomitant activation of the src-like tyrosine kinase $p56^{lck}$ (9). However, stimulation of $p56^{lck}$ by crosslinking CD8 alone was not sufficient to drive proliferation of these cells suggesting that a second signal was required. Since our initial studies were performed in either an antigen specific (tetanus toxoid) or allogeneic mixed cell culture system, the likeliest candidate for a second signaling molecule appeared to be the T cell receptor itself. This hypothesis was strengthened by the studies which demonstrated co-localization of the CD8 or CD4 molecule with the TCR complex during cellular activation. Class II/peptide complexes bind both the TCR and CD4 molecules forming this associated complex. Similarly the TCR on $CD8^+$ T cells binds to class I/peptide complexes which then associate with CD8. Whether formation of these new complexes altered the stimulus to the T cell was not determined in these initial studies.

By utilizing $F(ab)'_2$ bifunctional antibodies we hoped to address this issue. Interestingly $F(ab)'_2$ CD3/CD8 bifunctional mAbs were capable of stimulating increased IL-2 receptor expression and cell proliferation in the presence of IL-2. Monocytes played no role in this system as they were removed by nylon wool adherence (the lack of a T cell response to PHA attested to the successful depletion of these cells) and the absence of Fc on the bifunctional Abs

preclude their binding to Fc receptors. Interestingly, however, the addition of monocytes back to these cultures abrogated the requirement for exogenous IL-2, possibly by providing a CD28 signal. CD28 has been shown to be a potent co-activating signal for T cells, enhancing IL-2 production and secretion. In a normal immune response T cells bind to the peptide/MHC complex as well as to other accessory molecules on the surface of a conventional APC. Since macrophages can be induced to express the ligand for CD28, B7/BB1, this interaction typically results in IL-2 secretion (18). A possible surrogate for macrophage activation of T cells in our system was the bifunctional mAb CD3/CD28. In contrast to CD3/CD8 Abs, no exogenous IL-2 was required for proliferation induced by CD3/CD28 mAbs, and significant induction of IL-2 receptor expression was evident. The interesting findings related to the functional properties of the T cells activated by these two bifunctional mAbs. Whereas CD3/CD28 stimulation resulted in the generation of CTLs, CD3/CD8 stimulation activated cells which suppressed immune responses. Suppression was also seen mediated by CD3/CD28 stimulated T cells but this related to the killing of activated T cell blasts. No killing, in fact suppression of spontaneous IL-2 induced killing, was seen with CD3/CD8 activated T cells. Thus in the absence of a CD28 signal, suppression rather than cytotoxicity may result. As alluded to above, the conventional ligand for CD28, B7/BB1, is expressed in a limited fashion on antigen presenting cells. Thus far we have been unable to detect B7/BB1 on intestinal epithelial cells by immunohistochemical techniques (Panja, Mayer, unpublished). Therefore, in the mucosa where the epithelial cell

may be providing the initial signals to T cells, the presence of a CD8 ligand, crosslinking CD8, in the absence of B7/BB1 may account for the selective proliferation of $CD8^+$ antigen nonspecific suppressor cells.

Our findings are contrary to those described by Emmrich et al. Using CD3/CD8 crosslinking on a solid support, induction of CTLs was seen (19). However, these were not Fab fragments, but rather chemically linked anti-CD3 and anti-CD8 $F(ab)'_2$ mAbs. Clearly our system utilizing bifunctional mAbs would provide different signals, crosslinking CD3 to CD8 without CD3-CD3 or CD8-CD8 crosslinking. Our bifunctional mAbs formed chemically by linking two Fab fragments brings CD3 and CD8 molecules together to form a co-receptor. This may be more physiologic than fixation of two mAbs on a solid phase or linking two intact mAbs by a chemical spacer. Furthermore, we have shown that the addition of soluble intact mAbs (CD3 or CD8) inhibits the ability of the bifunctional Ab to activate T cells. These findings underscore the critical need for crosslinking CD3 to CD8 comparable to what one would expect of a class I or class I-like molecule expressed on IEC.

Analysis of the signal transduction pathway utilized by both intestinal epithelial cells and bifunctional antibodies confirms that both activate the protein tyrosine kinase $p56^{lck}$ which induces tyrosine phosphorylation of several cellular protein substrates. A protein tyrosine kinase inhibitor, genestein, is able to inhibit both intestinal epithelial cell and bifunctional antibody induced T cell proliferation. In summary, we have identified a pathway for activation of suppressor T cells which may mimic the response of T

cells to normal intestinal epithelial cells. The presence of some, but not other accessory cognate signals appear to dictate subpopulation specific expansion.

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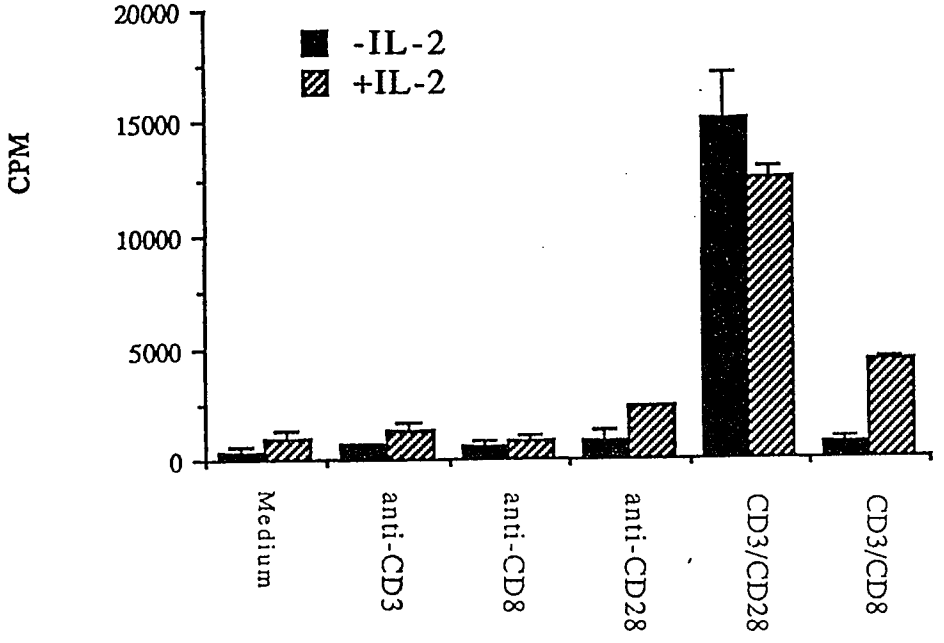


**Figure 1A. Stimulation of T cells with bifunctional antibodies CD3/CD8 and CD3/CD28.** Nylon wool purified T cells ($10^5$ cells/well) were cultured with bifunctional antibody anti-CD3/CD8 (410 ng/ml), anti-CD3/CD28 (350 ng/ml) or with monoclonal antibody 446 (anti-CD3, 500 ng/ml), OKT8 (anti-CD8, 500 ng/ml), 9.3 (anti-CD28, 500 ng/ml) in the presence or absence of IL-2 (100 U/ml) for 72 hours. $^3H$ thymidine was added 18 hours before harvest and counting.

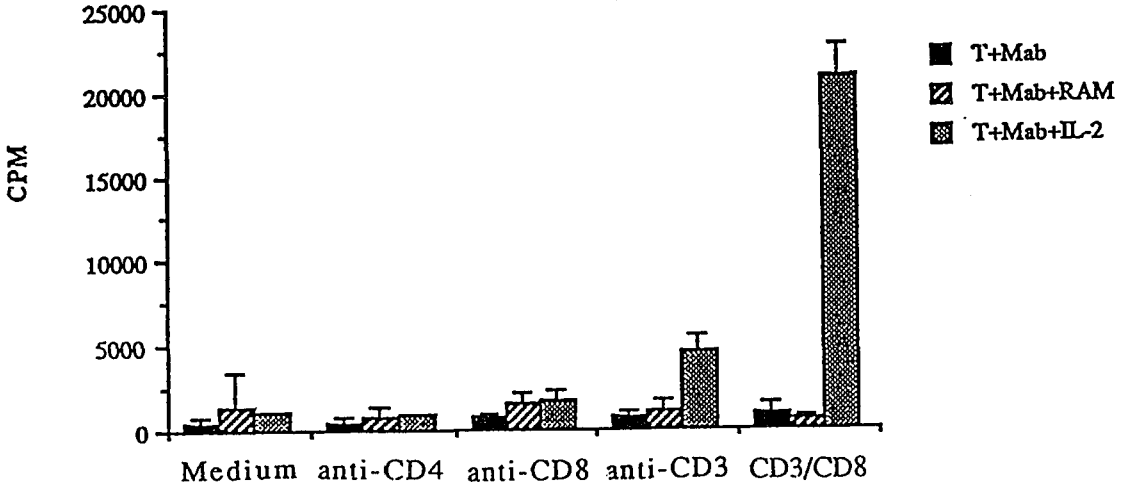


**Figure 1B. T cell proliferation induced by bifunctional antibodies in the presence of IL-2 or a cross-linking reagent.** Nylon wool purified T cells ($10^5$ cells/well) were cultured with anti-CD4 (500 ng/ml), anti-CD8 (500 ng/ml), anti-CD3 (500 ng/ml) or the bifunctional antibody CD3/CD8 (410 ng/ml) in the presence of either IL-2 (100 U/ml), cross-linking reagent (rabbit anti-mouse IgG, 1ug/ml) or medium alone for 72 hours. $^3H$ thymidine was added 18 hours before harvest and counting.

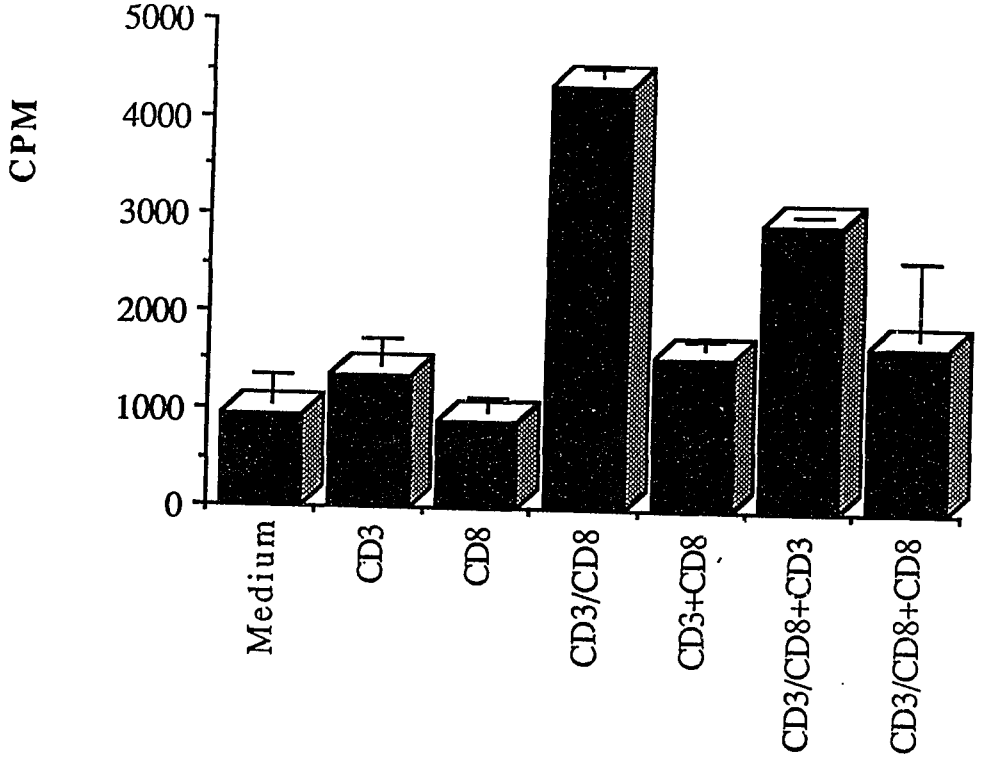


**Figure 2A. Blocking of T cell proliferation induced by bifunctional antibody CD3/CD8 in the presence of soluble antibodies.** Nylon wool purified T cells were incubated with either soluble monoclonal antibody against CD3, CD8 (alone or in combination), or the bifunctional antibody CD3/CD8. $^{3}H$ thymidine incorporation was measured at 72 hours.

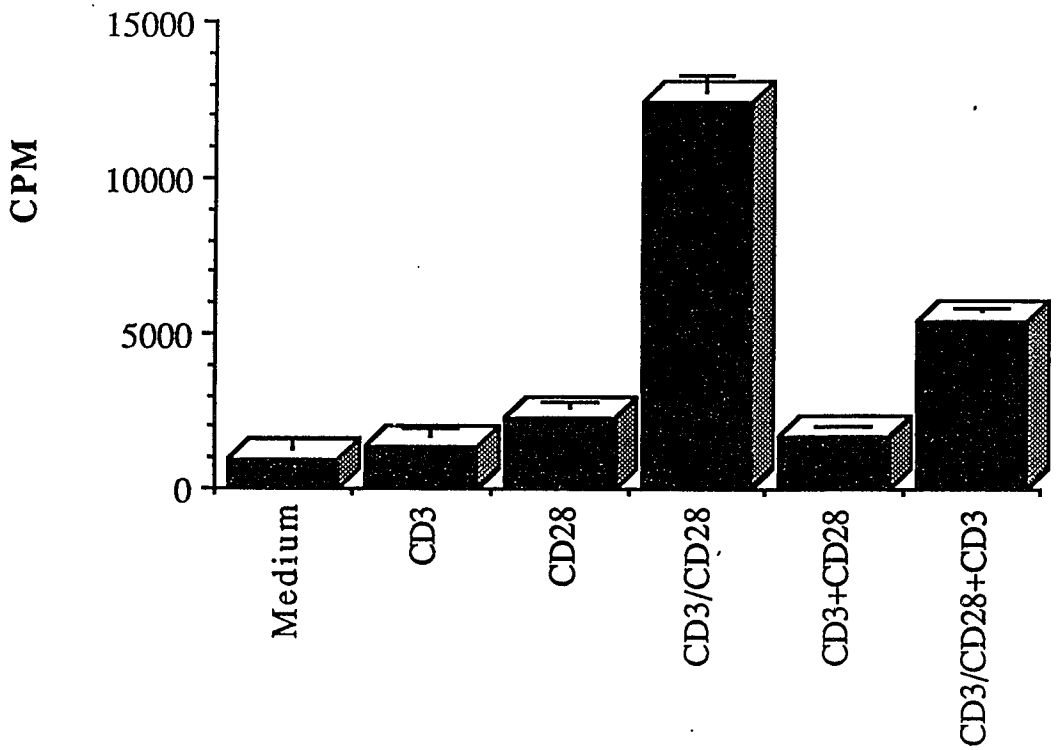


**Figure 2B. Blocking of T cell proliferation induced by bifunctional antibody CD3/CD28 in the presence of soluble antibodies.** Nylon wool purified T cells were incubated with soluble monoclonal antibody against CD3, CD28 (alone or in combination), or with the bifunctional antibody CD3/CD28. $^{3}H$ thymidine incorporation was measured at 72 hours.

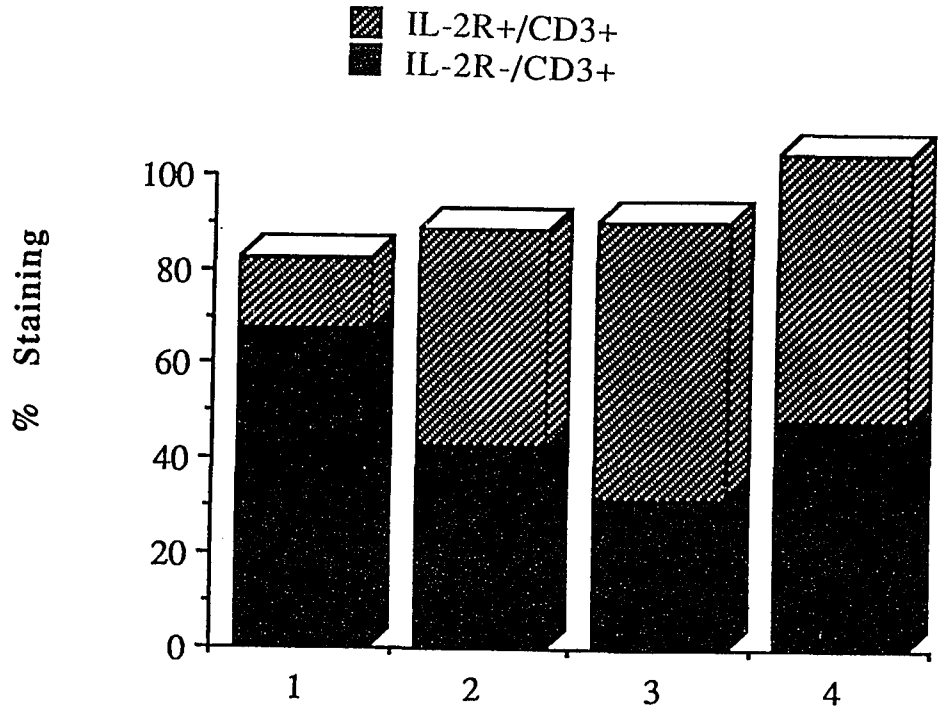


**Figure 3. Measurement of IL-2 receptor expression on bifunctional antibody activated T cells.** T cells were cultured with anti-CD3 mAb, bifunctional antibodies CD3/CD8 or CD3/CD28, or medium alone for 72 hours. Cells were then harvested and washed three times with PBS, and stained with FITC-conjugated anti-IL-2 receptor. Lane 1, unstimulated T cells; Lane 2, anti-CD3 activated T cells, Lane 3, CD3/CD8 activated T cells and Lane 4, CD3/CD28 activated T cells.

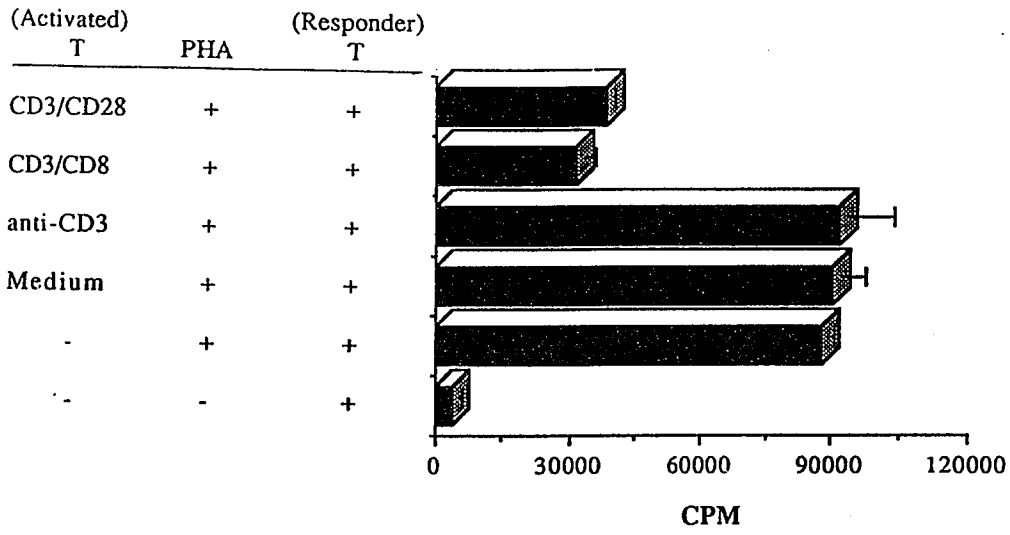


**Figure 4. Suppression of PHA stimulated T cell proliferation by bifunctional antibody activated T cells.** T cells were cultured with either anti-CD3 mAb, bifunctional antibody CD3/CD8 or CD3/CD28, or medium alone in the presence of IL-2 for 72 hours. Cells were then harvested, washed three times with PBS, irradiated (3000 rad) and added to freshly isolated T cells in the presence of 1% PHA (1 ug/ml). The cells were incubated for 48 hours and $^{3}H$ thymidine incorporation was measured.

**Table I: Cytotoxicity induced by bifunctional antibody activated T cells**

| | % Cytotoxicity* | |
|---|---|---|
| Source of effector cells | Exp. 1 | Exp. 2 |
| T | 62 | 63.5 |
| T+anti-CD3 | 84.3 | 95.7 |
| T+CD3/CD8 | 54.5 | 56.4 |
| T+CD3/CD28 | 96.5 | 92.8 |

* Nylon wool purified T cells were cultured with anti-CD3, CD3/CD8, CD3/CD28 or medium alone in the presence of IL-2 for three days. After harvesting and washing, cells were co-cultured at varying ratios with $^{51}Cr$ labeled P815 cells in the presence of monoclonal antibody OKT3 for 4 hours at 37°C and $^{51}Cr$ release was measured. The percent cytotoxicity was calculated by dividing the value (cpm) of test release minus spontaneous release by the value of maximum release minus spontaneous release.

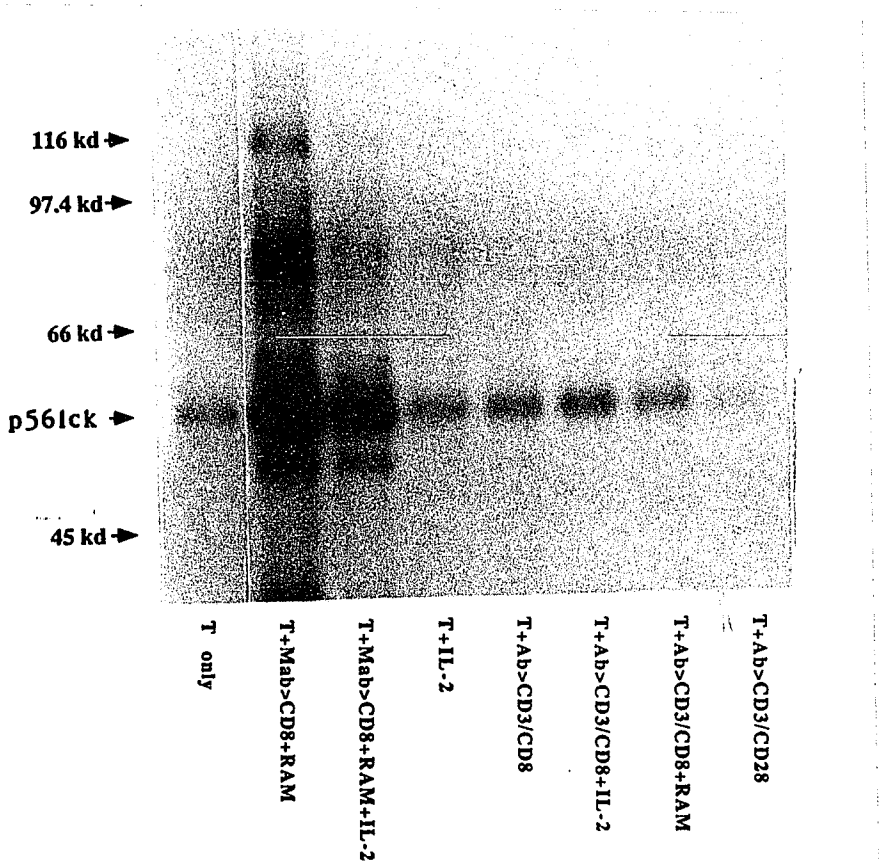


**Figure 5. Determination of $p56^{lck}$ enzyme activity induced by bifunctional antibodies.** Nylon wool purified T cells were stimulated with either anti-CD8 mAb plus RAM in the presence or absence of IL-2, anti-CD3/CD8 in the presence of absence of IL-2 +/- RAM, or anti-CD3/CD28 for 1 minute at 37°C. Cells were then lysed and p56lck was immunoprecipitated. ($\gamma$-$^{32}$p)ATP was added to the precipitates and the reaction product was resolved on SDS-PAGE. Autophosphorylation was measured by autoradiography.

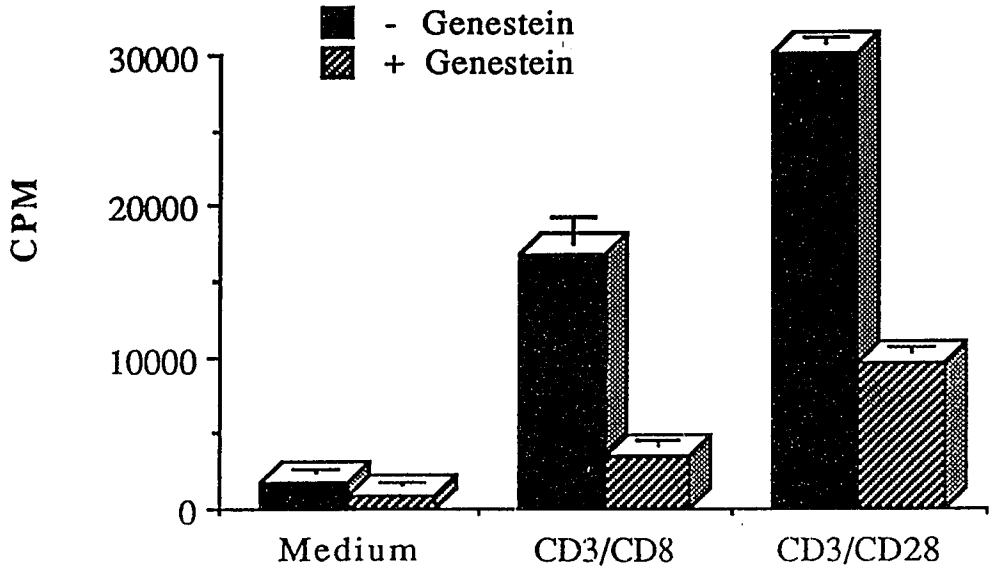


**Figure 6. Suppression of bifunctional antibody stimulated T cell proliferation in the presence of genestein.** Nylon wool purified T cells ($10^5$/well) were either cultured alone or pretreated with genestein (25 uM for 2 hours) prior to culture with bifunctional antibodies CD3/CD8 and CD3/CD28 for 72 hours.

# ======== General Discussion ========

Human intestinal epithelial cells have been demonstrated to be able to express HLA class II molecules, and process and present antigens to T cells (Mayer et al., 1987). Studies on antigen processing and cytokine secretion profiles, also support that intestinal epithelial cells can function as antigen presenting cells in a manner comparable to conventional antigen presenting cells, monocytes. The unique feature of intestinal epithelial cells is their capacity to selectively activate $CD8^+$ suppressor T cells among peripheral blood T cells. This phenomenon may be important for the gut to maintain appropriate homeostasis by downregulating immune responses.

In this thesis, we have identified that the activation signal delivered by epithelial cells to $CD8^+$ T cells was through CD8 and CD8-associated $p56^{lck}$. Blockade of the binding of epithelial cells to CD8 completely abrogates CD8-associated $p56^{lck}$ activation and $CD8^+$ T cell proliferation. Therefore, stimulation through CD8 provides a positive signal to T cells. Another interesting finding is that the CD8 ligand expressed on epithelial cells in our assay system was not class I, but a novel CD8 ligand recognized by either mAb B9 or L12, both of which could inhibit epithelial cell driven T cell proliferation. mAb B9 could also inhibit epithelial cell triggered CD8-associated $p56^{lck}$ activation on peripheral blood T cells. Since the presence of two distinct CD8 ligands expressed on epithelial cells is unlikely, most likely one mAb recognizes the CD8 ligand

whereas the other has blocking function either because of steric hindrance of cell:cell interactions or the recognization of another other regulatory protein that interacts with $CD8^+$ T cells. The final proof of these scenarios depends upon the specific isolation of the surface protein. We have identified a 43kd protein recognized by mAb L12 on both $^{35}S$ metabolic labelling and western blot, and a 55kd protein recognized by mAb B9 on $^{35}S$ metabolic labelling. Further determination of the amino acid sequence and expression of these proteins in vectors would allow us to test the direct binding between CD8 and the novel CD8 ligand.

Human intestinal epithelial cells acting as antigen presenting cells (APC) are distinct from conventional APC, monocytes/dendritic cells. The previous published results from our laboratory have indicated that antigen processing and presentation by epithelial cells is much slower than monocytes although the efficiency in turns of stimulation of T cells was similar (Mayer et al., 1987). In addition, these two kinds of APC activated two different T cell subsets, i.e. epithelial cells stimulated $CD8^+$ suppressor T cells and monocytes activated $CD4^+$ helper T cells. In this thesis, we found that epithelial cells selectively bound to CD8 and activated CD8-associated $p56^{lck}$. In contrast, monocytes selectively bound to CD4 and activated CD4-associated $p56^{lck}$. Furthermore, the kinetics of $p56^{lck}$ activation induced by the different cell types was distinct. Epithelial cells induced early $p56^{lck}$ activation and monocytes induced relatively late $p56^{lck}$ activation. Blocking of $p56^{lck}$ activation in the presence of a protein tyrosine kinase inhibitor, genestein, was required early in culture in order to inhibit

epithelial cell stimulated T cell proliferation whereas blocking of monocyte triggered T cell proliferation could be achieved by addition of genestein even two days following onset of culture.

Since the activation pattern of p56lck induced by epithelial cells and monocytes was different, the pattern of substrate phosphorylation by $p56^{lck}$ might be distinct. By employing $^{32}P$ metabolic labelling and an in vitro tyrosine kinase assay, we have identified a 97kd protein, which was phosphorylated by $p56^{lck}$ activated by both cross-linking CD8 stimulating and by epithelial cells, but not by monocytes. The phosphorylation of unique substrates might affect the direction of activation of T cells. Although activation through CD8 might stimulate $Ca^{2+}$ release and tyrosine phosphorylation of important intracellular mediators, such as PLC-γ1, Raf-1, GTP-binding protein, etc., our data suggested that stimulation through CD8 alone by cross-linking CD8 with antibodies is necessary but not sufficient for promoting T cell proliferation and that a second signal is requited.

Since our initial studies were performed in either an antigen specific (tetanus toxoid) or allogeneic mixed cell culture system, the likeliest candidate for the second signaling molecule appeared to be the T cell receptor itself. This hypothesis was strengthened by the studies which demonstrated co-localization of the CD8 or CD4 molecule with the TCR complex during cellular activation (Anderson et al., 1988). Coreceptor formation may also modify the signals received through the TCR alone. Therefore the second signal was most likely through the TCR/CD3 since the TCR/CD3 complex plays a central role in conventional antigen-specific T cell activation. By

employing specially made bifunctional antibodies, anti-CD3/CD8 (constructed by the chemical linking of two Fab hetero-fragments), we were able to see increased IL-2 receptor expression and significant T cell proliferative responses to this bifunctional antibody in the presence of IL-2. The requirement for cross-linking CD3 to CD8 was evidenced by the fact that isolated anti-CD3 and anti-CD8 mAbs separately or in combination failed to stimulate T cell proliferation. The bifunctional antibody brought CD3 and CD8 molecules together and to form a co-receptor in a more physiologic way. The inhibition of the binding of one arm of anti-CD3/CD8 by soluble mAb markedly reduced T cell responses. The formation of CD3/CD8 or CD3/CD4 microaggregates as T cells were activated has been well documented. Therefore, such an approach might help us define the true mechanism of T cell activation induced by antigen presenting cells. Furthermore, our bifunctional antibody anti-CD3/CD8 induced suppressor T cell proliferation whereas cross-linking CD3 and CD8 by mAbs fixed on solid phase has been previously reported to stimulate cytotoxic T cell subset. This is the first report of suppressor T cell activation and proliferation in vitro using antibodies. Thus, the application of this bifunctional antibody anti-CD3/CD8 should be helpful in understanding the mechanisms of the generation and function of the suppressor T cells.

Analysis of the signal transduction pathway utilized by both intestinal epithelial cells and bifunctional antibodies has shown that both activate the protein tyrosine kinase $p56^{lck}$ and induce tyrosine phosphorylation of abundant cellular protein substrates. A protein tyrosine kinase inhibitor, genestein, was able to inhibit both

intestinal epithelial cell and bifunctional antibody induced T cell proliferation. Therefore, we found that epithelial cells and bifunctional antibody anti-CD3/CD8 used similar tyrosine kinase activation as a signal transduction pathway.

T cell proliferation in response to the bifunctional antibody anti-CD3/CD28 was generally greater than that seen with CD3/CD8 and was independent of IL-2. These activated T cells possessed cytotoxic activity rather than the suppressor cell activity seen with CD3/CD8 stimulation.

CD28 is not expressed on suppressor T cells and expression of the CD28 ligand, B7/BB1, on monocytes has been demonstrated to be important for the induction of cytotoxic T cells (Li et al., 1990). Thus far we have been unable to detect B7/BB1 on intestinal epithelial cells even after IFN-γ induction for 24 hours. Therefore, in the mucosa where the epithelial cells may be providing the initial signals to T cells, the presence of a novel CD8 ligand, cross-linking CD8, in the absence of B7/BB1 may account for the selective proliferation of $CD8^+$ antigen nonspecific suppressor T cells.

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