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The Functional Characterization of gp180, a Novel CD8 Ligand Expressed by Intestinal Epithelial Cells

by

Nicola A. Campbell

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the Degree of Doctor of Philosophy, The City University of New York.

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ABSTRACT

The functional characterization of gp180, a novel CD8 ligand expressed by intestinal epithelial cells.

by

Nicola A. Campbell

Advisor: Professor Lloyd Mayer, MD

The immune system in the gastrointestinal tract has evolved to allow it to react to potential pathogens while at the same time maintaining a tolerant state to dietary antigen and normal luminal constituents. The overall tone of the mucosal immune system is one of suppression or down regulated immune responses. This is evident from the limited number of oral vaccines. Several unique phenomena underscore the difference between the mucosal and systemic immune systems. Most strikingly, an active non-responsiveness develops following oral antigen priming. This state is referred to as oral tolerance. It is believed that the activation of suppressor T cells in the intestinal tract is one of the mechanisms involved in the development of this tolerant state yet the manner in which this subset of T cells is activated is currently unknown.

One of the problems plaguing immunobiologists has been documenting the existence of suppressor T cells. While suppressor T cells have been reported in functional assays, defined populations have not been isolated or cloned. This may relate to the possibility that suppressor T cells are regulated in a manner distinct from conventional T cells. In such a model, either distinct intracellular

signaling pathways, unique restricting elements or both might regulate the activation of such cells. The rationale for the generation of suppressor T cells in the gut is clear. The absence of suppression could be costly, resulting in active immunity, inflammation, and the loss of functional integrity in the gastrointestinal tract. In this thesis, we set out to investigate the mechanisms and molecules which may be involved in the generation of this suppressive state in the gastrointestinal tract.

It has been previously demonstrated that intestinal epithelial cells can act as antigen presenting cells (APC) capable of stimulating primed T cells. Interestingly, despite the constitutive expression of class II MHC molecules on these cells, the T cells proliferating in these co-cultures are $CD8^+$. The subset of peripheral blood T cells that proliferate when co-cultured with intestinal epithelial cells are $CD8^+CD28^-$ T cells. These T cells appear to have suppressor activity as they inhibit primary, secondary and unrelated mixed lymphocyte reactions, as well as B cell responses *in vitro* in an antigen non-specific manner. The addition of monoclonal antibodies against CD8 to these co-cultures has documented that the CD8 molecule itself is important in the activation of suppressor T cells by IECs, with the activation of the CD8-associated protein tyrosine kinase $p56^{lck}$ being a necessary but not sufficient event. The activation of $CD8^+$ suppressor T cells appears to involve a novel 180 kd glycoprotein (gp180) expressed on the surface of intestinal epithelial cells. This molecule is heavily N-glycosylated and has two forms, an apically sorted GPI anchored form and a basolateral transmembrane form. Interestingly, purified

gp180 appears to be responsible for the activation of the CD8-associated protein tyrosine kinase $p56^{lck}$ but is not enough to induce $CD8^+$ T cell proliferation. Co-culture of T cells with intact intestinal epithelial cells results in the activation of both CD8-associated $p56^{lck}$ and TCR-associated $p59^{fyn}$, and results in the proliferation of $CD8^+$ T cells.

While investigating why intact intestinal epithelial cells, but not purified gp180, results in the activation of the TCR-associated protein tyrosine kinase $p59^{fyn}$, it was determined that CD1d associated with and aided gp180 in the activation of $CD8^+$ suppressor T cells. Furthermore, we demonstrated that gp180 directly binds to CD8 molecules through sites distinct from those bound by classical class I MHC. In our model, gp180 associates with the non-classical class I molecule CD1d on the surface of intestinal epithelial cells. More specifically, we found that each component of this complex has a unique function: gp180 binds to the CD8 molecule resulting in the activation of the CD8-associated kinase $p56^{lck}$ while CD1d interacts with the TCR causing the phosphorylation of the TCR-associated kinase $p59^{fyn}$. The gp180:CD1d complex thus appears more like classical class I, capable of interacting with the TCR:CD8 co-receptor complex.

Finally, we determined that this novel complex targets a unique population of $CD8^+$ T cells which appear to be pre-committed to becoming suppressor cells. During these studies, it was determined that $CD8^+$ T cells with both suppressor and cytolytic activity were activated via the MAP kinase cascade. However, the activation of this MAP kinase pathway in suppressor T cells was

accomplished via the nucleotide exchange factor vav rather than the PLC-γ1 pathway as was observed in CTL. This suggests that these subsets of $CD8^+$ T cells signal through distinct pathways. Finally, it was determined that soluble gp180 was not able to effectively inhibit CTL killing compared to anti-class I MHC and anti-CD8 mAbs. This suggests that gp180 binding to CD8 on CTL either does not interfere with the ability of class I MHC to bind and deliver a dominant signal, or that other molecules on CTL, such as CD28, deliver a signal which may downregulate the pathway mediated by gp180. Overall, these data suggest that $CD8^+$ T cells may be pre-committed to become either suppressor or cytolytic T cells.

In summary, our model relies on the finding that intestinal epithelial cells express gp180 and the non-classical class I molecule CD1d in an associated form. In addition to interacting with the TCR and phosphorylating TCR-associated $p59^{fyn}$, CD1d appears to be involved in T cell maturation. Data generated in this thesis indicate that suppressor T cells are restricted by the non-classical restriction element CD1d, unlike CTL which are class I MHC restricted. It now appears that IECs target a selected subpopulation of "pre-suppressor" cells which results in the CD1d:gp180 complex transducing a specific signal that promotes suppressor T cell activation.

Format of Thesis

This thesis is prepared according to the guidelines of Graduate School of Biological Sciences, Mount Sinai School of Medicine, City University of New York. This thesis has a general introduction with literature review, material and methods, three sections, general discussion and general references. Each section contains an abstract, introduction, methods, results, discussion and references.

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ABBREVIATIONS

APC:	antigen presenting cell
CTL:	cytotoxic T lymphocyte
FCS:	fetal calf serum
FBS:	fetal bovine serum
GALT:	gut associated lymphoid tissue
GPI:	glycosyl-phosphatidylinositol
IBD:	infammatory bowel disease
IEC:	intestinal epithelial cell
IEL:	intraepithelial cell
Ii:	invariant chain
KGF:	keratinocyte growth factor
LPL:	lamina propria lymphocyte
mAb:	monoclonal antibody
METR:	mixed epithelial cell/T cell reaction
MLR:	mixed lymphocyte reaction
NL:	normal
NWT:	nylon wool T cells
PBMC:	peripheral blood mononuclear cells
PBS:	phosphate buffered saline
PBT:	peripheral blood T cell
PIPLC:	phosphatidylinositol specific phospholipase C
PP:	peyer's patches
TCR:	T cell receptor

FOOTNOTES

*: a portion of the experiment was conducted by a laboratory colleague, Lisa Toy, MD.

INTRODUCTION

I. Phenomena unique to the mucosal immune system

The goal of the systemic immune system is to encounter foreign antigen and to generate a specific response that results in the elimination of the potential pathogen. In the gastrointestinal tract, however, the exposure to dietary and microbial antigens is magnified, yet there is a limited immune response. The unique yet sophisticated role of the mucosal immune system effectively sets up three distinct barriers to protect against microbial invasion: the physiologic barrier, the anatomic barrier and the immunologic barrier.

The physiologic barrier, including mucus, acid, luminal proteases as well as a number of bactericidal and bacteriostatic products, attempts to kill or disable pathogens before entry is achieved. In addition, antigens entering the intestinal lumen are affected by gastric acid and pepsin, alkaline pancreatic, intestinal and biliary secretions containing various proteases, lipases, amylases and bile salts. All of these alter the chemical properties of ingested antigens. The next barrier encountered by a pathogen is the single layer of mucosal epithelium that separates the internal environment (lamina propria) from the antigen rich external milieu of the intestinal lumen. This mucosal epithelium makes up the anatomical barrier which controls the entry of antigens through the mucosa to maintain homeostasis. This epithelial layer is joined by tight junctions which also helps to prevent antigen access. In addition, the epithelium is covered by a thick mucus coat in order to

prevent pathogen penetration between epithelial cells. Finally, the immunologic barrier, primarily IgA, attempts to both prevent entry and neutralize potential pathogens. Thus, mucosal surfaces have evolved highly efficient and effective mechanisms to prevent microbial invasion and infection.

Intestinal epithelial cells also appear to play a role in cellular immunity in the gastrointestinal tract. Normal absorptive IECs express class I and class II MHC on their surface and appear to be capable of antigen presentation (1-3). The ability to present antigen allows these cells to interact with nearby mucosal T cells, resulting in their activation. Thus, IECs appear to be involved in important regulatory mechanisms since alterations in the ability of IECs to present antigen may result in immune dysregulation and loss of suppression.

The establishment of distinct barriers in mucosal immunity are not the only features adapted by the gut to handle such enormous antigen loads. Other features, such as controlled inflammation and oral tolerance, which are unique to the mucosal immune system appear to play a significant role in the maintenance of suppression.

Controlled inflammation

The antigenic challenge to the intestinal immune system is enormous. It has been estimated that the number of microbial antigens in the intestine exceeds the total number of cells in the body. In addition, there are also the abundant number of antigens present in the diet. Exactly how the mucosal immune system deals with this challenge is not completely characterized, however, it is

apparent that the mucosal immune system is in a constant state of response as documented by the large number of plasma cells, and activated T and B cells, present throughout the intestine (4). The fact that this activated state is antigen driven is supported by studies using germ-free animals (5) where the mucosal lymphoid tissue is poorly developed. Once these animals are conventionalized (normal gut flora is introduced) lymphocytes rapidly populate the lamina propria and become activated. However, regardless of what is administered via the gastrointestinal tract, this inflammatory state remains constant with tissue damage. These observations have led to the concept of controlled or physiologic inflammation, in which the normal intestine is viewed as being in a state of inflammation due to the massive antigenic challenge but regulated from becoming overactive. The overall objective of the mucosal immune system is to maintain a careful balance by reacting to potential pathogens while at the same time maintaining a tolerant state to dietary antigen and normal luminal constituents.

Oral tolerance

Oral tolerance is the state of systemic immune hyporesponsiveness following antigenic priming via the gastrointestinal tract. This state of non-responsiveness is antigen specific and can be induced by oral administration of many different protein antigens prior to a systemic antigen challenge (6-17).

A classic experiment describing the phenomenon of oral tolerance involved the feeding of ovalbumin (OVA) to naive mice. Those mice fed with OVA prior to a systemic challenge did not

develop delayed-type hypersensitivity (DTH) or produce antibodies to OVA in contrast to the response seen when mice were immunized by intraperitoneal injections (9, 18, 19). Initial studies suggested that this systemic tolerance could be transferred to a naive mouse via $CD8^+$ splenocytes (20). It must be mentioned, however, that subsequent studies have shown that $CD4^+$ T cell, but not $CD8^+$ T cell, depletion inhibits the ability to induce tolerance by oral administration of ovalbumin (21), indicating that more than one mechanism may be responsible. Dissecting the mechanisms behind oral tolerance could be critical to an understanding of mucosal immunoregulation.

Although many mechanisms are involved in the induction of oral tolerance (clonal anergy (22), generation of tolerogenic molecules by luminal proteases (19, 23), and suppressive immune complexes (24, 25)), some data indicates that suppressor T cells transfer oral tolerance to a naive animal (20). Further evidence for the importance of suppressor T cells comes from the fact that pretreatment of ovalbumin fed mice with cyclophosphamide or 2'-deoxyguanosine, inhibiting suppressor T cell generation, abrogated the induction of oral tolerance (26, 27). More recently, several groups have described two forms of oral tolerance: high doses of fed antigen resulted in clonal anergy and deletion while low dose tolerance was the result of active suppression by $CD8^+$ T cells (19) as well as by regulatory cytokines (IL-4, IL-10, TGF-β) secreted by $CD4^+$ T cells in the Peyer's patch (28).

II. Antigen processing and presentation by intestinal epithelial cells

Attempts to define the mechanisms underlying the induction of oral tolerance led to studies relating to antigen handling in the gastrointestinal tract. There appear to be several ways in which antigen can enter the lamina propria of the gastrointestinal tract.

Antigen trafficking pathways

As in the systemic immune system, antigen is able to initiate an immune response in the mucosal immune system. There are three documented ways in which luminal antigen can enter the lamina propria. Firstly, antigen may enter through the membranous epithelial cell (M cells) which are specialized epithelial cells overlying the Peyer's patches. M cells are able to endocytose large particulate antigen and transport it to the subepithelial space. Although M cells are highly efficient in trafficking antigen (29-33), the limited number of M cells in the gut seems insufficient to handle the enormous antigen load in the gastrointestinal tract. Secondly, antigen may enter the lamina propria through the tight junctions between absorptive epithelial cells (paracellular transport) or, thirdly, through absorptive epithelium (transcellular transport) (34).

Paracellular antigen transport relates to antigen entry through the tight junctions between IECs. Under normal conditions, these tight junctions are not permeable to macromolecules, allowing only small ion transport. The continuous sheet of epithelial cells created by these tight junctions and desmosomes, acts as a physical barrier to the antigen load in the lumen of the gastrointestinal tract. It has

been shown, however, that this physical barrier can be disrupted by *Clostridium difficile* toxin A (35) and IFN-γ (36). In areas of active inflammation, such as in inflammatory bowel disease, a dramatic increase in IFN-γ results in the breakdown of tight junctions. This allows for the paracellular transport of antigen from either the lumen to the lamina propria or conversely, the lamina propria to the lumen. The presence of antigen in the lamina propria may then lead to overwhelming immune responses in an attempt to remove it.

Finally, transcellular transport of luminal antigen may occur through the absorptive epithelium. Normal absorption involves IECs that can transport non-immunogenic di-peptides and tri-peptides across the epithelial layer. It appears that approximately 2% of intact dietary antigen is transported into the lamina propria and systemic circulation (37, 38). Proteins endocytosed at the apical surface by non-specific endocytosis are apparently targeted to lysosomes (39), with a small fraction escaping and translocating to the basolateral surface. The absorptive epithelium may also play the role of an antigen presenting cell.

Antigen presentation in IECs

In the late 1970s, IECs were found to express class II MHC molecules thus indicating the potential immunoregulatory function of absorptive IECs (40). Over the next few decades, many laboratories have documented that enterocytes possess the ability and efficiency to process and present antigen in both human and rodent systems (1, 41). Mayer and Schlien have demonstrated that freshly isolated human IECs are able to present tetanus toxoid to

primed T cells (42). Processing of tetanus toxoid by enterocytes is required before the antigen can be presented, since the pretreatment of IECs with glutaraldehyde or paraformaldehyde block this activity (42). Like conventional APCs, antigen presentation by IECs could be blocked by agents which disrupt the acidic pH in endosomes such as chloroquine, ammonium chloride or monensin (3). Leupeptin, however, had no effect on IEC presentation (3), suggesting that processing enzymes may be different from those seen in macrophages. Overall, IECs appear to be less efficient at presenting antigen than conventional APCs.

Using fluorescein conjugated tetanus toxoid as a model, the efficiency of antigen processing by enterocytes has been further studied. When compared to conventional APCs, antigen uptake was shown to be slower and processing prolonged inside enterocytes (43). One explanation is that the processing and degradation machinery in the IEC is somewhat limited and therefore, IEC processing may rely on the preprocessing of antigens in the intestinal lumen by digestive enzymes. An alternative explanation may be that IECs process antigen in a unique manner, different from that of conventional APCs. The slow rate of processing might lead to persistence of antigen in the IEC, resulting in prolonged and continuous stimulation of a distinct set of intestinal lymphocytes. It is still not known if IEC processing generates the same set of peptides as conventional APCs. Furthermore, IECs produce a number of immunoregulatory cytokines such as IL-6 but fail to produce IL-1β (41, 44). This may reflect the difference in the functional requirements for IECs when serving as an APC as compared to

monocytes. IECs also express cytokine receptors, such as those for IL-1, IL-2, IL-3, IL-10 and IFN-γ (1, 36, 45-47) and express adhesion molecules such as LFA-3 and LFA-1, which are important in T cell interactions (41). It should be noted however, that IECs lack the important co-stimulatory molecules B7.1, B7.2 and CD40, indicating that IECs may represent an exception to the general rule in conventional antigen presentation.

Overall, IECs possess other requirements of an APC: the expression of class II MHC, expression of adhesion molecules, secretion of some accessory cytokines and expression of cytokine receptors. It was shown that T cells could be stimulated to proliferate by co-culturing them with IECs both in antigen specific and in allogeneic co-culture systems (2, 3, 42, 48). Interestingly, the subset expanded in these IEC:T cell co-cultures appeared to be $CD8^+CD28^-$ T cells with suppressor function (49). While IECs express class II MHC on their surface, the ability of IECs to induce the proliferation of $CD8^+CD28^-$ T cells was not inhibited by anti-class II MHC antibodies (50). Furthermore, this does not appear to relate to class I MHC antigen presentation by IECs since antibodies to MHC class I have no effect in this system. One molecule known to be involved in this interaction is CD8 since antibody to CD8 blocks $CD8^+$ suppressor T cell proliferation (42, 50).

To investigate other molecules which may be involved in the generation of the suppressive state in the gastrointestinal tract, a series of mAbs were generated against human IECs. These antibodies were screened for their ability to stain IECs and to inhibit $CD8^+$ suppressor T cell proliferation in an allogenic mixed

epithelial cell/T cell reaction system (METR). Two mAbs, B9 and L12, were found to be potent inhibitors of the METR (50). Both antibodies stained IECs, but did not recognize CD1d, class I MHC or class II MHC molecules. In addition, they did not stain peripheral blood T cells or B cells (50). It was then documented that the mAbs B9 and L12 recognize a 180 kd glycoprotein expressed on the surface of IECs. This molecule, called gp180, is heavily N-glycosylated (76 kd protein core) and appears to have two distinct forms: an apically distributed GPI-anchored form and a basolaterally distributed transmembrane form (51).

Several further findings support the hypothesis that novel epithelial cell surface molecules, such as gp180, play an important role in the induction of $CD8^+$ suppressor T cells in the gut. First, CD8 molecules themselves are a critical element in IEC induced $CD8^+$ suppressor T cell proliferation, since mAbs to CD8 inhibit such proliferation. Interaction of $CD8^+$ T cells with IECs results in intracellular protein tyrosine phosphorylation apparently via activation of CD8-associated $p56^{lck}$ and IECs selectively activate $p56^{lck}$ in human CD8α transfectants (3G8) but not human CD4 transfectants (3G4). Early inhibition of tyrosine kinase in this system by genestein or herbimycin completely abrogates IEC induced $CD8^+$ suppressor T cell proliferation. Thus, it appears that in IEC:T cell interactions, binding to CD8 by a molecule on IECs is essential and results in the activation of CD8-associated $p56^{lck}$ and downstream signaling components which eventually results in the proliferation of $CD8^+$ suppressor T cells.

Secondly, mAbs to gp180 (B9 and L12) significantly inhibit IEC induced activation of CD8-associated $p56^{lck}$ in T cells. This supports previous data which found that B9 and L12 are potent inhibitors of $CD8^+$ suppressor T cell proliferation induced by IECs, as the activation of $p56^{lck}$ is critical for IEC induced $CD8^+$ suppressor T cell proliferation in our system.

Lastly, studies in our laboratory have documented that the CD8-associated protein tyrosine kinase $p56^{lck}$, but not the TCR-associated kinase $p59^{fyn}$, is activated when T cells are stimulated by purified gp180 (51). This is in contrast to studies using intact IECs where activation of $p59^{fyn}$ was also seen. These data suggest that gp180 may be associated with a molecule that is capable of interacting with the TCR and activating $p59^{fyn}$. Since IECs express the non-classical class I molecule CD1d and IEC-induced $CD8^+$ T cell proliferation could be inhibited by anti-human CD1d mAbs (52), CD1d was considered an eligible candidate to aid gp180 in the activation of $CD8^+$ suppressor T cells in the gastrointestinal tract.

Non-classical MHC molecules

Although the role of classical restriction elements has been relatively clearly defined, the function of the structurally related class Ib proteins has not yet been thoroughly investigated. These molecules include the CD1 family of proteins. The human CD1 gene family is composed of five members, four of which (CD1a-d) are known to be expressed *in vivo*. The CD1 family of class I-like proteins is encoded outside the MHC and most members have limited homology with regard to classical class I molecules (53).

Interestingly, the CD1 family can bind lipid antigen as well as peptides approximately 20 to 50 amino acids in length. It is, therefore, suspected that these molecules may have a unique interaction with specialized populations of T cells. For example, CD1b has been reported to present nonpeptide lipoglycan mycobacterial antigen to human $CD4^-CD8^-$ T cells (54). In addition, murine CD1.1 (corresponding to human CD1d) has been shown to bind highly hydrophobic lipids (55) such as those derived from mycobacteria.

It has been found that CD1d is expressed predominantly by IECs (56) and hepatocytes. When CD1d is immunoprecipitated from the surface of IECs, it appears that two separate forms exist. The first is a 37 kd protein which is neither glycosylated nor associated with β_2-microglobulin, while the other is a 48 kd protein which appears to be β_2-microglobulin associated (56). The existence of two forms of CD1d suggests that two different CD1d processing pathways exist. It has been documented that a TAP-independent, β_2-microglobulin dependent molecule is responsible for the development of most $CD8\alpha\beta^+$, $\alpha\beta TCR^+$ IEL in the mouse (57). The expression of the β_2-microglobulin associated form of CD1d by IECs makes it an eligible candidate to fulfill this role. The role of the non-glycosylated, non-β_2-microglobulin associated form of CD1d in the intestinal mucosal immune system is investigated in this thesis.

The predominant expression of CD1d by human IECs suggests that CD1d may be a ligand for T cells in the intestinal epithelium. As previously noted, IEL are located in between IECs and the majority are $CD8^+$ T cells (58). It should be noted however that

CD1d does not bind CD8 molecules (59). Furthermore, investigations by this laboratory have indicated that freshly isolated IECs can efficiently stimulate the proliferation of allogeneic peripheral blood lymphocytes and that this stimulation is blocked by anti-human CD1d mAbs (52). Finally, anti-CD1d antibodies also inhibit IEL-mediated cytotoxicity of IECs.

Recent data have indicated that CD1d is expressed in a polarized fashion on IECs (60). CD1d localization to the apical and lateral membrane domains places CD1d in an ideal location to serve as a luminal antigen sampling and presentation molecule to laterally positioned IEL (61). In addition, CD1d has been found to congregate just beneath the apical surface (60) suggesting that CD1d may be cycling between the apical and basolateral regions and essentially sampling antigen from the lumen and presenting it to IEL. The non-polymorphic nature and class I-like structure make CD1d a candidate ligand for TCR mediated activation of $CD8^+$ T cells in the epithelium.

III. CD8+ T cell populations

The literature thus far has documented the importance of $CD8^+$ T cells in mucosal immunity. Furthermore, it has been shown by various groups that IECs selectively activate $CD8^+$ T cells. We were, therefore, interested in determining which T cell populations in the gut may interact with IECs and the different effector activity that these cells may possess.

Intraepithelial lymphocytes

Intraepithelial lymphocytes are a population of cells located between IECs near the basement membrane and are predominantly $CD8^+$ T cells, with $CD8\alpha\alpha$ cells comprising a significant percentage in the mouse (62). It has been postulated by some that these cells exist in this location either by specific homing to this site or by actually developing within the gastrointestinal tract (ie. extrathymic development). The ratio of IEL to IEC ranges from as high as 1:6 to as low as 1:100 and are found most abundantly in the jejunum (63). The cellular composition of this compartment is different from that in either the PP or the lamina propria. Plasma cells are not present, and B cells are absent or infrequent. In most mouse strains, about half bear the $\gamma\delta$ TCR and the other half $\alpha\beta$ TCR (63). In humans, the majority of these $CD8^+$ IEL express the adhesion molecule $\alpha_e\beta_7$ and only 2-5% of IEL express the $\gamma\delta$ TCR (64). Although IEL have a low rate of proliferation and do not respond to conventional lectins (PHA, ConA and pokeweed mitogens), IEL do proliferate in response to the IEC produced (as well as thymically derived) cytokine IL-7. Interestingly, IL-7 knockout mice lack IELs.

Furthermore, IEL proliferation can be stimulated by the combination of anti-CD2 and IL-2. This can also be achieved by culturing IEL with sheep red blood cells, which express the CD2 ligand LFA-3, in the presence of IL-2, thus resulting in IEL activation.

Due to the close proximity of IEL to the IEC, it is not surprising that each influences the other. It has been suggested that two distinct types of IEL exist, a granulated and a non-granulated form. Approximately 50% of IEL are devoid of the thymic marker Thy-1 which suggests that these cells are not thymically derived. It is primarily the granulated form of IEL that fits into this category (65, 66). It has been suggested that the intestinal epithelium can attract IEL precursors to the gut before TCR rearrangement has occurred and provide a microenvironment similar to the thymus which is conducive to differentiation. This theory has been supported by the finding of fully differentiated IEL in athymic mice (63). Another theory exists which states the IEL originate from precursors in the PP which migrate back to the mucosal epithelium after traveling through the mesenteric lymph nodes, thoracic ducts and systemic circulation (67). However, the majority of IEL express the memory marker CD45RO (in contrast to peripheral blood T cells which predominantly express a marker indicating a naive state, CD45RA) suggesting that IEL have previously encountered antigen (68, 69).

Although IEL appear to be primarily cytolytic, some evidence suggests that they are also capable of suppressing mucosal immune responses (70). *In vitro* data suggest that IEL effectively decrease the production of immunoglobulin, such as IgA, by subepithelial

plasma cells. In addition, most $CD8^+CD3^+$ IEL fail to express the cytolytic T cell marker H366 providing further evidence that IEL are not primarily CTL (71). Finally, functional suppressor T cells derived from leprosy patients also possess the machinery for cytotoxicity and can kill targets under certain experimental settings where the specific antigen is appropriately presented by the targets (72). This suggests that IEL may be able to act in either a cytotoxic or suppressive capacity.

Just as IECs have the potential to influence IEL, the IEL have an important impact on epithelial cells. *In vitro* analysis has determined that many IEL have potent cytolytic properties and produce cytokines such as IL-2, IL-3, IL-4, KGF, TGF-β, TNF-α and IFN-γ (36, 64, 73, 74). KGF has been shown to be important for IEC growth and differentiation. Among other activities, IFN-γ effectively breaks down the tight junctions involved in epithelial adherence (36, 75), enhances the production of secretory component (65, 74, 76) and dramatically increases the expression of classical restriction elements, particularly class I and class II MHC (77). Further increases in secretory component production can be obtained from IL-4 and TNF-α (65, 76, 78). Increased or altered expression of these molecules may result in significant immunologic dysregulation. Additionally, alterations in the expression of these various molecules by the epithelial cells may, in turn, effect the antigen presenting capabilities of these cells. Interestingly, IEL display significant oligoclonality, even expressing similar TCRs in distinct regions of the gastrointestinal tract. Given the difference in the luminal antigen environment in different sites, it is unlikely

that IELs recognize luminal antigens. Therefore, in all probability, IEL do not play a role in host defense but do play a critical role in keeping the mucosal immune system intact.

Lamina propria lymphocytes

Roughly one-third of the lamina propria T lymphocytes are $CD8^+$. The lamina propria is located beneath the epithelial layer in a loose connective tissue stroma. The lamina propria is composed of plasma cells (primarily IgA secreting), T cells, macrophages, and mast cells. When compared to IEL, lamina propria lymphocytes are significantly different. While IEL are predominantly T lymphocytes, LPL are composed of approximately equal numbers of T and B cells. As mentioned above, LPLs do express CD8, but there appears to be two-fold greater $CD4^+$ T cells present similar to that seen in the peripheral blood (79). Phenotypically, LPL express CD45RO (a memory marker), CD69 (an activation marker) and constitutively express the IL-2 receptor. These cells proliferate poorly to stimuli mediated through the TCR, however, 95% of LPL proliferate in response to anti-CD28 and anti-CD2 antibodies. The cytokine profile secreted by these lymphocytes appears to be TH2-like and suppressive in nature. Overall, it appears that lamina propria lymphocytes are a unique population that appear to be phenotypically positioned between peripheral blood T cells and IEL.

Suppressor T cells

A unique subset of $CD8^+$ T cells are the suppressor cells. Suppressor T cells are a class of lymphocytes thought to be distinct

from helper and cytolytic T lymphocytes, whose function is to inhibit the activation phase of immune responses. The existence of suppressor cells was first suggested in the 1960s and 1970s where functional assays of inhibition were established. In the classical oral tolerance experiments, initial studies suggested that systemic tolerance, induced by feeding of the antigen prior to systemic challenge, could be transferred to a naive mouse via $CD8^+$ splenocytes. It has been determined that suppressor T cells have several unique properties (80). First, the cells that inhibit many immune responses appear to be $CD8^+$ T cells and their growth and differentiation may be dependent on $CD4^+$ T cells (81, 82). Second, the role of the MHC in the development and activation of suppressor T cells is unclear. Early studies done in mice indicated that the stimulation of suppressor T cells was restricted by a region of the class II MHC called I-J. The I-J glycoprotein was thought to distinguish suppressor cells from all other cells. In addition, this same structure was thought to comprise part of secreted glycoproteins with suppressive activity. Antibodies were generated to I-J and were found to bind to both suppressor T cells and to the secreted glycoproteins (83). I-J was thought to be located between the I-A and I-E loci based on analyses of various inbred strains, however, sequencing of the entire mouse class II MHC has demonstrated that there is no DNA coding for a unique I-J molecule at this site, and attempts to demonstrate an I-J encoded cell surface glycoprotein have generally failed. Much controversy has surrounded the presence of I-J and it is still not clear whether a

molecule such as this exists. The explanation for this apparent artifact remains obscure.

A major problem in the study of suppressor T cells has been that attempts to purify these cells in numbers sufficient for biochemical analyses of receptors and secreted products, or to establish stable cloned lines and hybridomas with specific suppressive activity, have been largely unsuccessful. As a result, even basic questions, such as the nature of the receptors expressed by suppressor cells, are unresolved. There is evidence to suggest that suppressor T cells do exist, the problem now is determining how these cells are activated and the mechanisms by which their suppressor function is mediated.

Cytotoxic T cells

Cytotoxic T lymphocytes (CTL) are a subset of $CD8^+$ T cells that kill target cells expressing specific antigen. CTLs appear to be important effector cells in intracellular infections of non-phagocytic cells (such as viral infections or infections by intracellular bacteria such as *Listeria monocytogenes*), acute allograft rejection and rejection of tumors. The majority of CTL express the CD8 molecule and specifically recognize foreign peptides derived from intracellularly synthesized antigens associated with self class I MHC molecules.

CTL undergo thymic selection in a similar manner to $CD4^+$ T cells. Although CTL are not fully differentiated when they exit the thymus, they do express a functional $\alpha\beta$ T cell receptor and recognize antigen, however they lack cytolytic function. These pre-

CTL are class I MHC restricted and committed to the CTL lineage. Differentiation of pre-CTLs to functional CTLs requires at least two separate signals: the first is specific recognition of antigen on a target cell and the second depends on T cell derived cytokines (84). The precise cytokines required are not known. It is likely that IL-2 and IFN-γ are important but several other cytokines, including IL-4, IL-6, IL-7 and IL-12 have been found to play some role in some *in vitro* experiments (84). In addition, the identity of the T cell responsible for providing these cytokines is not well established. In most experiments, there appears to be a requirement for $CD4^+$ helper T cells that respond to antigens presented by class II on APCs (85). Indeed, it may be possible that such cytokines may be produced by CD8+ cells and act in an autocrine fashion, giving rise to "helper-independent" CTLs. The importance of the generation of $CD8^+$ cytotoxic T cells in the context of this thesis, is to gain a better understanding of the necessity of $CD8^+$ T cells to be pre-committed to a specific lineage.

IV. T cell signaling via the T cell antigen receptor/CD8 coreceptor complex

T lymphocytes play a central role in specific immune responses to protein antigen. Pools of predominantly normal self proteins are sampled by class I and class II MHC and displayed to the T cell repertoire, which surveys these samples for the rare foreign or mutant peptide. Antigen recognition by T cells is the initiating stimulus for T cell activation. In different T cells, activation leads to the secretion of cytokines, proliferation, and the performance of regulatory or cytolytic effector functions. The receptors on T cells that are responsible for the specific recognition of and response to antigen plus MHC are composed of several integral plasma membrane proteins. Some of the proteins in this complex mediate specific binding to peptide-MHC complexes on the surface of APCs or target cells. The subunits of this complex that are involved in antigen binding differ among T cells with different antigen specificities. Other proteins in the complex are invariant among all T cells and they have been implicated in T cell signal transduction. In addition, T cells express a number of other cell surface proteins which are collectively called accessory or co-stimulatory molecules. These molecules are important in cognate activation and effector phases of T cell responses.

The antigens recognized by TCRs are short peptides 8 to 20 amino acid residues in length which are bound to the antigen binding cleft of either class I MHC or class II MHC on antigen presenting cells (86). Conventionally, an appropriate class I MHC-peptide complex forms a bridge between the TCR/CD3 complex and the CD8

molecule on $CD8^+$ T cells, eliciting a cytotoxic immune response against endogenously processed antigens such as viruses (87). Class II MHC, on the other hand, forms a bridge between the TCR/CD3 complex and the CD4 molecule on $CD4^+$ T cells, and these cells function to aid in the generation of active immunity against exogenously processed antigens (87). Adhesion molecules such as LFA-1, LFA-2 and ICAM-1 serve to enhance the avidity of the interaction between the APC and the T cell (88). Upon optimal stimulation of the T cell, protein tyrosine kinases (PTKs) associated with the CD4/CD8 molecule and the TCR are independently phosphorylated (89). It is the activation of these kinases that initiates other downstream signaling cascades leading to the translocation of transcriptional regulatory factors into the nucleus and the eventual transcription of cytokine genes in the T cell which regulate immune responses.

T cell receptor and the CD3 molecule

The TCR is the apparatus on the T cell capable of antigen recognition. The receptor for peptide-MHC complexes on the majority of T cells including MHC-restricted helper T cells and CTLs, is a heterodimer consisting of two polypeptide chains, designated α and β, non-covalently linked to each other by disulfide bonds. Another less common type of TCR expressed on a small subset of $\alpha\beta$-negative peripheral blood T cells, IEL and thymocytes, is composed of a δ and γ chain (90). The $\alpha\beta$ TCR heterodimer provides T cells the ability to recognize peptide antigens bound to MHC molecules, but both the cell surface expression of TCR and their

function are dependent on five other proteins that non-convalently associate with the αβ heterodimer (91). Together these proteins form the functional T cell receptor.

Three members of the complex are called CD3 proteins and include a 25 kd γ chain, a 20 kd δ chain and a 20 kd non-glycosylated ε chain. The CD3 molecule is also associated with a ζζ homodimer in 90% of T cells while the remaining are associated with a ηζ heterodimer. Both antigen receptor chains (α/β and γ/δ) and CD3 chains (ε, γ, δ) belong to the Ig supergene family (92) while the ζ chain, as well as its alternatively spliced product η, are related to the γ chain of the high affinity IgE receptor (92, 93).

Antigen receptor chains (α/β and γ/δ) are responsible for ligand binding and antigen recognition. They have short cytoplasmic domains of approximately only 5 residues which do not appear to have enzymatic activity and appear to be responsible for association with the CD3 and ζ chains (89, 92, 93). The invariant CD3 and ζ chains appear to have significantly larger cytoplasmic domains ranging from 40 to 113 amino acids and are thought to be responsible for signal transduction (93, 94). The cytoplasmic tails of each of the CD3 proteins contains a sequence motif also found in the cytoplasmic tails of several other membrane proteins involved in signaling including the Igα and Igβ proteins associated with IgM and IgD. This motif, called immunoregulatory tyrosine activation motif (ITAM), is composed of an approximately 17 amino acid sequence in which the sequence tyrosine-X-X-leucine occurs twice separated by 6 to 8 amino acids (89). One ITAM is found in each of the CD3 chains while three ITAMs are found on each of the ζζ

homodimer or ηζ heterodimer chains, reflecting signal amplification, functional redundancy or distinct downstream molecules with which ITAMs interact (89, 93, 94). It has been proposed that ITAMs directly interact with cytoplasmic protein tyrosine kinases. One PTK shown to be capable of interacting with the TCR is $p59^{fyn}$ (95).

TCR-associated p59fyn

Phosphorylation of tyrosine residues of proteins has previously been shown to be an essential component of signal transduction. These phosphorylation events, mediated by phosphotyrosine kinases, are detectable within the first minute after ligand binding to the TCR (93). Phosphorylation of tyrosine residues on a protein has two general consequences. First, it allows other proteins that contain specific phosphorylated tyrosine sites to bind to the phosphorylated protein (93). These binding sites are structurally conserved and are call src-homology-2 (SH2) domains. Therefore, phosphorylation of tyrosine residues in the cytoplasmic tail of a membrane protein may lead to binding of SH2-containing proteins to that site. Different SH2 domains may bind to different amino acid sequences containing phosphorylated tyrosine, and phosphorylation of multiple tyrosines in the same membrane protein can lead to assembly of multiprotein complexes. Second, certain enzymes may be activated by phosphorylation of tyrosine residues. Many of these enzymes, such as src, contain SH2 domains and activation occurs after the enzyme docks at a phosphotyrosine containing membrane receptor (92).

As a member of the src family of protein tyrosine kinases, p59fyn has a catalytic domain with tyrosine kinase activity (SH1 domain), an SH2 domain which binds to amino acid sequences containing a phosphorylated tyrosine, an SH3 domain which interacts with proteins carrying proline-rich sequences, and a unique N-terminal sequence (96). There are three amino acid residues which may be related to the function and regulation of $p59^{fyn}$: 1) an N-terminal myristylated glycine at position 2 that enables membrane anchoring. Myristylation is required, but not sufficient, for the kinase to associate with the inner surface of the plasma membrane (96); 2) a tyrosine residue at the C-terminal side of the SH1 domain involved in regulating kinase activity. Dephosphorylation of this tyrosine residue by CD45 *in vitro* activates $p59^{fyn}$ kinase activity (97, 98). Nevertheless, the majority of cellular $p59^{fyn}$ does not appear to be regulated by CD45 (99); 3) a second tyrosine residue, located within the catalytic domain of the kinase, does exist and can be autophosphorylated. Autophosphorylation of this site allows the recruitment of other SH2 domain containing molecules, such as ZAP70 and PLC-γ1 (92).

CD4 and CD8 molecules

CD4 and CD8 molecules on T cells are very important for T cell development and activation. The structure of CD4 is invariant as it exists only as a 55 kd monomer (100, 101). The structure of the CD8 molecule varies depending upon the location and stage of development of the T lymphocyte. Usually, CD8 exists as either a CD8$\alpha\beta$ heterodimer or, less frequently, as a CD8$\alpha\alpha$ homodimer (102).

Peripheral blood T cells predominantly express CD8 as a heterodimer while some murine IEL appear to express the CD8αα homodimer (32, 103-105). When associated, CD8 is approximately a 66 kd molecule (100).

It has been shown that CD4 and CD8 can associate with the TCR-CD3 complex forming a co-receptor complex in antigen recognition and T cell activation (87). The CD8 molecule is capable of interacting with MHC class I via the monomorphic class I α_3 domain, while CD4 interacts with MHC class II via the β_2 domain of this molecule (86). This binding stabilizes the TCR/peptide-MHC interaction. Furthermore, CD4 or CD8 molecules may transduce signals into T cells (87). Similar to the chains of the TCR, CD4 and CD8 molecules do not contain intrinsic protein tyrosine kinase activity. Nevertheless, CD4 and CD8 have been shown to associate with $p56^{lck}$, another member of the src family of protein tyrosine kinases which is predominantly expressed in T cells. In our system, $CD8^+$ T cells are primarily activated, thus it is these cells on which we will focus.

CD8-associated p56lck

CD8 molecules appear to serve two functions. First, CD8 serves as a cell-cell adhesion molecule by binding to the α_3 domain of class I MHC molecules. This interaction serves to stabilize the TCR-MHC association, as the T cell normally expresses a receptor with low affinity for specific MHC-peptide complexes. A second function of CD8 is signal transduction. The CD8α cytoplasmic domain is associated with the protein tyrosine kinase, $p56^{lck}$, which

is phosphorylated upon interactions between the TCR and MHC-peptide complexes (106-110). The activation of this kinase along with the TCR-associated kinase, $p59^{fyn}$, enhances signal transduction in order to produce an optimal T cell response. In addition, absence of $p56^{lck}$ has been reported to block downstream T cell signal transduction (89, 93). During T cell activation, CD8-associated $p56^{lck}$ interacts with substrates that have been phosphorylated. It has been shown that CD8-associated $p56^{lck}$ is capable of phosphorylating γ, δ, ε, and ζ chains of CD3 complex on tyrosine residues (108). Tyrosine phosphorylation of ζ chains and CD3 chains may be important for these molecules to either associate with or induce downstream signaling pathways.

T cells that express CD8 molecules can have either suppressor or cytolytic properties. It has been found by our laboratory that IECs can stimulate suppressor T cells which non-specifically inhibit primary, secondary and unrelated mixed lymphocyte reactions and show no cytolytic activity (50). These suppressor T cells can also inhibit mitogen-induced T cell proliferation and B cell differentiation in the presence of T cells and pokeweed mitogen. It was noted that cells activated by IECs appear to express CD8 but not CD28, and appear to have a suppressor function (49). Furthermore, the ability of IECs to selectively induce $CD8^+$ T cell activation may in some manner correlate with the predominance of $CD8^+$ IEL.

Downstream T cell signaling

The activation of the signaling cascade in T cells is a highly complex process which is not fully understood. The activation of the CD8-associated PTK $p56^{lck}$ and the TCR-associated PTK $p59^{fyn}$ results in the recruitment of ZAP70, via its SH2 domain, to the membrane receptor complex (111). This triggers downstream events which include the activation of the phosphatidylinositol pathway, activation of Ras and the activation of several serine/threonine protein kinases (such as Raf-1) which trigger a kinase cascade involving MAPK (112). The activation of Ras following TCR stimulation is the result of both protein kinase C (PKC) dependent and independent mechanisms. Ras activity is regulated in a PKC-independent manner through its interactions with guanine nucleotide exchange proteins, such as vav (113). The exchange protein vav is tyrosine phosphorylated (114) and actually appears to have increased guanine nucleotide exchange activity with Ras following TCR stimulation (115).

The PKC-dependent pathway also appears to be involved in the activation of $CD8^+$ T cells. Stimulation of the TCR/CD8 molecules in $CD8^+$ cytolytic T cells has been shown to induce the PLC-γ1 pathway (116). The antigen recognition motifs of the CD3 and ζ chains are tyrosine phosphorylated after TCR stimulation. This leads to docking of the enzyme phospholipase C-γ1 which is then rapidly tyrosine phosphorylated, causing the activation of enzymatic activity. The tyrosine phosphorylation of PLC-γ1 results in the hydrolysis of the plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), resulting in the

production of 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). The breakdown of PIP_2 is followed by a rise in the cytoplasmic Ca^{2+} concentration, thought to be the result of the IP_3 stimulated release of membrane sequestered intracellular calcium stores, and the activation of PKC by DAG. The mechanism by which PLC-γ1 is phosphorylated is not clear, however it has been shown that $p56^{lck}$ co-immunoprecipitates with PLC-γ1 through an interaction of the PLC-γ1 SH2 domain and $p56^{lck}$ (117).

In summary, protein tyrosine phosphorylation is important in the initiation of cellular responses by T cell antigen receptor. The activation of T cells through the TCR reflects an alteration in the equilibrium between PTKs and protein tyrosine phosphatases (PTPases). The net result of these changes in balance is critical in the activation of downstream signaling components. The identification of other downstream effectors that regulate cellular responses promises the future possibility of connecting the complex pathway from the plasma membrane to the nucleus in T lymphocytes. This will aid in our understanding of the mechanisms responsible for lymphocyte development and differentiation.

MATERIALS AND METHODS

Cell lines

T84, DLD-1, CaCo-2 and HT29 are malignant IEC lines obtained from the American Type Culture Collection (ATCC, Rockville, MD). These cells were maintained in RPMI supplemented with 10% FCS, 1% L-glutamine, 1% penicillin/streptomycin.

3G4 and 3G8 are murine T cell hybridoma transfectants which constitutively express human CD4 and CD8α, respectively. Their functional properties have been previously described (118, 119). The cell lines were the kind gift of Dr. Steven Burakoff (Dana-Farber, Boston, MA).

FO-1 cells transfected with full length human CD1d cDNA (FO-1 D5) have been previously described (120). These transfected cells were maintained in RPMI supplemented with 10% FCS, 20 mM Hepes, 1% non-essential amino acids, 1% L-glutamine, 1% penicillin/streptomycin and 3 mg/ml G418 (Sigma). Untransfected control FO-1 cells were cultured in similar media without G418 and served as a negative control.

Monoclonal antibodies

B9 is a murine IgG1 anti-human IEC mAb which appears to recognize an epitope on the carbohydrate side chains of the novel IEC surface molecule gp180. Ascites was generated at a concentration of 1 mg/ml and used at a 1:1000 dilution for western blotting while 10 μg was used for immunoprecipitation studies. An irrelevant

murine IgG1 antibody was used as a negative control whenever necessary.

Four mAbs were used to detect CD1d. 3C11 and 1H1 are rat IgM anti-mouse CD1 mAbs, which have been previously shown to cross-react with human CD1d (75, 78). Supernatants were harvested from the 1H1 or 3C11 hybridomas and adjusted to a concentration of 10 μg/ml. D5 is a murine anti-human CD1d mAb generated against a glutathione-S-transferase fusion protein of CD1d (121). This antibody was used at a concentration of 5 μg/ml. 51.1.3 is a mouse anti-human CD1d mAb raised against an Fc-fusion protein of CD1d (122).

OKT3, OKT4, OKT8 and W6/32 are hybridomas obtained from the American Type Culture Collection (ATCC, Rockville, MD) which produce mAbs against CD3, CD4, CD8 and a non-polymorphic domain of class I MHC, respectively.

Isolation of human intestinal epithelial cells

IECs were isolated by a method described previously (42, 52). Surgical specimens from patients undergoing operative procedures for cancer were obtained directly from the operating room. These specimens from cancer patients were obtained from an area at least 10 cm from the tumor. Specimens were washed extensively with PBS containing 1% penicillin/streptomycin and 1% fungizone (Flow Laboratory Inc., McLean, VA). The mucosa was stripped from the submucosa, cut into small pieces and placed in 1 mM dithiothreitol (Sigma, St. Louis, MO) for 5 minutes at room temperature to remove the mucus. The pieces were then washed in PBS and incubated in

dispase (3 mg/ml in RPMI 1640, Boehringer Mannheim, GmbH, Germany) for 30 minutes in a 37°C shaking incubator. This was repeated four times. The tissue pieces were removed and the cell suspension collected, pooled and centrifuged on a Percoll density gradient (Pharmacia, Piscataway, NJ). Enterocytes located at the 0-30% interface were washed three times with PBS and resuspended in RPMI with 0.1% BSA for co-culture experiments. Preparations of purified enterocytes were >90% viable, free of macrophages and B cell contamination as determined by staining with anti-CD14 and anti-CD20 mAbs (Coulter Corp., Hialeah, FL) and contaminated with only 2-4% IEL ($CD3^+$ cells).

Isolation of peripheral blood lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated from leukocyte concentrate packs and separated into T and non-T cells using rosetting and density gradient centrifugation as previously described (52). Heparinized venous blood was collected from normal donors and aliquoted into 50 ml tubes (10 ml per tube) and 30 ml PBS was added. Ficoll-Paque (Pharmacia, Piscataway, NJ) was layered beneath the blood and centrifuged at 1600 rpm for 30 minutes. The PBMCs were collected from the interface, washed in PBS and centrifuged at 1600 rpm for 10 minutes. This was repeated two more times prior to resuspending the cells in RPMI containing 2 mM L-glutamine and 1% penicillin/streptomycin. The number of lymphocytes were counted using a hemacytometer. The cells were then separated using one of two techniques.

T cells and non-T cells were isolated from PBMCs by a rosetting method using neuraminidase-treated sheep red blood cells followed by Ficoll-Paque density gradient centrifugation. The cells were diluted to $5x10^6$ cells/ml with neuraminadase treated sheep RBC. These tubes were then spun for 5 minutes at 1000 rpm to make a loose pellet and refrigerated overnight. The pellets were then resuspended into one 50 ml tube, layered onto 12 ml Ficoll and spun at 1600 rpm for 25 minutes. The non-T cells, located at the interface between the RPMI and ficoll, were washed thoroughly in RPMI while the T cells, located with the RBC at the bottom of the tube, were treated with 0.7% NH_4Cl to lyse the RBCs.

In order to isolate inactivated T cells, another method involving nylon wool was employed. This method of T cell isolation takes advantage of the fact that B cells and monocytes adhere to the nylon wool, thereby allowing non-adherent T cells to pass through the column. A nylon wool column was made by inserting 0.8 g nylon wool (Polysciences, Inc., Warrington, PA) into a 10 ml syringe which is then autoclaved. Using a 50 ml tube, the nylon wool is soaked and any air pockets removed. The column was then filled with warm RPMI and incubated for 45 minutes in a humidified, 37oC, 5% CO_2 incubator. 2 ml ($2\text{-}3x10^7$ cells/ml) of the lymphocyte suspension was loaded into the pre-warmed column and incubated for 45 minutes in an humidified incubator. The first 15 ml of T cell enriched cells was then collected from the column. This approach has the advantage of greater monocyte depletion and, as previously mentioned, no T cell activation of T cells through CD2 (sRBC express LFA-3 which crosslinks CD2 and induces some baseline activation).

Isolation of T cell populations

Five subsets of T cells were isolated using specific antibody-bound magnetic beads. These subsets included: PBTs, $CD4^+$ T cells, $CD8^+$ T cells, $CD8^+CD28^+$ T cells and $CD8^+CD28^-$ T cells. To isolate $CD4^+$ and $CD8^+$ Tcells, T cells were negatively selected for either CD4, leaving a $CD8^+$ population, or CD8, leaving a $CD4^+$ population. This was accomplished using DYNABEADS (DYNAL, Lake Success, NY) conjugated with either CD4 or CD8 mAb. These beads were washed several times with PBS and incubated at RT for 30 minutes at 4°C with PBTs. The beads were then collected using a Magnetic Particle Concentrator (MPC) and washed 4 times with washing buffer (PBS, 10% FCS). The supernatant was collected as it contained the negatively selected T cells. These cells were washed several times in PBS. The $CD8^+$ T cells were then subjected to another round of selection. Using CD28-conjugated beads, $CD28^+$ cells were positively selected while at the same time negatively $CD28^-$ cells. The same technique was used as described above. To remove the bound beads from the $CD28^+$ T cells, the Dynabeads detach after 18 hour incubation at 37°C. The cells were then ready to be co-cultured with freshly isolated IECs, purified gp180.

PIPLC treatment of cells

IECs ($2x10^7$) were washed, resuspended in 1 ml RPMI with 1 unit PIPLC (Sigma) and incubated for 45 minutes at 37°C. gp180 has previously been shown to exist in two forms; a GPI-anchored, apically sorted form and a transmembrane basolateral form (51).

The cell suspension was centrifuged for 10 minutes at 1400 rpm and the cells and supernatant were analyzed for released gp180 (GPI-anchored form) through cell staining and western blot. This approach yielded soluble gp180.

Purification of B9 and gp180

Supernatant was collected from the B9 hybridoma which produced a mAb recognizing a 180 kd glycoprotein on the surface of IECs. The supernatant was collected by centrifugation at 1400 rpm for 10 minutes, filtered and stored at 4°C. The mAb B9 was purified from culture supernatant by recombinant protein G-Sepharose 4B affinity chromatography. Five ml of protein G sepharose CL-4B (Pharmacia Biotech, Inc., Piscataway, NJ) was packed into a column. The column was washed with 200 ml PBS (5.4 mM KCl, 1.5 mM KH_2PO_4, 140 mM NaCl, 8 mM Na_2HPO_2, pH7.4, all from Sigma Chemical Co., St. Louis, MO). 500 ml of mAb supernatant was passed through the column for 18 hours at 4°C. The column was washed with 300 ml of PBS. The mAb was eluted with 25 ml 0.1 M glycine (Sigma Chemical Co.), pH2.7. One-tenth the volume of 1 M Tris solution (pH 6.8) was added to the eluate after the elution. The purified antibody was dialyzed against two changes of 2 L PBS and concentrated using centriprep (Amicon Inc., Beverly, MA). The concentration of protein was determined by spectrophotometry. The column was balanced with 50 ml PBS. The purity of the mAb was determined using a minigel.

The mAb B9 was then conjugated to Sepharose 4B using a procedure supplied by Pharmacia. Briefly, CNBr-activated sepharose

4B beads (Pharmacia Biotech Inc., Piscataway, NJ) were swollen for 15 minutes in 1 mM HCl and washed with 1 mM HCl (200 ml/1 g dry sepharose) on a glass filter (G3 porosity). The beads were subsequently washed with 5 ml coupling buffer (0.1 M $NaNCO_3$, 0.5 M NaCl, pH8.3) per gram of dry gel. After washing, the gel was transferred to coupling buffer containing 5 mg/ml gel of mAb B9, and incubated at 4°C overnight on an end-to-end rotator. The gel was spun down and the supernatant removed. Ten gel volumes of blocking buffer (0.1 M glycine, pH8.0) was added into the gel and the incubation was continued at room temperature for two more hours. The coupled gel was then washed with 0.1 M sodium acetate buffer, pH4.0 (50 ml/ml gel), then with coupling buffer (50 ml/ml gel) and eventually balanced by PBS (50 ml/ml gel). The gel was stored at 4°C with the addition of 0.1% NaN_3 as a preservative. The OD of the residual supernatant defined the degree of binding of the mAb to the beads. Typically, greater than 90% of the protein was bound.

Released gp180 obtained from PIPLC treatment of IECs was incubated on the B9 affinity column for 18 hours at 4°C. The column was washed with 300 ml of PBS. The gp180 was eluted with 25 ml 0.1 M glycine (Sigma Chemical Co.), pH2.7. One-tenth the volume of 1 M Tris solution (pH 6.8) was added to the eluate after the elution. The gp180 was dialyzed against two changes of 2 L PBS and concentrated using a centricon vial (Amicon Inc., Beverly, MA). The concentration of protein was expressed in terms of the cell equivalents it was isolated from.

Construction of Fusion Proteins

Fusion proteins containing the α_3 domain of class I MHC and β_2 domain of class II MHC (which has been shown to be the CD4 binding domain and, thus used as a negative control) were generated using a cDNA coding sequence for full-length HLA-A2.1 in a M13 RF subclone designated M13mp18/A2.1 (123). An EcoRI insert, which encompassed the entire HLA-A2.1 cDNA sequence, was subcloned into the EcoRI site of the expression vector pBluescript II KS$^-$ (Stratagene) to generate pHLA-A2.1/BT. A partial cDNA encoding the α_3 domain of HLA-A2.1 only was generated by PCR using 5'-TAGGATCCATGGACGCCCCCAAAA-3' and 5'-TAGAATTCTCACCATCTCAGGTGAGG-3' as 5' and 3' primers, respectively, and pHLA-A2.1/BT plasmid as template. A start codon and a BamHI restriction endonuclease site were incorporated into the 5'-end of the 5' primer, and a stop codon and an EcoRI restriction endonuclease site were added to the 5' end of the 3' primer. PCR reactions were performed under mineral oil in the presence of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 1.25 mM of each deoxynucleotide triphosphate, 0.1 nmoles of each primer and 2.5 U AmpliTaq DNA polymerase (U.S. Biochemical, Cleveland, OH). Reaction thermocycling (30 cycles at 94°C for 1 min, stepped to 50°C for 2 min, stepped to 72°C for 2 min) were performed in a PTC-100 thermocycler (M.J. Research, Watertown, MA). Following organic extraction, the digested α_3 cDNA insert was subcloned between the BamHI and EcoRI sites in the multiple cloning site of the baculovirus vector pVL1393, remobilized with BamHI/Bg1II digestion, and subcloned into the prokaryotic expression vector

pMALp2 (New England Biolabs). This generated a chimeric coding sequence in which α_3 and maltose binding protein (MalE) are linked in-frame in a vector designated pA2.1α_3/MALp2. For large scale purification, α_3/MalE was purified from 2 liter cultures of *E. coli* DH5 α transformants. Bacteria were grown in LB medium containing 0.2% glucose and 100 μg/ml ampicillin up to OD_{600}=0.4 and induced with IPTG. Following induction, bacteria were pelleted, washed with PBS, resuspended in 400 ml of 30 mM Tris-HCl pH 8.0, 20% sucrose, 1 mM EDTA, and incubated 10 min at room temperature. After centrifugation, the cell pellet was subjected to osmotic lysis for 10 min with 400 ml of cold 5 mM $MgSO_4$. The supernatant was collected after centrifugation (8000g for 20 min) and the pH of this solution was adjusted by the addition of 8 ml 0.5 M sodium phosphate buffer, pH 7.2. The fusion protein in the supernatant was bound to a 75 ml amylose resin column (NE Biolabs). The column was washed and the fusion protein was eluted with high salt column buffer (10 mM sodium phosphate, 0.5 M NaCl, 10 mM 2-ME, 1 mM EDTA) containing 10 mM maltose (Sigma). Fractions were analyzed by spectrophotometry and SDS-PAGE. Protein containing fractions were dialysed overnight against PBS and concentrated with Centriprep-30 microconcentrators (Amicon). A similar process was used to generate the β_2 fusion protein.

The CD1d-GST fusion protein was constructed using the α1-α3 domains of CD1d in a pGEX vector (Pharmacia, Piscataway, NJ) (121). For large scale preparations of CD1d-GST fusion protein, 10 ml of transformed bacteria grown freshly overnight was inoculated into 500 ml of LB media with ampicillin selection. This was

incubated for 90 minutes at 37°C. After adding 0.1 mM IPTG, the bacterial culture was grown for an additional 4 hours. The culture was spun in a Sorvall centrifuge for 10 min at 5000 rpm at room temperature and resuspended in 6 ml of STE buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA). It was then spun in a tabletop centrifuge for 10 min at 3500 rpm. The pellet was resuspended in 24 ml of 100 mg/ml lysozyme in STE along with protease inhibitors (0.1 mg/ml PMSF, and 20 μg/ml aprotinin) and incubated on ice for 15 min prior to adding 5 mM DTT and 1.5% sarkosyl in STE. Bacterial cell walls were broken using a tissue homogenizer for 15 min and pellets were obtained by spinning in a Sorvall SS-34 rotor at 14,000 rpm for 10 min. After adding 4% Triton X-100, 2.5 ml of glutathione agarose beads (Sigma) were incubated with the lysate for 2 hours at 4°C on a orbital rocker. The beads were spun down, transferred to microcentrifuge tubes and washed 6-8x with 1 ml cold PBS containing 0.5% Triton X-100. The final wash was with PBS alone and CD1d-GST protein was eluted by rocking at 4°C for 10 min in 0.75 ml of elution buffer (75 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM DTT, 0.1% Triton X-100) containing 100 mM reduced glutathione. The supernatant (eluant) was saved and another 200 μl of elution buffer was added to elute the remaining fusion protein. When the eluant was resolved by SDS-PAGE and stained with Coomasie, a single band at 65 KD was seen, the approximate weight of the CD1d-GST fusion protein. The fusion proteins CD8-Fc and CD40-Fc were generated using a similar method.

Cell staining and flow cytometry

1-2x10^5 cell per condition were stained in a V-bottom 96-well plate. The cells were washed 3x with 0.1% BSA in PBS. The cells were incubated with 10 μg/ml isotype control or specific mAbs on ice for 45 minutes. The cells were washed three times with PBS-BSA solution (1% BSA in PBS), and resuspended in 10 μg/ml FITC-conjugated goat anti-mouse IgG (Tago, Inc., Burlingame, CA) and then incubated on ice for another 45 minutes. The cells were then washed again three times and finally resuspended in 400 μl PBS for flow cytometric analysis. The percentage of positive cells and mean fluorescence was analyzed by an Epics Profile III flow cytometer.

ELISA

Enzyme-linked immunosorbant assays were used in this thesis to determine the ability of the novel glycoprotein, gp180, to bind to various other molecules expressed by either IECs or PBTs. In performing these assays, gp180 was purified using a B9 affinity column and diluted in coating buffer (dH_20, 0.015 M Na_2CO_3, 0.03 M $NaHCO_3$, 3 mM NaN_3). An optimal dilution of gp180 (4x10^5 cell equivalents) was used to coat 96-well Nunc ELISA plates overnight at 4°C. The plates were washed 5x with 100 ml/well of ELISA buffer and blocked with 1% BSA-PBS for 1 hour at RT. After washing, 1 μg/ml various fusion proteins or controls (CD1d-GST, GST, CD8-Fc or CD40-Fc) was added to the plates and incubated at RT for 1 hour. The plates were then washed five times. Binding was

detected by incubating the plates with 5 μg/ml primary antibody for 1 hour followed by an hour incubation with 10 μg/ml HRP-conjugated goat anti-mouse Ig secondary antibody. When Fc fusion proteins were used, only the HRP-conjugated goat anti-mouse Ig was used. After 65 μl HRP substrate (dH_20, 0.2 M $NaPO_4$, 0.2 M Na_2PO_4, 0.001% H_2O_2, 2 mM phenol, 1.2 mM 4-aminoantipyrine) was added, the plate was read by a Genetic Systems microplate ELISA reader at 490 nm.

Cell:cell co-culture experiments

Allogeneic T cells and IECs were resuspended to achieve a concentration of $1x10^7$ cells/ml in 0.1% BSA-RPMI 1640. Cells were prewarmed in a 37°C water bath. For each reaction, one million T cells (or ten million when performing immunoprecipitations) were placed in a 1.5 ml eppendorf tube and centrifuged for 5 seconds. The supernatant was removed and the pellet loosened. One million IECs, FO-1 D5 or untransfected FO-1 cells were mixed with the T cells, spun quickly for 20 seconds and placed in a 37°C water bath. After 1, 2 or 5 minutes, 1 ml of ice cold stop buffer (PBS with 10 mM sodium orthovanadate) (Sigma, St. Louis, MO) was added. The pellet was resuspended in 100 μl lysis buffer (20% PBS, 80% dH_2O, 100 μM Na_2VO_3, 1 mM PMSF, 5 mM iodoacetamide, 20 μg/ml leupeptin and 20 μg/ml aprotinin) and vortexed at the start and end of a 30 minute incubation on ice. As a positive control, one million T cells were incubated with 10 μg/ml anti-CD8 antibody (OKT8) for 30 minutes at 4°C and crosslinked with 10 μg/ml rabbit anti-mouse IgG antibody for 2 minutes at 37°C. Time zero tubes were prepared by adding stop buffer to individual tubes containing T cells and either epithelial

cells, FO-1 D5 or untransfected cells. The lysate from these two tubes were then combined into one tube to account for all constitutive kinases and substrates in both cell types. 25 µl reducing buffer (50 mM Tris-HCl pH6.8, 5% 2-ME, 10% glycerol, 1% SDS) was added to all reactions prior to boiling for 5 minutes and the resulting samples were run on a 10% SDS-PAGE gel for western blot.

In the presence of blocking monoclonal antibodies

When blocking antibodies were used, PBTs were incubated with 1 µg/ml of the desired antibody (such as OKT8 mAbs specific to different CD8 epitopes) for 45 minutes on ice. The cells were then washed several times in RPMI. An allogeneic mixed cell culture experiments was performed as described previously.

In the presence of fusion proteins

When fusion proteins were used to determine the effect of a specific molecule in the ability of gp180 to induce T cell activation, IECs were incubated with the fusion protein for 45 minutes on ice. The cells were then washed several times in RPMI. An allogeneic mixed cell culture experiments was performed as described previously.

Immunoprecipitation and kinase assay

In some experiments, the lysate from these co-culture conditions were immunoprecipitated with a rabbit anti-human $p59^{fyn}$ or rabbit anti-human $p56^{lck}$ antibody which were covalently bound to sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA). These conjugated antibodies were rotated with co-culture lysates

(pre-cleared with rabbit serum coated sepharose 4B beads) for 1 hour at 4°C. The beads were washed four times with PBS and 75 µl reducing buffer (50 mM Tris-HCl pH6.8, 5% 2-ME, 10% glycerol, 1% SDS) was added prior to boiling for 5 minutes. The resulting samples were then run on a 10% SDS-PAGE gel, transferred to nitrocellulose paper (Schleicher & Schuell Inc., Keene, NH), and an anti-phosphotyrosine mAb (4G10-HRP) (Upstate Biotechnology Incorporated, Lake Placid, NY) western blot was then performed.

In other experiments, lysates of IECs were immunoprecipitated with murine IgG1 (isotype control), anti-gp180 mAb B9 or the anti-class I mAb W6/32 (negative control) to determine if an association between CD1d and gp180 existed. Protein A sepharose beads were coated with 10 µg/ml rabbit anti-mouse IgG immunoglobulin followed by 10 µg/ml primary antibody (isotype control, anti-gp180 or anti-class I MHC). Each of these steps were performed for 1 hour at room temperature while rotating. The beads were then washed thoroughly with wash buffer (2 M Tris pH7.4, 0.5 M EDTA, 4 M NaCl) and epithelial cell lysate was rotated with the coated beads overnight at 4°C. The beads were washed 5 times with RIP buffer (10 mM Tris pH8.0, 1.0 mM EDTA, 0.5% NP-40, 0.1 M NaCl, 1 mg/ml ovalbumin and 0.02% Na azide) and resolved on SDS-PAGE. After transferring to nitrocellulose an anti-CD1d, D5 (10 µg/ml) followed by an anti-gp180, B9 (10 µg/ml) western blot was performed.

When kinase assays were performed, beads were resuspended in 30 µl kinase buffer (10 mM $MnCl_2$, 50 mM Tris, pH7.4) and mixed with 10 µCi (γ-32)ATP (Amersham, Arlington Heights, IL) for 30 minutes at room temperature. The enzyme reaction was stopped by

adding 15 μl 4x reducing buffer (as previously described) and boiled 5 minutes.

Western blot

A 10% SDS-PAGE gel was prepared and one million cells were loaded per lane. The protein was transferred from the gel to nitrocellulose paper at 65V for four hours in transfer buffer (20% methanol, 150 mM glycine, 25 mM Tris, pH8.3). The transfer sandwich was prewet in transfer buffer and set up in the following order: 1) a porous polyethylene foam sheet; 2) one sheet of 3M filter paper (Whatman International Ltd., Maidstone, England); 3) the gel; 4) a nitrocellulose membrane sheet (0.2 μm, Schleicher & Schuell, Inc., Keene, NH); 5) another sheet of 3M filter paper; 6) another sheet of porous polyethylene foam. After the transfer, the nitrocellulose was blocked with 5% milk for 1 hour at RT. The membrane was washed once in PBS and then incubated with primary antibody (1-10 μg/ml) overnight at 4°C. The membrane was washed with PBS several times and incubated with an HRP-conjugated secondary antibody (1-2 μg/ml) for 1 hour at RT. The membrane was finally washed 3-5 times in washing buffer (0.05% Tween-20 in PBS) for 5 minutes each time. The membrane was developed using an enzyme linked chemiluminescence (Dupont NEN, Boston, MA) reagent.

Mixed Lymphocyte Reactions in the presence of blocking antibodies

Prior to setting up an mixed cell proliferation assay, isolated IECs were irradiated (2800 rads). These stimulator cells were then

plated in a round-bottom 96-well plate at a concentration of $1x10^5/100\mu l$. PBTs were incubated with 10 μg/ml blocking antibody for 45 minutes on ice. The cells were washed thoroughly and incubated with the plated stimulators at a 1:1 ratio. In all cases, IECs and PBTs were incubated alone as control conditions. The cells were cultured together for 5 days at 37°C in a humidified incubator. At this point, all conditions were pulsed with 1 μCi ^3H-tritiated thymidine (Dupont NEN, Boston, MA) and harvested 18 hours later. Incorporated counts were measured by a Wallac Microbeta counter.

Cytotoxicity assay in the presence of blocking antibodies and substrates

To make effector cytolytic T cells (CTLs), T and non-T cells were separated using the procedure previously described in this thesis. Cultures were performed in 25 cm^2 flasks, with responders and stimulators (targets) at a 1:1 ratio at a concentration of $1x10^6$/ml. These cells were cultured for 6 days in RPMI with 1% penicillin, 1% L-glutamine and 10% FCS. On the same day, $5x10^6$ target cells were set up at a concentration of $1x10^6$ cells/ml and stimulated with 10 μl/ml PHA. On day 3, these same target cells were spun down, counted and resuspended at a concentration of $2x10^5$ cells/ml in RPMI constituted with 10% FCS and 5% biotest (Biotest Diagnostics Corp., Dreieich, Germany). On day 6, the targets were removed from the flasks and pooled in 50 ml centrifuge tubes. The cells were spun at 1500 rpm for 5 minutes and the supernatant was removed. The cells were resuspended in 150 μCi ^{51}Cr and incubated at 37°C for 90 minutes. These cells were then carefully

washed to prevent chromium release. For some conditions, the target cells were then incubated in varying concentrations of anti-class I mAb for 30 minutes at RT. During this 90 minute incubation, the effector cells were washed and set up in 96-well round bottom plates at a concentration of 10^5 cell/100 µl. In some wells, the effector cells were incubated with varying concentrations of anti-CD8 mAb or purified gp180 for 45 minutes at RT. The cells were washed before target cells were added to the plate. In all cases, the target cells were diluted to achieve a 100:1 E:T ratio in 100 µl RPMI. The total volume in each well was always 200 µl. In some wells, the targets were incubated only in RPMI to determine the spontaneous release of chromium while others were incubated in 1% NP-40 to determine the maximal release of chromium. The target:effector reactions were incubated for 4 hours at 37°C at which time 100 µl was transferred to another plate and dried overnight. The counts were then measured using a Beckman Gamma 5500B counter. The percentage of killing was determined by the following formula:

$$\frac{\text{(experimental count - spontaneous count)}}{\text{(maximal count - spontaneous count)}} \times 100 = \%\ \text{cytotoxicity}$$

SECTION I

The novel CD8 ligand, gp180, binds to CD8 at sites distinct from those used by class I MHC.

ABSTRACT

The activation of $CD8^+$ suppressor T cells by normal IECs in antigen-specific or allogeneic mixed cell culture systems has significant implications for the regulation of mucosal immune responses. A 180 kd glycoprotein (gp180) has been identified and shown to be important in $CD8^+$ T cell activation by IECs. In this study, we examine, in further detail, the role that the CD8 molecule plays in this interaction. It has been previously shown that gp180 expression on IECs is critical for the activation of CD8-associated $p56^{lck}$. Although indirectly suggested by this data, there was no evidence that the activation of this protein tyrosine kinase was a direct result of gp180 interacting with the CD8 molecule. In this study, we documented that soluble gp180 was able to bind to CD8-Fc fusion proteins and that this interaction was mediated via a carbohydrate:protein interaction. Furthermore, the sites used for binding by gp180 were distinct from those used by the conventional CD8 ligand, class I MHC. Thus, gp180 is a novel CD8 ligand which plays an important role in the activation of kinases associated with this molecule.

INTRODUCTION

The mucosal immune system appears to utilize unique regulatory mechanisms from those of the systemic immune system. The phenomenon which underscores this difference is the induction of tolerance following oral antigen priming and the characteristic immunologically suppressed tone in the gut (1-12). $CD8^+$ suppressor T cells appear to play, in part, a role in regulating such mucosal immune responses and it appears that IECs are capable of presenting antigens to and activating $CD8^+$ suppressor T cells in a non-class I, non-class II restricted manner (13). The mechanisms underlying the activation of these $CD8^+$ T cells by IECs has not been completely delineated. Thus, the molecules involved in T cell:epithelial cell interactions were studied.

Initial studies conducted in our laboratory indicated that the induction of $CD8^+$ T cells by IECs appeared to be linked to the binding of CD8 molecules and the activation of its associated kinase, $p56^{lck}$ (13). It was clearly determined that this activation is neither mediated by class I nor class II MHC on the epithelial cell surface but does involve the CD8 molecule itself since the addition of anti-CD8 mAbs inhibits this activation. To further investigate the role of CD8 and its associated kinase, murine transfectants 3G4 and 3G8, transfected with human CD4 and CD8α cDNA respectively, were used. Co-culture of 3G8 cells with IECs but not monocytes resulted in the activation of $p56^{lck}$ while CD4-associated $p56^{lck}$ was activated by monocytes and not IECs in 3G4 cells (13).

To study the molecule on IECs that was involved in the interaction with $CD8^+$ T cells, two epithelial cell specific mAbs, B9

and L12, were generated. The initial screen for these anti-IEC mAbs was their ability to stain IECs, block the proliferation of $CD8^+$ T cell in IEC:T cell co-cultures, and inhibit the activation of CD8-associated $p56^{lck}$. Both mAbs recognize a 180 kd epithelial membrane glycoprotein (gp180) which is heavily N-glycosylated and has two forms, an apically sorted GPI anchored form and a basolateral transmembrane form (13). Functional studies of purified gp180 found that it binds to PBT cells and activates CD8-associated $p56^{lck}$. It, therefore, appears that gp180 is a novel mucosal immune regulator capable of activating, either directly or indirectly, CD8-associated $p56^{lck}$ on T cells.

The focus of this section will be to determine if gp180 is capable of directly binding CD8 and activating CD8-associated $p56^{lck}$, and to determine the nature of this interaction. The primary goal is to confirm and elaborate on data previously generated with the intent to achieve a greater understanding of the role of IEC-activated $CD8^+$ T cells in mucosal immunity.

MATERIAL AND METHODS

Cell lines

$HepG_2$ is a human hepatocellular carcinoma cell line which has an epithelial morphology and expresses a high level of gp180. It is cultured in Eagles's MEM with 10% FCS, 1% penicillin/streptomycin and1% L-glutamine in a 37°C, 5% CO_2, humidified incubator.

Monoclonal antibodies

OKT3, OKT4, OKT8 and W6/32 are hybridomas obtained from the American Type Culture Collection (ATCC, Rockville, MD) which produce mAbs against CD3, CD4, CD8 and a non-polymorphic domain of class I MHC, respectively.

Seven mAbs reactive with the CD8 molecule were the kind gift of Dr. W.E. Biddison (Washington University, St. Louis, MO). These antibodies were the result of three separate fusions (14). CAF_1 mice (Jackson Laboratories, Bar Harbor, ME) were immunized intraperitoneally at 2 to 3 week intervals with either $2x10^7$ thymocytes (OKT8B and OKT8C), ConA activated sheep red blood cell rosetting cells (OKT8A) or ConA activated sheep red blood cells depleted of $CD4^+$ cells by OKT4 and rabbit complement (OKT8E, OKT8F, $OKT8I_1$ and $OKT8I_2$). Four days after the third injection splenocytes were fused with P3 x 63 Ag8U1 myeloma cells. Hybridomas producing antibodies reactive with an $OKT8^+$ population and unreactive with an $OKT4^+$ population were selected for subcloning and ascites production. Antibodies were purified from ascites by column chromatography prior to use.

Isolation of human intestinal epithelial cells

IECs were isolated by a method described previously (15, 16). Surgical specimens from patients undergoing operative procedures for cancer were obtained directly from the operating room. These specimens were obtained from an area at least 10 cm from the tumor. Specimens were washed extensively with PBS containing 1% penicillin/streptomycin and 1% fungizone (Flow Laboratory Inc., McLean, VA). The mucosa was stripped from the submucosa, cut into small pieces and placed in 1 mM dithiothreitol (Sigma) for 5 minutes at room temperature to remove the mucus. The pieces were then washed in PBS and incubated in dispase (3 mg/ml in RPMI 1640, Boehringer Mannheim, GmbH, Germany) for 30 minutes in a 37°C shaking incubator. This was repeated four times. The tissue pieces were removed and the cell suspension collected, pooled and centrifuged on a Percoll density gradient (Pharmacia, Piscataway, NJ). Enterocytes located at the 0-30% interface were washed three times with PBS and resuspended in RPMI with 0.1% BSA for co-culture experiments. Preparations of purified enterocytes were >90% viable, free of macrophages and B cell contamination as determined by staining with anti-CD14 and anti-CD20 mAbs (Coulter Corp., Hialeah, FL) and contaminated with only 2-4% IEL ($CD3^+$ cells).

Isolation of peripheral blood lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated from leukocyte concentrate packs and separated into T and non-T cells using rosetting and density gradient centrifugation as previously

described (16). Briefly, heparinized venous blood was collected from normal donors and separated by Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradient centrifugation. T cells and non-T cells were isolated from PBMCs by a rosetting method using neuraminidase-treated sheep red blood cells followed by Ficoll-Paque density gradient centrifugation.

PIPLC treatment of cells

$2x10^7$ IECs were washed, resuspended in 1 ml RPMI with 1 unit PIPLC (Sigma) and incubated for 45 minutes at 37°C. gp180 has previously been shown to exist in two forms; a GPI-anchored, apically sorted form and a transmembrane basolateral form (13). The cell suspension was centrifuged for 10 minutes at 1400 rpm and the cells and supernatant were analyzed for released gp180 (GPI-anchored form) through cell staining and western blot.

Purification of gp180

gp180 obtained from PIPLC treatment of IECs was incubated on a mAb B9 affinity column for 18 hours at 4°C. The column was washed with 300 ml of PBS. The gp180 was eluted with 25 ml 0.1 M glycine (Sigma Chemical Co.), pH2.7. One-tenth the volume of 1 M Tris solution (pH 8.6) was added to the eluate after elution. The gp180 was dialyzed against two changes of 2 L PBS and concentrated using a centricon vial (Amicon Inc., Beverly, MA). The amount of protein was determined based on cell equivalents that the gp180 was generated from.

Construction of Fusion Proteins

Fusion proteins containing the α_3 domain of class I MHC and β_2 domain of class II MHC (which has been shown to be the CD4 binding domain and, thus used as a negative control) were generated using a cDNA coding sequence for full-length HLA-A2.1 in a M13 RF subclone designated M13mp18/A2.1 (17). An EcoRI insert, which encompassed the entire HLA-A2.1 cDNA sequence, was subcloned into the EcoRI site of the expression vector pBluescript II KS$^-$ (Stratagene) to generate pHLA-A2.1/BT. A partial cDNA encoding the α_3 domain of HLA-A2.1 only was generated by PCR using 5'-TAGGATCCATGGACGCCCCCAAAA-3' and 5'-TAGAATTCTCACCATCTCAGGTGAGG-3' as 5' and 3' primers, respectively, and pHLA-A2.1/BT plasmid as template. A start codon and a BamHI restriction endonuclease site were incorporated into the 5'-end of the 5' primer, and a stop codon and an EcoRI restriction endonuclease site were added to the 5' end of the 3' primer. PCR reactions were performed under mineral oil in the presence of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 1.25 mM of each deoxynucleotide triphosphate, 0.1 nmoles of each primer and 2.5 U AmpliTaq DNA polymerase (U.S. Biochemical, Cleveland, OH). Reaction thermocycling (30 cycles at 94°C for 1 min, stepped to 50°C for 2 min, stepped to 72°C for 2 min) were performed in a PTC-100 thermocycler (M.J. Research, Watertown, MA). Following organic extraction, the digested α_3 cDNA insert was subcloned between the BamHI and EcoRI sites in the multiple cloning site of the baculovirus vector pVL1393, remobilized with BamHI/Bg1II digestion, and subcloned into the prokaryotic expression vector

pMALp2 (New England Biolabs). This generated a chimeric coding sequence in which α_3 and maltose binding protein (MalE) are linked in-frame in a vector designated pA2.1α_3/MALp2. For large scale purification, α_3/MalE was purified from 2 liter cultures of *E. coli* DH5 α transformants. Bacteria were grown in LB medium containing 0.2% glucose and 100 μg/ml ampicillin up to OD_{600}=0.4 and induced with IPTG. Following induction, bacteria were pelleted, washed with PBS, resuspended in 400 ml of 30 mM Tris-HCl pH 8.0, 20% sucrose, 1 mM EDTA, and incubated 10 min at room temperature. After centrifugation, the cell pellet was subjected to osmotic lysis for 10 min with 400 ml of cold 5 mM $MgSO_4$. The supernatant was collected after centrifugation (8000g for 20 min) and the pH of this solution was adjusted by the addition of 8 ml 0.5 M sodium phosphate buffer, pH 7.2. The fusion protein in the supernatant was bound to a 75 ml amylose resin column (NE Biolabs). The column was washed and the fusion protein was eluted with high salt column buffer (10 mM sodium phosphate, 0.5 M NaCl, 10 mM 2-ME, 1 mM EDTA) containing 10 mM maltose (Sigma). Fractions were analyzed by spectrophotometry and SDS-PAGE. Protein containing fractions were dialysed overnight against PBS and concentrated with Centriprep-30 microconcentrators (Amicon). Exactly the same process was used to generated the β_2 fusion protein. The CD8-Fc (18) and CD40-Fc fusion proteins were constructed using full length cDNA for both CD8 and CD40 in a similar manner.

ELISA

Enzyme-linked immunosorbant assays were used to determine the ability of gp180 to bind to various other molecules expressed by either IECs or PBTs. In performing these assays, gp180 was purified using a mAb B9 affinity column and diluted in coating buffer (dH_20, 0.015 M Na_2CO_3, 0.03 M $NaHCO_3$, 3 mM NaN_3). An optimal dilution of gp180 ($4x10^5$ cell equivalents) was used to coat 96-well Nunc ELISA plates overnight at 4°C. The plates were washed 5x with 100 µl/well of ELISA buffer and blocked with 1% BSA-PBS for 1 hour at RT. After washing, 1 µg/ml CD8-Fc fusion protein or an irrelevant CD40-Fc fusion protein was added to the plates and incubated at RT for 1 hour. The plates were then washed five times. Binding was detected by incubating the plates with 10 µg/ml HRP-conjugated goat anti-mouse Ig antibody which recognized the mouse Fc portion of the fusion protein. After 65 µl HRP substrate (dH_20, 0.2 M $NaPO_4$, 0.2 M Na_2PO_4, 0.001% H_2O_2, 2 mM phenol, 1.2 mM 4-aminoantipyrine) was added, the plate was read by a Genetic Systems microplate ELISA reader at 490 nm.

Cell:cell co-culture experiments

Allogeneic T cells and IECs or $HepG_2$ cells were resuspended to achieve a concentration of $1x10^7$ cells/ml in 0.1% BSA-RPMI 1640. Cells were prewarmed in a 37°C water bath. For each reaction, one million T cells (or ten million when performing immunoprecipitations) were placed in a 1.5 ml eppendorf tube and centrifuged for 5 seconds. The supernatant was removed and the pellet loosened. One million IECs, or $HepG_2$ were mixed with the T

cells, spun quickly for 20 seconds and placed in a 37°C water bath. After 1, 2 or 5 minutes, 1 ml of ice cold stop buffer (PBS with 10 mM sodium orthovanadate) (Sigma, St. Louis, MO) was added. The pellet was resuspended in 100 µl lysis buffer (20% PBS, 80% dH_2O, 100 µM Na_2VO_3, 1 mM PMSF, 5 mM iodoacetamide, 20 µg/ml leupeptin and 20 µg/ml aprotinin) and racked 20x at the start and end of a 30 minute incubation on ice. As a positive control, one million T cells were incubated with 10 µg/ml anti-CD8 antibody (OKT8) for 30 minutes at 4°C and crosslinked with 10 µg/ml rabbit anti-mouse IgG antibody for 2 minutes at 37°C. Time zero tubes were prepared by adding stop buffer to individual tubes containing T cells and either epithelial cells or $HepG_2$. The lysate from these two tubes were then combined into one tube to account for all constitutive kinases and substrates in both cell types. 25 µl reducing buffer (50 mM Tris-HCl pH6.8, 5% 2-ME, 10% glycerol, 1% SDS) was added to all reactions prior to boiling for 5 minutes and the resulting samples were run on a 10% SDS-PAGE gel for western blot.

In the presence of fusion proteins

The α_3 and β_2 fusion proteins (17) were used to determine the effect of either molecule to alter gp180's ability to induce T cell activation. IECs were incubated with the fusion protein for 45 minutes on ice. The cells were then washed several times in RPMI. An allogeneic mixed cell culture experiment was performed as described previously.

Western blot

A 10% SDS-PAGE gel was prepared and one million cells were loaded per lane. The protein was transferred from the gel to nitrocellulose paper at 65V for four hours in transfer buffer (20% methanol, 150 mM glycine, 25 mM Tris, pH8.3). The transfer sandwich was prewet in transfer buffer and set up in the following order: 1) a porous polyethylene foam sheet; 2) one sheet of 3M filter paper (Whatman International Ltd., Maidstone, England); 3) the gel; 4) a nitrocellulose membrane sheet (0.2 μm, Schleicher & Schuell, Inc., Keene, NH); 5) another sheet of 3M filter paper; 6) another sheet of porous polyethylene foam. After the transfer, the nitrocellulose was blocked with 5% milk for 1 hour at RT. The membrane was washed once in PBS and then incubated with primary antibody (1-10 μg/ml) overnight at 4°C. The membrane was washed with PBS several times and incubated with an HRP-conjugated secondary antibody (1-2 μg/ml) for 1 hour at RT. The membrane was finally washed 3-5 times in washing buffer (0.05% Tween-20 in PBS) for 5 minutes each time. The membrane was developed using enzyme linked chemiluminescence (Dupont NEN, Boston, MA) reagent.

Mixed Cell Proliferation Assay in the presence of blocking antibodies

Prior to setting up an mixed cell proliferation assay, isolated IECs were irradiated (2800 rads). These stimulator cells were then plated in a round-bottom 96-well plate at a concentration of $1x10^5/100\mu l$. PBTs were incubated with 10 μg/ml of the various anti-CD8 blocking antibodies (these mAbs are described in the

antibody section) for 45 minutes on ice. The cells were washed thoroughly and incubated with the plated stimulators at a 1:1 ratio. In all cases, IECs and PBTs were incubated alone as control conditions. The cells were cultured together for 5 days at 37°C in a humidified incubator. At this point, all conditions were pulsed with 1 μCi ^{3}H-tritiated thymidine (Dupont NEN, Boston, MA) and harvested 18 hours later. Incorporated counts were measured by a Wallac Microbeta counter.

RESULTS

gp180 binds to CD8-Fc fusion proteins.

Previous data generated in our laboratory indicated that IECs were able to interact with PBTs resulting in both the activation of CD8-associated $p56^{lck}$ and T cell proliferation (13). Furthermore, it was determined that the IEC glycoprotein gp180 was regulating these two events. Since anti-CD8 mAbs inhibit the ability of IECs to induce $CD8^+$ T cell proliferation, it was also known that the CD8 molecule itself was important in this interaction, however, it was not known whether or not gp180 directly interacted with the CD8 molecule. We, therefore, set out to determine if gp180 directly binds to CD8 molecules.

An enzyme-linked immunosorbant assay was used to determine the ability of gp180 to bind to CD8-Fc fusion proteins. gp180 ($4x10^5$ cell equivalents) was used to coat 96-well Nunc Elisa plates. CD8-Fc fusion protein or CD40-Fc (used as a control fusion protein) was added to the plates and incubated at RT for 1 hour. Binding of both CD8-Fc and CD40-Fc was detected using an HRP-conjugated goat anti-mouse Ig antibody which recognizes the mouse Fc portion of the fusion protein. As seen in Figure 1, it appears that gp180 directly binds to CD8 molecules. This appears to explain how intact IECs and purified gp180 are able to induce the phosphorylation of CD8-associated $p56^{lck}$.

The carbohydrate side chains of gp180 appear to be involved in IEC-induced activation of CD8-associated $p56^{lck}$.

Since gp180 is a heavily N-glycosylated glycoprotein, we wanted to determine whether or not the carbohydrate side chains were involved in gp180's ability to bind the CD8 molecule. To do this, we treated cells expressing high levels of gp180 ($HepG_2$) with tunicamycin, to inhibit carbohydrate production by the cell, thus allowing for the expression of a non-glycosylated form of gp180 on the cell surface.

$HepG_2$ cells were cultured to 80% confluence and incubated with 1.5 μg/ml tunicamycin in 10 ml fresh media for 36 hours. The cells were then harvested from the cell culture flask with cell dissociation media (Sigma, St. Louis, MO). Cell viability was assessed to be greater than 90% in all experiments. Since the mAb B9 recognizes the carbohydrate side chains of gp180, mAb B9 staining of tunicamycin-treated $HepG_2$, compared to untreated cells, confirmed a decrease in the expression of gp180 by $HepG_2$. The cells were then co-cultured with PBTs for 0, 1, 2, or 5 minutes. The T cell lysates were run on a 10% SDS-PAGE gel, transferred to nitrocellulose and subjected to an anti-phosphotyrosine western blot.

As seen in Figure 2, it appears that treating these gp180-expressing cells with tunicamycin prevented these cells from properly glycosylating gp180, thereby effectively interfering with the ability of these cells to induce the phosphorylation of CD8-associated $p56^{lck}$. Importantly, the phosphorylated band seen at 70

kd indicates that tunicamycin did not interfere with the ability of $HepG_2$ to activate other kinases or substrates. The band observed at 70 kd may be ZAP70. This kinase could be phosphorylated through interaction with the TCR.

This suggests that the carbohydrate side chains are important in the ability of gp180 to bind to CD8 and trigger the activation of $p56^{lck}$. It is conceivable, however, that non-glycosylated gp180 either fails to get to the cell surface or that de-glycosylation may affect the conformation of this molecule. Similar studies could not be performed using N-glycanase since the reaction requires detergent.

A class I MHC fusion protein (α_3 fusion protein) does not block the activation of $p56^{lck}$ by intestinal epithelial cells.

We have previously shown that CD8:class I MHC interactions do not result in the activation of CD8-associated $p56^{lck}$, thus gp180 and class I MHC might bind to CD8 at distinct sites. To determine whether the class I MHC binding sites of CD8 overlap with the sites used by gp180, an MHC class I α_3 fusion protein was used to block the MHC class I binding domain of CD8. Importantly, the α_3 domain of class I MHC has been shown to contain the CD8 binding sites.

Fusion proteins from the α_3 domain of class I MHC and β_2 domain of class II MHC (which has been shown to be the CD4 binding domain and, thus used as a negative control) were incubated with $1x10^6$ peripheral T cells for 30 minutes on ice. Both fusion protein were the generous gift of Dr. Mark Tykocinski (Case Western Reserve

University, Cleveland, OH). The coated T cells were then incubated with IECs for 0, 1, 2 or 5 minutes and the reactions stopped by adding 1 ml of ice cold stop buffer. As a positive control, T cells were incubated with 10 μg/ml OKT8 mAb (anti-CD8 mAb) for 30 minutes on ice and then washed with cold PBS. The positive control was then incubated with 10 μl/ml rabbit anti-mouse Ig antibody for 2 minutes in a 37°C water bath. All samples were lysed in 100 μl lysis buffer and run on a 10% SDS-PAGE gel and subsequently, blotted with an anti-phosphotyrosine (4G10) antibody (1 μg/ml). An HRP-conjugated secondary antibody was used to detect the binding of the primary antibody. Although occupying the class I MHC binding site of the CD8 molecule with the α_3 fusion protein slightly decreased the ability of gp180 to interact with the CD8 molecule, and induce the phosphorylation of CD8-associated $p56^{lck}$, this effect was not significant (Figure 3).

Anti-CD8 antibodies recognizing non-class I MHC binding sites of CD8 inhibit the proliferation of T cells by intestinal epithelial cells.

Another approach was used to confirm whether or not similar CD8 sites are involved in gp180 and class I MHC binding. To test which sites on the CD8 molecule were involved in the binding to gp180, various mAbs against distinct CD8 epitopes were used (OKT8, OKT8B, OKT8C, OKT8E, OKT8F, $OKT8I_1$ and $OKT8I_2$). These antibodies have been previously characterized and some identify epitopes involved in the interaction of class I MHC with CD8 (14). These mAbs (10 μg/ml) were incubated with PBTs for 45 minutes on ice

before being washed. Irradiated stimulator cells (IECs) were incubated with T cells at a 1:1 ratio. In some cases, IECs and PBTs were incubated alone as negative controls. The cells were cultured for 5 days at 37°C in a humidified incubator. At this point, all conditions were pulsed with 1 μCi ^{3}H-tritiated thymidine and harvested 18 hours later.

Four anti-CD8 antibodies (OKT8B, OKT8E, OKT8F and OKT8I$_1$), recognizing different CD8 epitopes, inhibited IEC-induced T cell proliferation (Figure 4). This may point to critical amino acid sequences in the CD8 molecule that are involved in gp180 interactions. Importantly, others have reported that anti-CD8 mAbs OKT8A, OKT8C, OKT8I$_1$ and OKT8I$_2$ recognize sites of CD8's interaction with class I MHC. This was assessed by the inhibition of CTL responses. The fact that OKT8I$_1$ inhibited the ability of IECs to trigger the proliferation of CD8$^+$ T cells suggests that there may be at least one overlapping region in the class I MHC and gp180 binding sites of CD8 or that these antibodies bind CD8 epitopes which are close to epitopes bound by gp180 therefore inhibiting by steric hinderance. Overall, gp180 appears to bind to distinct sites on CD8 from those bound by class I MHC.

DISCUSSION

In the gastrointestinal tract, the demands upon the immune system are different from those of the systemic immune system. There is a marked difference in antigen load, with the intestine constantly exposed to dietary, viral and bacterial products, antigens that would evoke active immunity if administered systemically. There are several unique features of mucosal immunity. The state of systemic nonresponsiveness that can be induced by feeding antigen to a host (oral tolerance) is often the most intriguing feature of the mucosal immune system. Although the mechanisms involved in the induction of oral tolerance may be multifold, several groups have reported that $CD8^+$ T cells play a role in transferring tolerance to naive animals (5, 6). Recent studies have suggested that antigen presentation by IECs results in the activation of $CD8^+CD28^-$ T cells which have suppressor and not cytolytic activity (15, 19). This suggests that the IEC may play a role in the activation of a subset of T cells which is involved in the suppression of mucosal and, potentially, systemic immune responses.

Two mAbs, B9 and L12, identify a novel 180 kd IEC glycoprotein, gp180, which is capable of interacting with the CD8 molecule. In this paper, we have shown that gp180 binds to CD8 molecules, which would explain the earlier observation that IECs induce the activation of the CD8α chain associated, but not the CD4-associated, protein tyrosine kinase $p56^{lck}$ (20). When the anti-gp180 monoclonal antibody B9 is added to IEC:T cell co-cultures, no IEC induced $p56^{lck}$ activation or proliferation of $CD8^+$ T cells is observed. This supports the concept that gp180 is critical to the

activation of these $CD8^+$ T cells and that the activation of $CD8^+$ suppressor T cells by IECs appears to involve the CD8 molecule. Functionally, the cells that proliferate in these cultures are noncytolytic and suppress broadly in an antigen-unrestricted fashion, in man. Phenotypically, these cells appear to be $CD8\alpha^+$, $CD28^-$, $IL\text{-}2R^+$ (20). MAbs against CD8 but not CD4 inhibit the IEC-induced proliferation of suppressor T cells. Interestingly, antibodies recognizing conventional restriction elements do not inhibit IEC-induced activation and proliferation of CD8+ T cells (20).

The nature of the interaction between CD8 and gp180 appears to be mediated by the carbohydrate side chains of gp180. Furthermore, this interaction involves sites on the CD8 molecule that are, for the most part, distinct from those bound by classical class I MHC molecules. Thus, these data provide further evidence for unique molecular events involved in T cell:epithelial cell interactions. These interactions may help to explain the activation of distinct regulatory subsets in the gut and the development of the immunologically suppressed tone in the gastrointestinal tract.

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FIGURE LEGENDS

FIGURE 1: CD8-Fc fusion proteins appear to be capable of binding to gp180.

Purified gp180 ($4x10^5$ cell equivalents) was used to coat a Nunc ELISA plate. Binding of gp180 was assessed using the fusion proteins CD8-Fc (1 µg/ml) and CD40-Fc (1 µg/ml). Detection was determined using an HRP-conjugated goat anti-mouse mAb. Lane 1: control lane representing GAM-HRP without coating of the plate with gp180. Lane 2: control lane representing CD8-Fc and GAM-HRP without coating of the plate with gp180. In lanes 3 to 5, $4x10^5$ cell equivalents gp180 is used to coat the wells. Lane 3: gp180 is incubated with GAM-HRP to determine the degree of non-specific binding. In lane 4: gp180 is incubated with CD40-Fc followed by GAM-HRP to determine the degree of non-specific binding due to the use of a mouse Fc fusion protein. Lane 5: the CD8-Fc fusion is added to the gp180 coated wells followed GAM-HRP. This figure is representative of three separate experiments.

FIGURE 2: The carbohydrate side chains of gp180 are involved in the activation of CD8-associated p56lck.

$HepG_2$ cells were treated with tunicamycin for 36 hours prior to conducting the experiments. Staining of the treated cells confirmed a decrease in gp180 glycosylation. Treated and untreated cells were co-cultured with PBT cells for varying time periods. Lane 1: negative control of T cells alone. Lane 2: positive control of T cells incubated with anti-CD8 mAb (OKT8) and crosslinked with RAM for 2 minutes. Lane 3: negative control of $HepG_2$ cells alone. Lanes 4 to

7: untreated $HepG_2$ co-cultured with PBT for 0, 1, 2 or 5 minutes. Lanes 8 to 11: $HepG_2$ cells treated with tunicamycin for 36 hours prior to being co-cultured with T cells for 0, 1, 2 or 5 minutes. This figure is representative of two experiments.

FIGURE 3: The class I MHC fusion protein (α_3 fusion protein) does not block the activation of p56lck by intestinal epithelial cells.

PBTs were incubated with either the α_3 domain of class I MHC or the β_2 domain of class II MHC prior to being co-cultured with IECs for varying time periods. The T cell lysates were run on a 10% SDS-PAGE gel, transferred to nitrocellulose and subjected to an anti-phosphotyrosine western blot. Lane 1: negative control representing unstimulated T cell lysate. Lane 2: this positive control represents PBT cells stimulated with 10 μg/ml anti-CD8 monoclonal antibody (OKT8) and crosslinked with rabbit anti-mouse antibody. Lane 3 to 6: these lanes represent PBT cells pre-treated with the α_3 domain of class I MHC prior to being co-cultured with IECs for 0', 1', 2' or 5', respectively. Lanes 7 to 10: these lanes are the same as lanes 3 to 6 except that the PBTs are pre-treated with the β_2 domain of class II MHC. This figure is representative of two experiments.

FIGURE 4: Anti-CD8 antibodies recognizing non-class I MHC binding sites of CD8 inhibit the proliferation of T cells by intestinal epithelial cells.

PBTs were treated with various anti-CD8 antibodies recognizing different CD8 epitopes for 45 minutes prior to being cultured with irradiated IECs. The cells were cultured for 5 days at which time they were pulsed with tritiated thymidine and harvested 18 hours later. Lane 1: negative control of irradiated IECs alone. Lane 2: T cells alone. Lane 3: control of T cells cultured with IECs in presence of an isotype control mAb. Lanes 4-10: PBTs incubated with OKT8B, C, E, F, I_1, I_2 and A, respectively, prior to being cultured with IECs. Responses are depicted as a % of the positive control of T cells co-cultured with IECs. This figure is representative of two experiments.

Figure 1

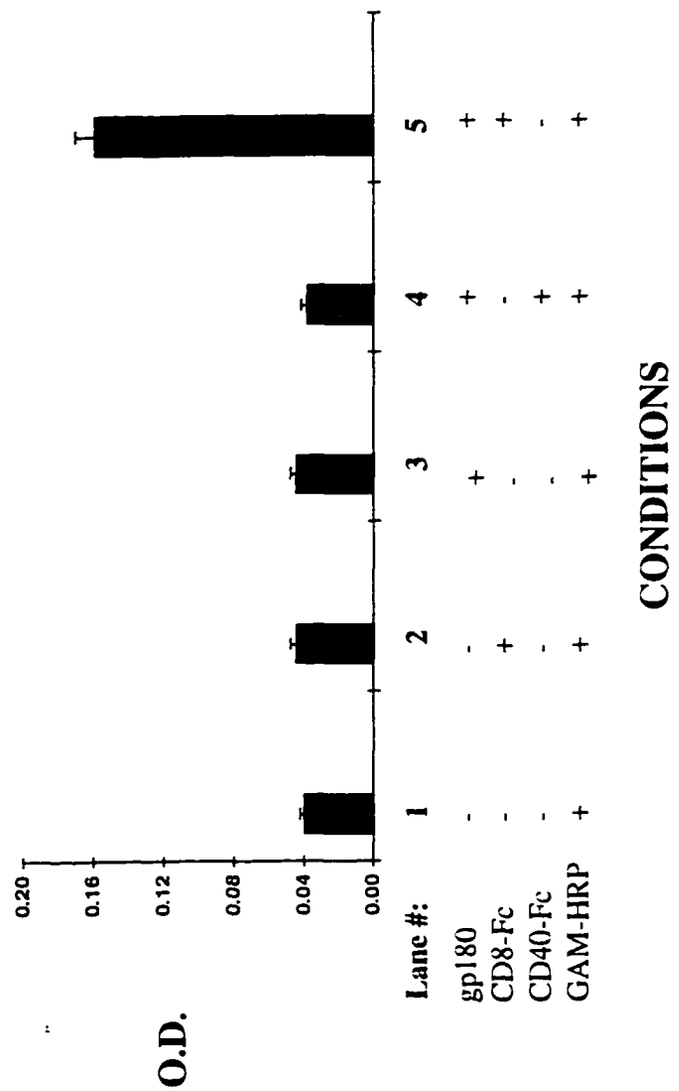


Figure 2

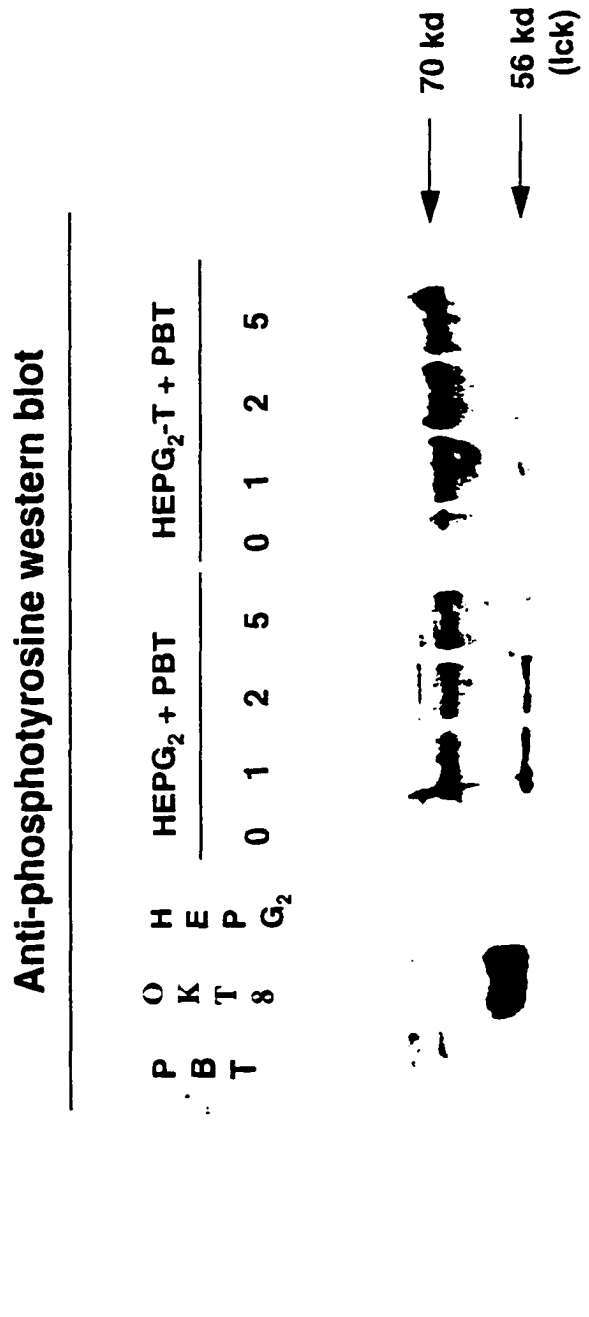
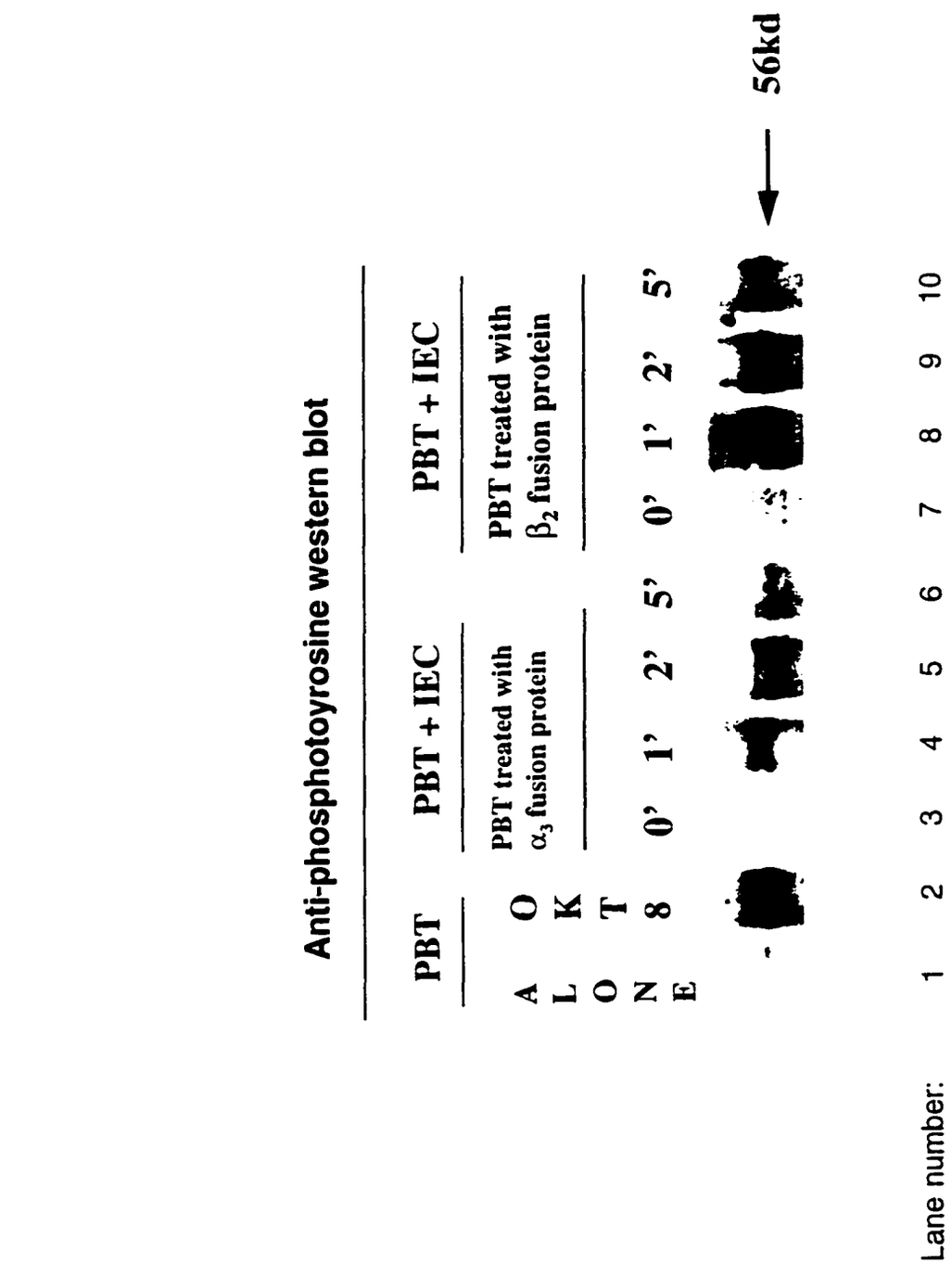
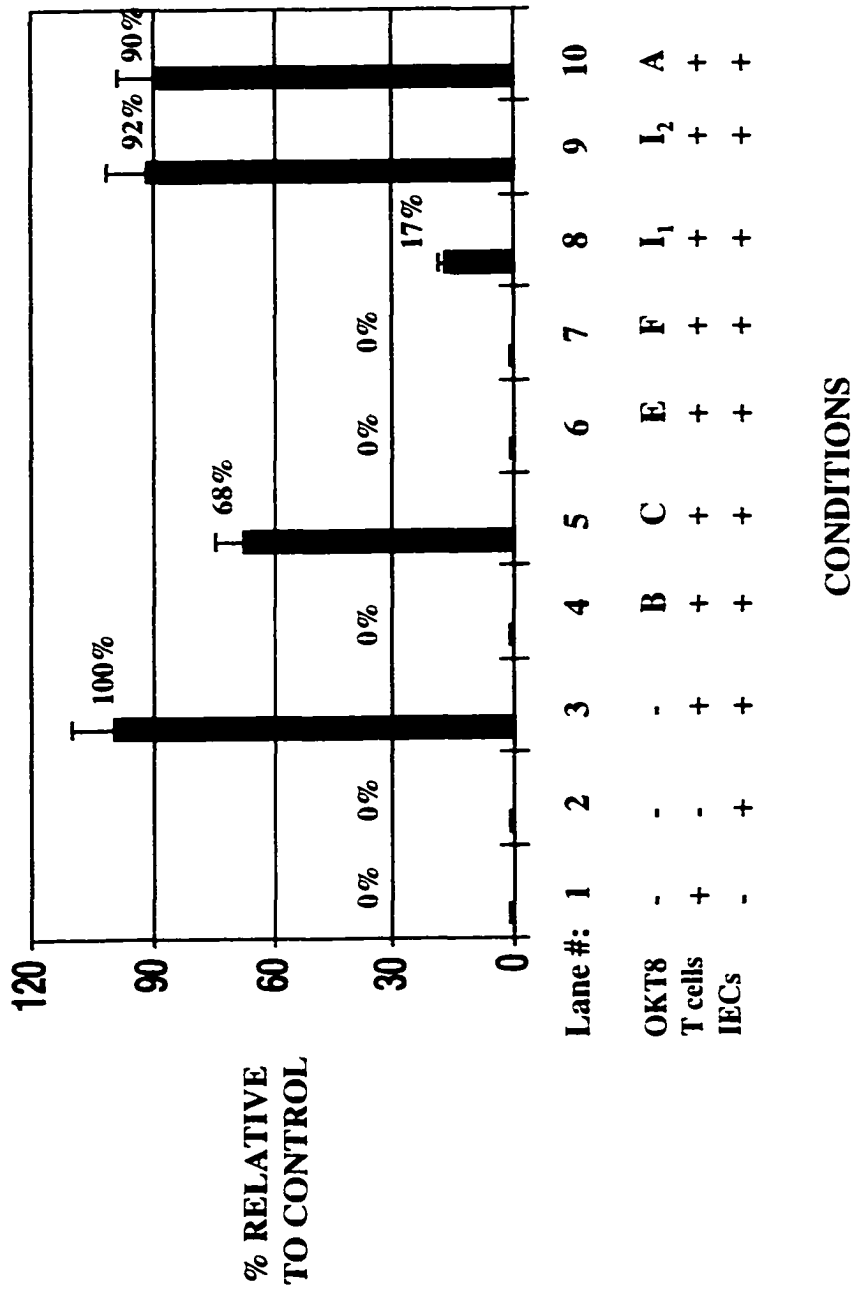


Figure 3



*** Figure 4**



SECTION II

The non-classical class I molecule CD1d associates with the novel CD8 ligand gp180 on intestinal epithelial cells

ABSTRACT

Previous studies have shown that normal IECs (IECs) are able to selectively activate $CD8^+$ suppressor T cells inducing proliferation associated with the activation of both the CD8-associated kinase $p56^{lck}$ and the TCR-associated kinase $p59^{fyn}$. This process appears to relate in part to a 180 kd IEC surface glycoprotein, gp180, which binds to CD8 and activates CD8-associated $p56^{lck}$. However, purified gp180 alone is unable to induce T cell proliferation and does not activate $p59^{fyn}$. Since the class Ib molecule CD1d is expressed by IECs and mAbs against CD1d inhibit IEC-induced proliferation of $CD8^+$ T cells, immunoprecipitation studies were performed and demonstrated an association of gp180 and CD1d on the IEC surface. This association was confirmed by ELISA using gp180 as the capture antigen. Interestingly, the activation of $p59^{fyn}$ in IEC:T cell co-cultures was blocked by the anti-CD1d mAb D5 but not by the anti-gp180 mAb B9. Conversely, treatment of IECs with mAb B9 inhibited IEC-induced activation of $p56^{lck}$ but not $p59^{fyn}$. More directly, a human CD1d cDNA (FO-1 D5) transfectant was able to activate $p59^{fyn}$ but not $p56^{lck}$. However, FO-1 D5 cells pulsed with soluble gp180 was capable of activating of both $p56^{lck}$ and $p59^{fyn}$. These data

suggest that the CD1d:gp180 complex on the surface of IECs can be recognized by the TCR:CD8 co-receptor resulting in the activation of $CD8^+$ T cells.

INTRODUCTION

The goal of the systemic immune system is to generate specific responses against foreign pathogens that result in their elimination. In contrast, while antigen exposure in the gastrointestinal tract appears to be magnified due to dietary and microbial antigen load, the immune response is limited or suppressed. This state of non-responsiveness to orally administered antigens is referred to as oral tolerance (1-12). While there have been several mechanisms involved for the development of this state, many studies have shown that the activation of suppressor T cells is crucial for the existence of oral tolerance (5, 6). Unfortunately, the mechanisms responsible for the activation of this subset of T cells have not been completely defined.

Previous studies have demonstrated that IECs can act as antigen presenting cells (APC) capable of stimulating primed T cells (13-16). Interestingly, despite the constitutive expression of class II MHC molecules on these cells, the T cells proliferating in these co-cultures are $CD8^+$(17, 18). The subset of $CD8^+$ T cells that proliferate when co-cultured with IECs are functional suppressor T cells (19) and inhibit primary, secondary and unrelated mixed lymphocyte reactions, as well as B cell responses *in vitro* in an antigen non-specific manner. The addition of mAbs against CD8 to these co-cultures have documented that the CD8 molecule itself is important in the activation of suppressor T cells by IECs with the activation of the CD8-associated protein tyrosine kinase $p56^{lck}$ being a necessary but not sufficient event (20). Interestingly, the addition of mAbs against classical restriction elements, class I and

class II MHC, do not inhibit the activation of these $CD8^+$ suppressor T cells suggesting some novel form of interaction.

It is known that IECs express the non-classical restriction elements CD1d in man and CD1 and TL in mouse (21-23). Interestingly, the CD1d transcripts are only observed within intestinal crypt cells while protein expression appears to be localized to the intestinal villus (24). Previous studies have documented that cytolytic IEL can be restricted by these class Ib molecules (25) and that the proliferation of T cells in IEC:T cell co-cultures can be inhibited by mAbs to CD1d in a human system (26). This molecule may, therefore, serve as a restriction element for IEC:T cell interactions. However, in contrast to the conventional restriction element class I MHC, CD1d fails to bind CD8. Thus, in order for a TCR:CD8 co-receptor complex to form in this system, another cell surface interaction must exist.

Studies in our laboratory have reported on two mAbs generated against IECs that were capable of inhibiting the IEC-induced proliferation of $CD8^+$ T cells and activation of the CD8-associated kinase $p56^{lck}$ (20). Both of these mAbs, B9 and L12, recognize a 180 kd glycoprotein, termed gp180, which is expressed on epithelial cells in various organs. While L12 recognizes a molecule expressed on the entire epithelium of the gastrointestinal and respiratory tract, B9 recognizes gp180 expressed by the entire gastrointestinal epithelium, cortical thymic epithelium and placental syncytiotrophoblasts. The 180 kd IEC surface glycoprotein is a novel molecule capable of binding to CD8 and activating the CD8-associated protein tyrosine kinase $p56^{lck}$, but not the TCR-

associated kinase $p59^{fyn}$. This is in contrast to studies using intact IECs where activation of $p59^{fyn}$ is also seen (27). These data suggest that gp180 may be associated with a molecule that is capable of interacting with the T cell receptor and activating TCR-associated $p59^{fyn}$.

Given the findings reported above, CD1d might be an eligible candidate to aid gp180 in the activation and proliferation of $CD8^+$ suppressor T cells. In the present investigation, we demonstrate that gp180 associates with CD1d and that this gp180:CD1d complex is involved in the activation of $CD8^+$ suppressor T cells.

MATERIALS AND METHODS

Cell Isolation, Cell Lines and Culture Conditions.

FO-1 cells transfected with CD1d cDNA (FO-1 D5) have been previously described (28). These transfected cells were maintained in RPMI supplemented with 10% FCS, 20 mM Hepes, 1% non-essential amino acids, 1% L-glutamine, 1% penicillin/streptomycin and 3 mg/ml G418 (Sigma). Untransfected control FO-1 cells were cultured in similar media without G418 and served as a negative control.

Peripheral blood mononuclear cells (PBMC) were isolated from leukocyte concentrate packs and separated into T and non-T cells using rosetting and density gradient centrifugation as previously described (26). Briefly, heparinized venous blood was collected from normal donors and separated by Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradient centrifugation. T cells and non-T cells were isolated from PBMCs by a rosetting method using neuraminidase-treated sheep red blood cells followed by Ficoll-Paque density gradient centrifugation.

Enterocytes were isolated by a method described previously (17, 26). Surgical specimens were obtained from the operating room. Specimens were washed extensively with PBS containing 1% penicillin/streptomycin and 1% fungizone (Flow Laboratory Inc., McLean, VA). The mucosa was stripped from the submucosa, cut into small pieces and placed in 1 mM dithiothreitol (Sigma) for 5 minutes at room temperature to remove the mucus. The pieces were then washed in PBS and incubated in dispase (3 mg/ml in RPMI 1640, Boehringer Mannheim, GmbH, Germany) for 30 minutes in a 37°C

shaking incubator. This was repeated four times. The tissue pieces were removed and the cell suspension collected, pooled and centrifuged on a Percoll density gradient (Pharmacia, Piscataway, NJ). Enterocytes located at the 0-30% interface were washed three times with PBS and resuspended in RPMI with 0.1% BSA for co-culture experiments. Preparations of purified enterocytes were >90% viable, free of macrophages and B cell contamination as determined by staining with anti-CD14 and anti-CD20 mAbs (Coulter Corp., Hialeah, FL) and contaminated with only 2-4% IEL ($CD3^+$ cells).

Antibodies.

B9 is a murine IgG1 anti-human IEC mAb which appears to recognize an epitope on the carbohydrate side chains of the novel IEC surface molecule gp180 (27). Ascites was generated at a concentration of 1 mg/ml and used at a 1:1000 dilution for western blotting while 10 μg was used for immunoprecipitation studies. An irrelevant murine IgG1 antibody was used as a negative control whenever necessary.

Four mAbs were used to detect CD1d. 3C11 and 1H1 are rat IgM anti-mouse CD1 mAbs, which have been previously shown to cross-react with human CD1d (21, 22, 28). Supernatants were harvested from the 1H1 or 3C11 hybridomas and adjusted to a concentration of 10 μg/ml of mAb. D5 is a murine anti-human CD1d mAb generated against a glutathione-S-transferase fusion protein of CD1d (29). This antibody was used at a concentration of 5 μg/ml. 51.1.3 is a mouse anti-human CD1d mAb raised against an Fc-fusion protein of CD1d (30).

OKT3, OKT8 and W6/32 are hybridomas obtained from the American Type Culture Collection (ATCC, Rockville, MD) which produce mAbs against CD3, CD8 and a non-polymorphic domain of class I MHC, respectively.

Isolation of Purified gp180.

$2x10^7$ IECs were washed, resuspended in 1 ml RPMI with 1 unit PIPLC (Sigma) and incubated for 45 minutes at 37°C. gp180 has previously been shown to exist in two forms; a GPI-anchored, apically sorted form and a transmembrane basolateral form. The cell suspension was centrifuged for 10 minutes at 1400 rpm and the cells and supernatant were analyzed for released gp180 (GPI-anchored form) through cell staining (to determine loss of gp180 from the cell surface) and western blot analysis (to determine the release of gp180 into the supernatant).

Construction of CD1d-GST Fusion Proteins.

The CD1d-GST fusion protein was constructed using the α1-α3 domains of CD1d in a pGEX vector (Pharmacia, Piscataway, NJ) (29). For large scale preparations of CD1d-GST fusion protein, 10 ml of transformed bacteria grown freshly overnight was inoculated into 500 ml of LB media with ampicillin selection. This was incubated for 90 minutes at 37°C. After adding 0.1 mM IPTG, the bacterial culture was grown for an additional 4 hours. The culture was spun in a Sorvall centrifuge for 10 min at 5000 rpm at room temperature and resuspended in 6 ml of STE buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA). It was then spun in a tabletop centrifuge for 10

min at 3500 rpm. The pellet was resuspended in 24 ml of 100 μg/ml lysozyme in STE along with protease inhibitors (0.1 mg/ml PMSF, and 20 μg/ml aprotinin) and incubated on ice for 15 min prior to adding 5 mM DTT and 1.5% sarkosyl in STE. Bacterial cell walls were broken using a tissue homogenizer for 15 min and pellets were obtained by spinning in a Sorvall SS-34 rotor at 14,000 rpm for 10 min. After adding 4% Triton X-100, 2.5 ml of glutathione agarose beads (Sigma) were incubated with the lysate for 2 hours at 4°C on a orbital rocker. The beads were spun down, transferred to microcentrifuge tubes and washed 6-8x with 1 ml cold PBS containing 0.5% Triton X-100. The final wash was with PBS alone and CD1d-GST protein was eluted by rocking at 4°C for 10 min in 0.75 ml of elution buffer (75 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM DTT, 0.1% Triton X-100) containing 100 mM reduced glutathione. The supernatant (eluant) was saved and another 200 μl of elution buffer was added to elute the remaining fusion protein. When the eluant was resolved by SDS-PAGE and stained with Coomasie blue, a single band at 65 KD was seen, the approximate weight of the CD1d-GST fusion protein.

Co-culture, Immunoprecipitation and Kinase Assays.

T cells and IECs were resuspended to achieve a concentration of $1x10^7$ cells/ml in 0.1% BSA-RPMI 1640. Cells were prewarmed in a 37°C water bath. For each reaction, one million T cells were placed in a 1.5 ml eppendorf tube and centrifuged for 5 seconds. The supernatant was removed and the pellet loosened. One million IECs, FO-1 D5 or untransfected FO-1 cells were mixed with the T cells,

spun quickly for 20 seconds and placed in a 37°C water bath. After 1, 2 or 5 minutes, 1 ml of ice cold stop buffer (PBS with 10 mM sodium orthovanadate) (Sigma, St. Louis, MO) was added. The pellet was resuspended in 100 ml lysis buffer (20% PBS, 80% dH_2O, 100 mM Na_2VO_3, 1 mM PMSF, 5 mM iodoacetamide, 20 µg/ml leupeptin and 20 µg/ml aprotinin) and racked 20x at the start and end of a 30 minute incubation on ice. As a positive control, one million T cells were incubated with 10 µg/ml anti-CD3 antibody (OKT3) or 10 µg/ml anti-CD8 antibody (OKT8) for 30 minutes at 4°C and crosslinked with 10 µg/ml rabbit IgG anti-mouse antibody for 2 minutes at 37°C. Time zero tubes were prepared by adding stop buffer to individual tubes containing T cells and either epithelial cells, FO-1 D5 or untransfected cells. The lysate from these two tubes were then combined into one tube to account for all constitutive kinases and substrates in both cell types. 25 µl reducing buffer (50 mM Tris-HCl pH6.8, 5% 2-ME, 10% glycerol, 1% SDS) was added to all reactions prior to boiling for 5 minutes and the resulting samples were run on a 10% SDS-PAGE gel for western blot.

In some experiments, the lysate from these co-culture conditions were immunoprecipitated with a rabbit anti-human $p59^{fyn}$ or rabbit anti-human $p56^{lck}$ antibody which were covalently bound to sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA). These conjugated antibodies were rotated with co-culture lysates (pre-cleared with rabbit serum coated sepharose 4B beads) for 1 hour at 4°C. The beads were washed four times with PBS and 75 µl reducing buffer (50 mM Tris-HCl pH6.8, 5% 2-ME, 10% glycerol, 1% SDS) was added prior to boiling for 5 minutes. The resulting

samples were then run on a 10% SDS-PAGE gel and transferred to nitrocellulose paper (Schleicher & Schuell Inc., Keene, NH). A mAb 4G10 anti-phosphotyrosine (Upstate Biotechnology Incorporated, Lake Placid, NY) western blot was then performed.

In other experiments, lysates of IECs were immunoprecipitated with murine IgG1 (isotype control), anti-gp180 mAb B9 or the anti-class I mAb W6/32 (negative control) to determine if an association between CD1d and gp180 existed. Protein A sepharose beads were coated with 10 μg/ml rabbit IgG anti-mouse immunoglobulin followed by 10 μg/ml primary antibody (isotype control, anti-gp180 or anti-class I MHC). Each of these steps were performed for 1 hour at room temperature while rotating. The beads were then washed thoroughly with wash buffer (2 M Tris pH7.4, 0.5 M EDTA, 4 M NaCl) and epithelial cell lysate was rotated with the coated beads overnight at 4°C. The beads were washed 5 times with RIP buffer (10 mM Tris pH8.0, 1.0 mM EDTA, 0.5% NP-40, 0.1 M NaCl, 1 mg/ml ovalbumin and 0.02% Na azide) and resolved on SDS-PAGE. After transferring to nitrocellulose an anti-CD1d (D5) followed by an anti-gp180 (B9) western blot was performed.

When kinase assays were performed, beads were resuspended in 30 μl kinase buffer (10 mM $MnCl_2$, 50 mM Tris, pH7.4) and mixed with 10 μCi (γ-32)ATP (Amersham, Arlington Heights, IL) for 30 minutes at room temperature. The enzyme reaction was stopped by adding 15 μl 4x reducing buffer (as previously described) and boiled 5 minutes.

Western Blots.

A 10% SDS-PAGE gel was prepared and one million cells were loaded per lane. The protein was transferred from the gel to nitrocellulose paper and blocked with 5% milk for 1 hour at RT. The membrane was then incubated in 1 μg/ml of primary antibody (4G10, B9, D5 or isotype control) overnight at 4°C. The membrane was washed with PBS several times and incubated in 1 μg/ml of HRP-conjugated goat anti-mouse Ig (Cappel, Durham, NC) for 1 hour at RT. In some cases, 1 μg/ml directly conjugated 4G10-HRP was used. The membrane was developed using enzyme linked chemiluminescence (Dupont NEN, Boston, MA) reagent.

Enzyme-Linked Immunosorbant Assays.

gp180 was purified using a B9 affinity column and diluted in coating buffer (dH_20, 0.015 M Na_2CO_3, 0.03 M $NaHCO_3$, 3 mM NaN_3). An optimal dilution of gp180 ($4x10^5$ cell equivalents) was used to coat 96-well Nunc ELISA plates overnight at 4°C. The plates were washed 5x with 100 μl/well of ELISA buffer and blocked with 1% BSA-PBS for 1 hour at RT. After washing, 1 μg/ml CD1d-GST fusion protein or GST alone was added to the plates and incubated at RT for 1 hour. The plates were then washed five times. Binding was detected by incubating the plates with 5 μg/ml mouse anti-GST antibody for 1 hour followed by an hour incubation with 10 μg/ml HRP-conjugated goat anti-mouse Ig secondary antibody. After 65 μl HRP substrate (dH_20, 0.2 M $NaPO_4$, 0.2 M Na_2PO_4, 0.001% H_2O_2, 2 mM phenol, 1.2 mM 4-aminoantipyrine) was added, the plate was read by a Genetic Systems microplate ELISA reader at 490 nm.

RESULTS

CD1d associates with gp180 on the surface of intestinal epithelial cells.

Our previous studies have shown that co-culturing IECs with PBTs results in the activation of the TCR-associated $p59^{fyn}$ and CD8-associated $p56^{lck}$ tyrosine kinases. Purified gp180 was found to be responsible for the activation of the protein tyrosine kinase $p56^{lck}$, but not $p59^{fyn}$ (27). It was, therefore, hypothesized that another molecule might associate with gp180 and interact with the TCR. Since anti-CD1d antibodies have been shown to inhibit IEC-induced proliferation of $CD8^+$ suppressor T cells, CD1d was an eligible candidate to aid gp180 in the activation of $CD8^+$ T cells. Thus, the hypothesis that gp180 associates with CD1d was studied.

Initial studies were performed to confirm that normal IECs express both gp180 and CD1d on their surface. $1x10^5$ cells per condition were stained with B9, D5 and appropriate isotype controls to confirm cell surface expression of gp180 and CD1d, respectively. The cells were analyzed by an Epics Profile III flow cytometer. Figure 1a illustrates that freshly isolated IECs do express both of these molecules. A co-immunoprecipitation study was then performed using these same freshly isolated IECs. A lysate was prepared by lysing $1x10^7$ IEC in lysis buffer containing 1% digitonin. The lysate was immunoprecipitated with a murine IgG1 mAb isotype control, mAb B9 or mAb W6/32 (anti-class I MHC as a negative control). An anti-CD1d (D5) followed by an anti-gp180 (B9) western blot was then performed. Figure 1b documents the association of gp180 (180 kd) and CD1d (37 kd). The B9 mAb immunoprecipitated a

180 kd band consistent with gp180 (lane 3), as well as a doublet at 37 kd, consistent with the form of CD1d isolated from IECs (28). The 37 kd, 48 kd, and 95 kd bands were detected only after the anti-CD1d (D5) western blot, while the 180 kd band was detected only upon incubation with the mAb B9. While this figure provides data with IECs lysed in a digitonin based lysis buffer, there appears to be no difference in those cells lysed with either NP-40 or Brij 97 (data not shown). Thus, the interaction between gp180 and CD1d appears to be quite strong. In addition, it appears that neither gp180 nor CD1d co-precipitates with the conventional restriction element class I MHC. The additional bands evident in the B9 IP lane, such as the ~95kd band, may reflect a trimer of CD1d, which has been previously described (28). Disruption of this multimer may require strong conditions of alkylation and reduction (28). A glycosylated form of CD1d, which has been described and is likely to be present on IECs (22), may account for the 48 kd band observed.

In order to confirm these findings more directly, an enzyme-linked immunosorbant assay was performed. An optimal concentration ($4x10^5$ cell equivalents) of gp180 was used to coat ELISA plates and 1 μg/ml CD1d-GST or control proteins were added to the wells. A mouse anti-GST antibody (5 μg/ml) was then added followed by an HRP-conjugated goat anti-mouse IgG antibody to detect binding. As seen in Figure 2, the CD1d-GST fusion protein bound gp180 to a significantly greater extent in comparison to the control GST protein. Thus by two experimental approaches, CD1d appears to be capable of associating with gp180.

CD1d alone does not activate the CD8-associated protein tyrosine kinase $p56^{lck}$ but does appear to activate the TCR-associated kinase $p59^{fyn}$.

Initial experiments conducted in our laboratory suggest that CD1d is not involved in the activation of CD8-associated $p56^{lck}$ in IEC:T cell co-cultures. This was determined by mAb inhibition studies where neither 3C11 nor 1H1 inhibited IEC activation of $p56^{lck}$. However, in order to confirm that CD1d was not involved in the activation of $p56^{lck}$ more directly, FO-1 cells stably transfected with human CD1d cDNA (FO-1 D5), were co-cultured with PBTs for varying incubation periods (0', 2' or 5'). The reactions were stopped with cold lysis buffer. The T cell lysates were immunoprecipitated with mouse IgG anti-human $p56^{lck}$ antibody, the proteins resolved on a SDS-PAGE gel, and an anti-phosphotyrosine western blot performed. In this experiment (Figure 3a), no phosphorylation of the CD8-associated protein tyrosine kinase $p56^{lck}$ was seen. As a control, untransfected FO-1 cells were cultured with PBTs and subjected to the same conditions as the FO-1 D5 cells. Equivalent amounts of precipitated lck were noted in all conditions (inset).

In order to document that the CD1d expressed by the transfectant was functional, we utilized the same cell lysate to determine whether CD1d could activate the TCR-associated kinase $p59^{fyn}$. FO-1 D5 cells were co-cultured with PBTs for varying incubation periods (0', 2' and 5') in the presence or absence of the mouse IgG anti-human CD1d mAb, D5. As a control, untransfected FO-1 cells were cultured with PBTs and subjected to the same conditions as the FO-1 D5 cells. All reactions were stopped with

cold lysis buffer. The T cell lysates were immunoprecipitated with rabbit anti-human $p59^{fyn}$ antibody and an anti-phosphotyrosine western blot was performed. As seen in Figure 3b, activation of $p59^{fyn}$ is readily seen in FO-1 D5:T cell co-cultures and this was blocked by the mAb D5. FO-1 cells do not express either CD1d or gp180 thus explaining the inability of this cell line to activate $p56^{lck}$ or $p59^{fyn}$. In addition, the ability of FO-1 D5 cells to activate $p59^{fyn}$ indicates that the non-β_2-microglobulin associated forms of CD1d expressed by FO-1 D5 is functional (28). Equal amounts of $p59^{fyn}$ protein (inset) in these immunoprecipitates were seen in all conditions.

Antibodies against CD1d do not inhibit CD8-associated kinase $p56^{lck}$, but do inhibit the TCR-associated kinase $p59^{fyn}$ in freshly isolated intestinal epithelial cell:T cell co-cultures.

We have previously shown that co-culturing purified gp180 with $CD8^+$ T cells results in the activation of CD8-associated $p56^{lck}$. The studies represented in Figures 3a and 3b appear to indicate that CD1d may be responsible for the activation of the TCR-associated kinase $p59^{fyn}$ but not of the CD8-associated kinase $p56^{lck}$. Since the use of a CD1d transfectant may not actually reflect *in vivo* events, we set out to confirm this in our isolated IEC:T cell co-culture system.

An antibody to CD1d (D5) was incubated with IECs prior to being co-cultured with T cells. Excess antibody was removed by washing three times with RPMI 1640. After varying incubation

periods at 37°C, the T cells were lysed, immunoprecipitated with rabbit anti-human $p56^{lck}$, resolved on SDS-PAGE and subjected to an anti-phosphotyrosine western blot. As seen in Figure 4a, the D5 mAb was capable of only slightly inhibiting the activation of $p56^{lck}$. To determine the impact that this same antibody has on the activation of the TCR-associated $p59^{fyn}$ kinase, T cell lysates from IEC:T cell co-cultures treated with D5 were immunoprecipitated with the rabbit anti-human $p59^{fyn}$ antibody covalently bound to sepharose beads (Santa Cruz). As can be seen in Figure 4b, the anti-CD1d mAb D5 is capable of inhibiting the activation of the TCR-associated protein tyrosine kinase $p59^{fyn}$ in this co-culture system. These results suggest that CD1d is capable of interacting with the TCR and activating the TCR-associated $p59^{fyn}$ kinase but was incapable, directly or indirectly, of activating $p56^{lck}$. It therefore appeared that CD1d was capable of interacting with the T cell receptor and activating $p59^{fyn}$ in both *in vitro* (CD1d transfectants) and *ex vivo* (freshly isolated IECs) systems.

Since the anti-CD1d mAb, D5 appeared to inhibit the ability of IECs to phosphorylate $p59^{fyn}$ in T cells, we next asked whether this observation could be confirmed using another anti-CD1d mAb, 51.1.3. IECs were incubated with either 51.1.3 or D5 mAb (10 μg/ml) for 30 minutes on ice and then washed thoroughly. The IECs were co-cultured with PBTs for 0, 2 or 5 minutes at 37°C. The T cells were lysed, immunoprecipitated with a rabbit anti-$p59^{fyn}$ antibody, the protein resolved by SDS-PAGE, transferred to nitrocellulose and subjected to an anti-phosphotyrosine western blot. As seen in Figure 5, the relative intensities of the bands resolved at 59 kd

indicated that whereas the D5 mAb almost completely inhibited the ability of IECs to activate the TCR-associated $p59^{fyn}$ kinase, the anti-CD1d mAb 51.1.3 inhibited $p59^{fyn}$ activation by 50% compared to the isotype control. These results further support the notion that CD1d on IECs was capable of triggering the phosphorylation of $p59^{fyn}$ in $CD8^+$ T cells.

gp180 is able to bind extracellularly to CD1d.

Experiments conducted to this point indicated that CD1d associates with gp180 on the surface of IECs and that this complex is involved in the activation of $CD8^+$ T cells. We wanted to then determine if gp180 was capable of binding to CD1d extracellularly or if membrane anchoring was required. PBTs were cultured with FO-1 cells (control transfection cells), FO-1 D5 cells (L cells transfected with human CD1d cDNA), purified gp180, or both for 0 or 2 minutes at 37°C. To test the ability of soluble gp180 to bind CD1d, purified gp180 was incubated with FO-1 or FO-1 D5 cells and left unwashed (U) or washed thoroughly (W), prior to being co-cultured with PBTs. The T cells were lysed and immunoprecipitated with an anti-$p56^{lck}$ antibody. The protein was resolved by SDS-PAGE and subjected to an anti-phosphotyrosine western blot. As seen in Figure 6, the relative intensities of the bands resolved at 56 kd indicated that soluble gp180 was capable of binding to membrane bound CD1d. Using this system we were able to reconstitute the signal events induced by the co-culture of PBT cells with intact IECs. These results further support the notion that gp180 is capable

of associating with CD1d, and that this complex serves to activate T cells.

DISCUSSION

Although the role of classical restriction elements has been relatively clearly defined, the function of the structurally related class Ib proteins has not yet been thoroughly investigated. Recent studies from several groups suggest that class Ib molecules may be involved in stress responses or in T cell responses against non-protein antigens (31, 32). These molecules include the CD1 family of proteins. The CD1 family of non-classical class I-like proteins are encoded outside the MHC and most members have limited homology to classical class I restriction elements (21, 22, 28, 31, 33). CD1d is a nonpolymorphic isoform of the CD1 family which is expressed predominantly by IECs. The mouse CD1d homologue has been shown to be capable of binding peptides generally larger than those bound by classical restriction elements (33), however the recent X-ray crystallographic structure of CD1d (34) suggests that it may also bind lipids similar to CD1b and CD1c. In addition, CD1d may be endosomally localized like CD1b (35), hence peptide and/or lipid (34) association with CD1d can occur within classical class II loading compartments (exogenous Ag pathway) or on the cell surface. The immunoregulatory function of CD1d has not been clearly defined but its presence on the surface of IECs lends support to the notion that it may serve as a regulator of mucosal immune responses. Indeed, previous studies have shown that IEL killing of IEC lines can be CD1d restricted and PBT cell proliferation induced by IEC is inhibited by anti-CD1d antibodies (25, 26).

Mucosal surfaces, such as those found in the gastrointestinal tract, are continuously exposed to antigen, however, immune

responses are not mounted to most dietary and microbial antigens. The state of systemic nonresponsiveness that can be induced by feeding antigen to a host (oral tolerance) is a unique feature of the mucosal immune system. Although the mechanisms involved in the induction of oral tolerance may be multifold, several groups have reported that $CD8^+$ T cells play a role in transferring tolerance to naive animals. Recent studies have demonstrated that antigen presentation by IECs results in the activation of $CD8^+CD28^-$ T cells which clearly have suppressor activity (17, 19). This suggests that the IEC may play a role in the activation of a subset of T cells which is involved in the suppression of mucosal and, potentially, systemic immune responses.

The activation of $CD8^+$ suppressor T cells by IECs appears to involve the CD8 molecule. MAbs against CD8 but not CD4 inhibit IEC-induced proliferation of suppressor T cells. Interestingly, antibodies recognizing conventional restriction elements do not inhibit IEC-induced activation and proliferation of $CD8^+$ T cells (20). Thus, other molecular interactions were analyzed.

Two mAbs, B9 and L12, identify a novel 180 kd IEC glycoprotein, gp180, which is capable of interacting with the CD8 molecule. We have shown that gp180 binds to CD8 molecules and activates the CD8α chain associated protein tyrosine kinase $p56^{lck}$ (27). When the anti-gp180 mAb B9 is added to IEC:T cell co-cultures, no IEC induced activation or proliferation of $CD8^+$ T cells is observed. This supports the concept that gp180 is critical to the activation of these $CD8^+$ T cells.

Data generated in this study clearly indicate that gp180 does not act alone in the activation and proliferation of $CD8^+$ suppressor T cells. Since it has been previously determined that anti-CD1d antibodies inhibit the proliferation of $CD8^+$ T cells by IECs (26), we examined the role that CD1d played in the activation of these cells. It was clearly demonstrated that CD1d associates with the glycoprotein gp180. Furthermore, it was shown that CD1d is not responsible for the activation of the CD8-associated kinase $p56^{lck}$ but is responsible for the activation of the TCR-associated kinase $p59^{fyn}$. This is consistent with previous studies which failed to show an association of CD1d with CD8 molecules.

The key question relates to the nature of the antigen presented by the non-polymorphic molecule, CD1d, in our allogeneic system. Several possibilities exist. First, alloreactive T cells may recognize processed class I or class II fragments in the antigen binding cleft of CD1d. Alternatively, proteins found in serum (both human serum and fetal calf serum used in cell culture) may be taken up by a non-polymorphic restriction element, CD1d, expressed on IECs and presented to T cells. Thus, CD1d on cell lines and freshly isolated IECs could be constantly loaded with peptides derived from serum. Finally, it may be that CD1d has limited polymorphisms not previously appreciated. Wilson et al (34) suggested that CD1 is incapable of binding peptide due to its deep hydrophobic pocket, however, Castano et al. using a peptide display library showed that CD1 was capable of associating with peptides larger that those bound by classical class I and class II molecules (33). The response to CD1d presented peptide (or non-peptide) may be limited but it is

present. The other issue relates to the cell population activated. The subpopulations of $CD8^+$ T cells expanded in our co-culture system may exist as "pre-suppressor" cells. If such regulatory cells arise from a distinct subpopulation, it could well explain the difficulty in clearly identifying suppressor T cells in the past.

In our postulated model, gp180 associates with the non-classical class I molecule CD1d on the surface of IECs. More specifically, we postulate that each component of this complex has a unique function: gp180 binds to the CD8 molecule resulting in the activation of the CD8-associated kinase $p56^{lck}$ while CD1d interacts with the TCR causing the phosphorylation of the TCR-associated kinase $p59^{fyn}$. The gp180:CD1d complex thus appears more like classical class I, capable of interacting with the TCR:CD8 co-receptor complex. This postulated model may account for several features observed in the gastrointestinal tract. The existence of this complex could explain the presentation of exogenous antigen to $CD8^+$ T cells by IECs, the presence of predominantly $CD8^+$ T cells in the IEC compartment, and the poor activation of mucosal lymphocytes by conventional antigen presenting cells which lack CD1d. The necessity for the activation of these suppressor T cells in the gut is clear. Any inability to do so is costly, resulting in inflammation and the loss of functional integrity in the gastrointestinal tract. The use of distinct molecules and restriction elements provides further evidence for the differences between systemic and mucosal immunity. This dichotomy may evolve from the differences in antigen load and the requirement for controlled rather than active immune responses in the gastrointestinal tract.

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FIGURE LEGENDS

Figure 1a: Freshly isolated intestinal epithelial cells express surface gp180 and CD1d.

Freshly isolated IECs ($1x10^5$ per condition) were stained with an isotype control (mouse IgG1 or IgG2b), mouse IgG1 anti-human gp180 (B9) or mouse IgG2b anti-human CD1d (D5) mAbs. FITC-conjugated goat anti-mouse Ig antibody was used as the secondary detection antibody. Histogram (A): control well cells were stained with 10 μg/ml IgG1 or IgG2b isotype controls. Histogram (B): cells were stained with 10 μg/ml D5 mAb. Histogram (C): cells were stained with 10 μg/ml B9 mAb. This figure indicates that the IECs used for Figure 1b express both gp180 and CD1d on their cell surface.

FIGURE 1b: gp180 associates with the class Ib molecule, CD1d.

IEC lysate ($1x10^7$ cell equivalents) was immunoprecipitated with mouse IgG1 mAb (control), anti-class I MHC antibody (W6/32) or mouse IgG1 anti-gp180 (B9) and the immunoprecipitated proteins were run on a 10% SDS-PAGE gel. An anti-CD1d (D5) followed by an anti-gp180 western blot was then performed. All bands, except the one observed at 180 kd, were observed following the D5 western blot. Lane 1: IEC lysate was immunoprecipitated with mouse IgG1 mAb. Lane 2: IEC lysate immunoprecipitated with W6/32 mAb. In addition to acting as a control, this also determines if gp180 is capable of associating with classical class I MHC molecules. Lane 3: IEC lysate immunoprecipitated with mouse IgG1 anti-gp180 mAb (B9). This figure shows that gp180 does not associate with class I

MHC molecules but does appear to associate with CD1d which is represented by a 37 kd doublet.

FIGURE 2: gp180 binds to a CD1d-GST fusion protein.

The wells of Nunc ELISA plates were coated with purified gp180 or left uncoated. CD1d-GST or control GST fusion proteins were added to assess binding of CD1d to gp180. Lane 1: GST, anti-GST and GAM-HRP in the absence of coating the plate with gp180. Lane 2: control where no gp180 is coating the plate and CD1d-GST, anti-GST and GAM-HRP are added. In lanes 3 to 5, an optimal concentration ($4x10^5$ cell equivalents) of gp180 was used to coat the wells. Lane 3: the GAM-HRP lane indicates that GAM-HRP does not bind to gp180 directly (gp180 + GAM-HRP). In lane 4: the GST fusion protein is added to the gp180 coated wells, followed by an anti-GST mAb and GAM-HRP. Lane 5: the CD1d-GST fusion is added to the gp180 coated wells, followed by an anti-GST mAb and GAM-HRP. There is a dramatic difference seen between the wells containing CD1d-GST alone and those with gp180 plus CD1d-GST.

FIGURE 3a: CD1d does not activate CD8-associated protein tyrosine kinase p56lck.

Either the cell line transfected with CD1d cDNA, FO-1 D5, or the untransfected control FO-1 was cultured with PBTs for 0, 2 or 5 minutes. The cells were then lysed and were immunoprecipitated with an anti-$p56^{lck}$ antibody (Santa Cruz) and an anti-phosphotyrosine western blot was performed. Lane 1: negative control of T cells alone. Lane 2: control of T cells stimulated with

anti-CD3 (OKT3) mAb and crosslinked with RAM for 2 minutes. Lane 3: positive control of T cells stimulated with anti-CD8 (OKT8) mAb and crosslinked with RAM for 2 minutes. Lanes 4 to 6: FO-1 cells co-cultured with PBTs for 0, 2 or 5 minutes. Lanes 7 to 9: FO-1 cells treated with anti-CD1d mAb D5 for 30 minutes prior to being co-cultured with PBTs for 0, 2 or 5 minutes. Lanes 10 to 12: FO-1 D5 cells co-cultured with PBTs for 0, 2 or 5 minutes. Lanes 13 to 15: FO-1 D5 cells treated with anti-CD1d mAb D5 for 30 minutes prior to being co-cultured with PBTs for 0, 2 or 5 minutes. Inset: anti-lck western blot of these lysates serves as a control for equal loading of protein given the absence of bands. Compared to the positive control where T cells were incubated with anti-CD8 antibodies, CD1d alone does not appear to activate the CD8-associated protein tyrosine kinase $p56^{lck}$. This experiment was repeated three times.

FIGURE 3b: CD1d appears to activate the TCR-associated protein tyrosine kinase p59fyn.

Lysates from FO-1 D5 or FO-1:PBT co-cultures (treated or untreated with the anti-CD1d D5 mAb, as described in 3(a), were immunoprecipitated with an anti-$p59^{fyn}$ antibody (Santa Cruz) and an anti-phosphotyrosine western blot was performed. Lane 1: negative control of T cells alone. Lane 2: positive control of T cells stimulated with anti-CD3 (OKT3) mAb and crosslinked with RAM for 2 minutes. Lane 3: control of T cells stimulated with anti-CD8 (OKT8) mAb and crosslinked with RAM for 2 minutes. Lanes 4 to 6: FO-1 cells co-cultured with PBTs for 0, 2 or 5 minutes. Lanes 7 to

9: FO-1 cells treated with anti-CD1d mAb D5 for 30 minutes prior to being co-cultured with PBTs for 0, 2 or 5 minutes. Lanes 10 to 12: FO-1 D5 cells co-cultured with PBTs for 0, 2 or 5 minutes. Lanes 13 to 15: FO-1 D5 cells treated with anti-CD1d mAb D5 for 30 minutes prior to being co-cultured with PBTs for 0, 2 or 5 minutes. Inset: anti-fyn western blot of these lysates serves as a control for equal loading of protein given the absence of bands. Only the CD1d cDNA transfected cell line was capable of activating TCR-associated $p59^{fyn}$ and this was blocked by the addition of the anti-CD1d mAb D5. This figure is representative of 4 experiments.

FIGURE 4a: Anti-CD1d antibodies do not appear to be capable of inhibiting IEC-induced activation of p56lck.

Lysates from IEC:PBT co-cultures (treated or untreated with the anti-CD1d D5 mAb) were immunoprecipitated with anti-$p56^{lck}$ antibody (Santa Cruz) and an anti-phosphotyrosine western blot was performed. Lane 1: negative control of T cells alone. Lane 2: negative control of T cells stimulated with anti-CD3 mAb and crosslinked with RAM for 2 minutes. Lane 3: positive control of T cells stimulated with anti-CD8 mAb and crosslinked with RAM for 2 minutes. Lanes 4 to 8: IECs co-cultured with PBTs in the isotype control for 0, 1, 2, 5 or 10 minutes. Lanes 9 to 13: IECs pre-incubated with anti-CD1d antibody (D5) then co-cultured with PBTs for 0, 1, 2, 5 or 10 minutes. This figure is representative of three experiments.

FIGURE 4b: Antibodies against CD1d appear to inhibit the activation of TCR-associated protein tyrosine kinase p59fyn.

Lysates from IEC:PBT co-cultures (treated or untreated with the anti-CD1d D5 mAb) were immunoprecipitated with anti-$p59^{fyn}$ antibody and an anti-phosphotyrosine western blot was performed. Lane 1: negative control of T cells alone. Lane 2: negative control of T cells stimulated with anti-CD8 mAb and crosslinked with RAM for 2 minutes. Lane 3: positive control of T cells stimulated with anti-CD3 mAb and crosslinked with RAM for 1 minute. Lane 4: positive control of T cells stimulated with anti-CD3 mAb and crosslinked with RAM for 2 minutes. Lanes 5 to 8: IECs co-cultured with PBTs for 0, 1, 2 or 5 minutes. Lanes 9 to 12: IECs pre-incubated with anti-CD1d antibody (D5) then co-cultured with PBTs for 0, 1, 2 or 5 minutes. This figure is representative of four experiments.

FIGURE 5: Anti-CD1d antibody 51.1.3 partially inhibits the ability of intestinal epithelial cells to activate TCR-associated p59fyn kinase in CD8+ suppressor T cells.

Lysates from IEC:T cell co-cultures treated with isotype control, anti-CD1d D5 mAb or anti-CD1d 51.1.3 mAb were immunoprecipitated with an anti-$p59^{fyn}$ antibody, resolved by SDS-PAGE and subjected to an anti-phosphotyrosine western blot. Lane 1: negative control of T cells alone. Lane 2: positive control of T cells stimulated with anti-CD3 mAb and crosslinked with RAM for 2 minutes. Lane 3: negative control of T cells stimulated with anti-CD8 mAb and crosslinked with RAM for 2 minutes. Lanes 4 to 6:

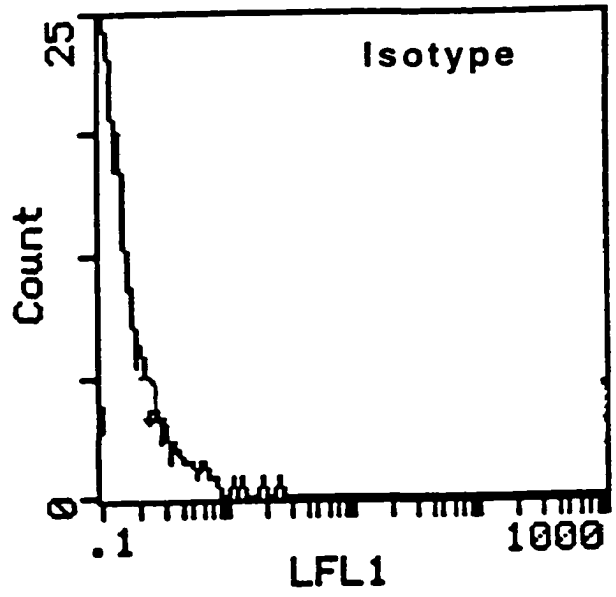
IECs pre-incubated with an irrelevant control mAb were co-cultured with PBTs for 0, 2 or 5 minutes. Lanes 7 to 9: IECs pre-incubated with anti-CD1d antibody (D5) and co-cultured with PBTs for 0, 2 or 5 minutes. Lanes 10 to 12: IECs pre-incubated with anti-CD1d antibody (51.1.3) and co-cultured with PBTs for 0, 2 or 5 minutes. The relative intensity depicted represents the percent of density of phosphorylated $p59^{fyn}$ in antibody treated conditions compared to the isotype control lanes.

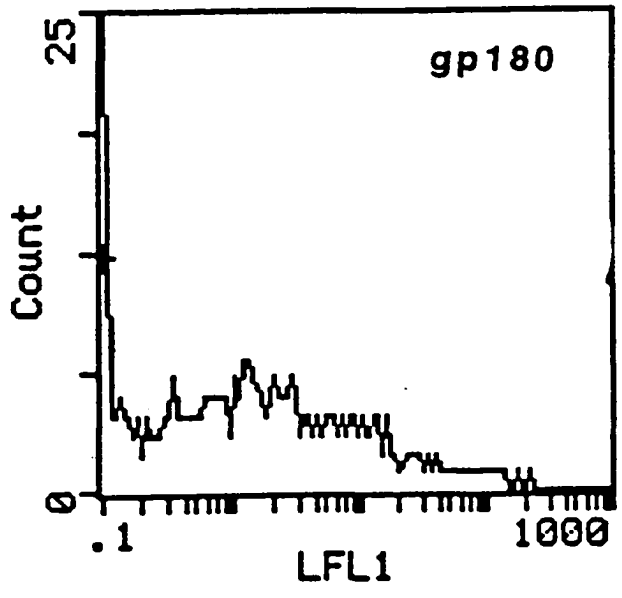
FIGURE 6: gp180 is able to bind extracellularly to CD1d.

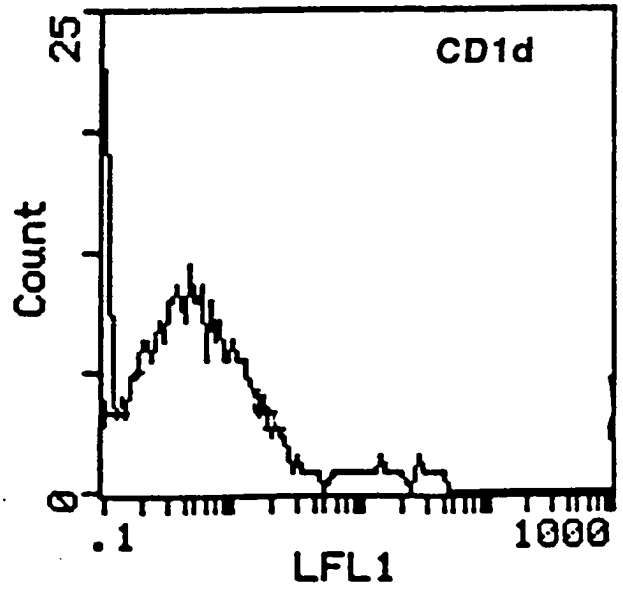
Lysates from PBTs treated with FO-1 cells (control transfection cells), FO-1 D5 cells (melanoma cells transfected with human CD1d cDNA), purified gp180, or both were immunoprecipitated with an anti-$p56^{lck}$ antibody, resolved by SDS-PAGE and subjected to an anti-phosphotyrosine western blot. Lane 1: negative control of T cells alone. Lane 2: negative control of T cells stimulated with anti-CD3 mAb and crosslinked with RAM for 2 minutes. Lane 3: positive control of T cells stimulated with anti-CD8 mAb and crosslinked with RAM for 2 minutes. Lanes 4 and 5: PBTs cultured with untransfected FO-1 cells for 0 or 2 minutes. Lanes 6 and 7: PBTs were co-cultured with human CD1d cDNA transfected cells (FO-1 D5) for 0 or 2 minutes. Lanes 8 and 9: PBTs cultured with purified gp180 for 0 or 2 minutes. Lane 10: FO-1 D5 cells were pre-incubated with purified gp180 prior to being co-cultured with PBT for 2 minutes. Lane 11: FO-1 D5 cells were pre-incubated with purified gp180 and washed four times prior to being co-cultured with PBT for 2 minutes. Lanes 12: FO-1 cells were pre-incubated

with purified gp180 prior to being co-cultured with PBT for 2 minutes. Lane 13: FO-1 cells were pre-incubated with purified gp180 and washed four times prior to being co-cultured with PBT for 2 minutes. The absence of a band in this lane confirms the effectiveness of the washing procedure. This figure is representative of two separate experiments.

Figure 1a







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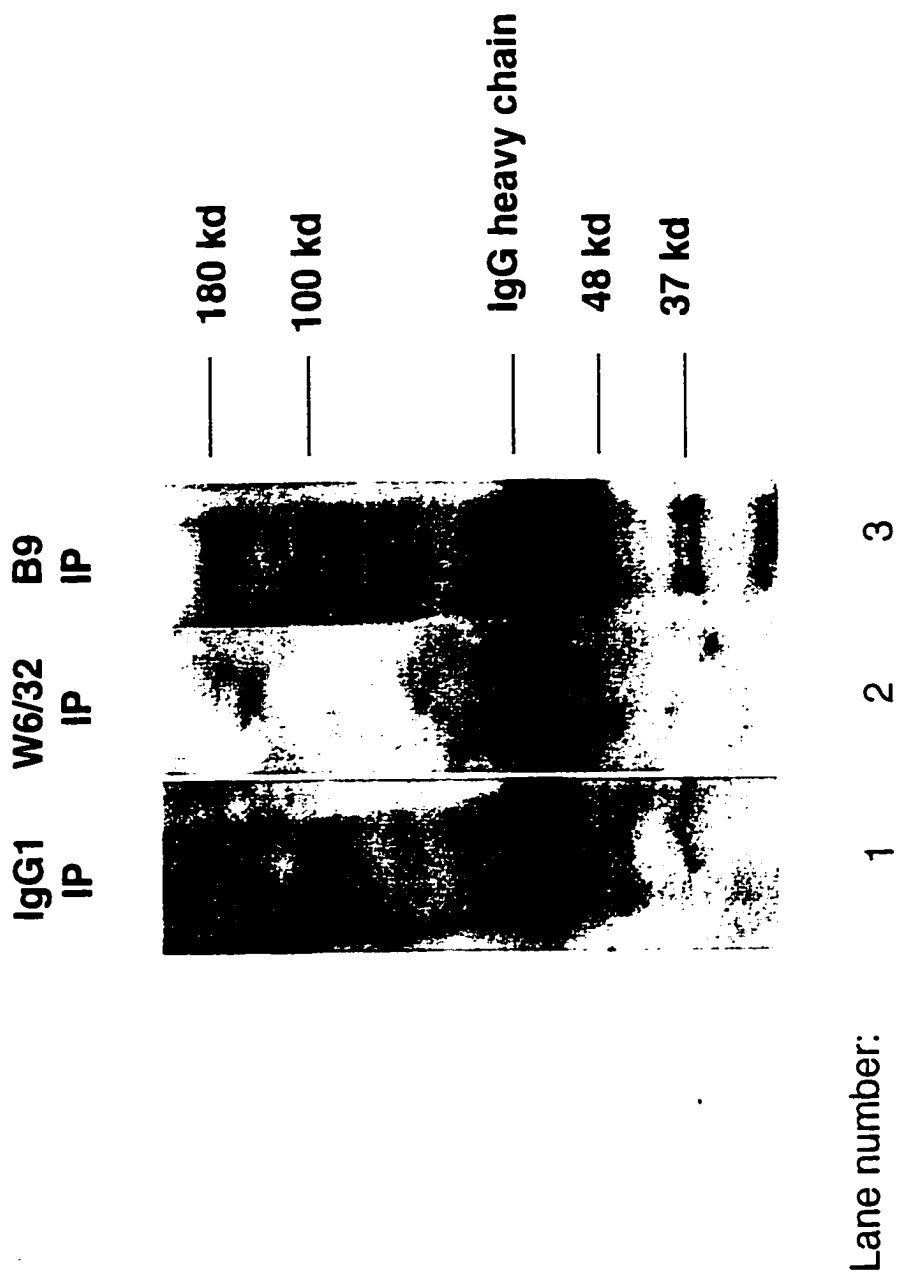

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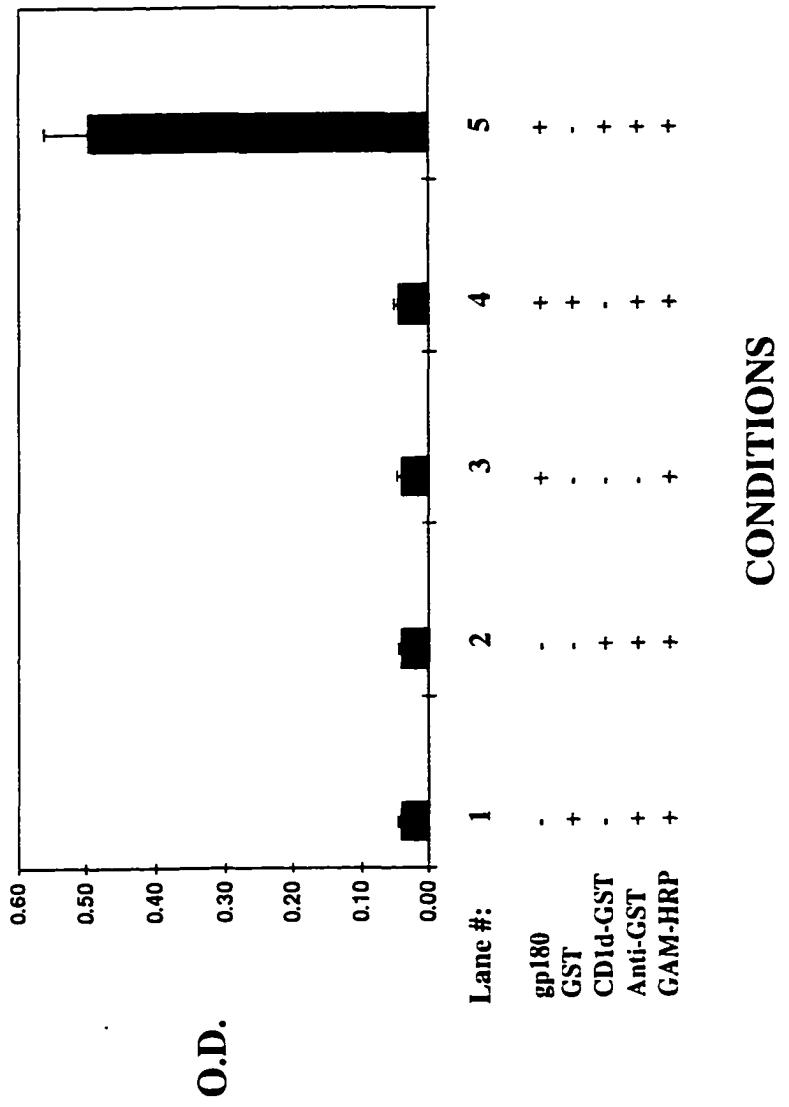

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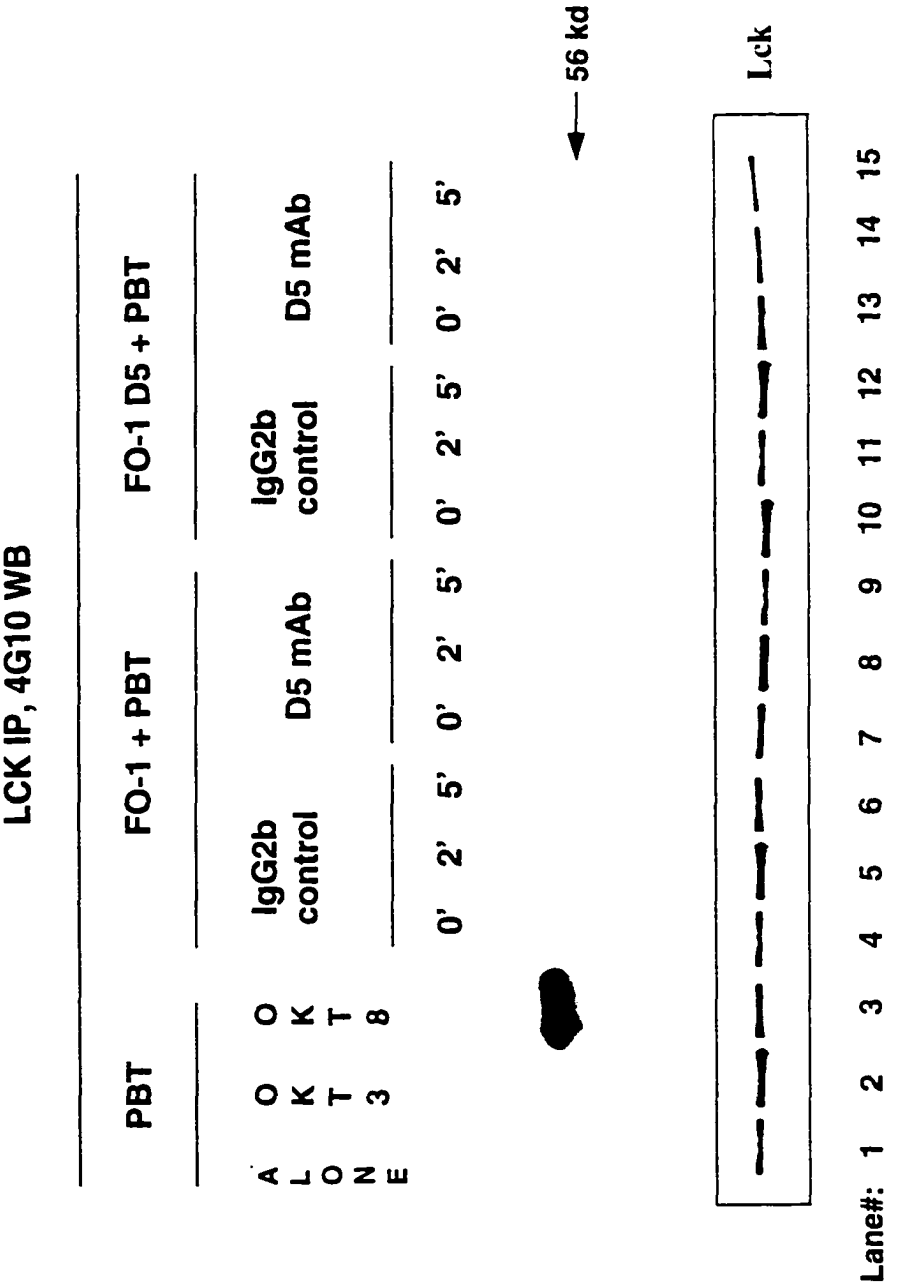

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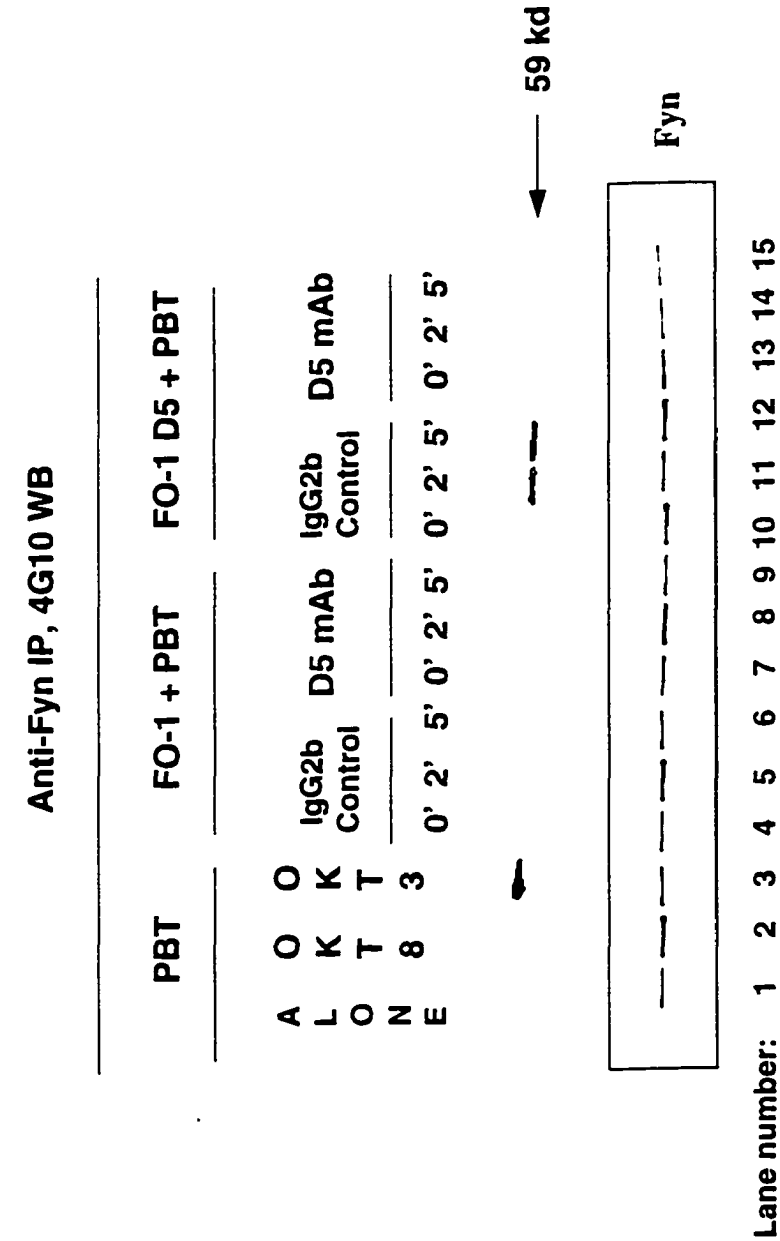

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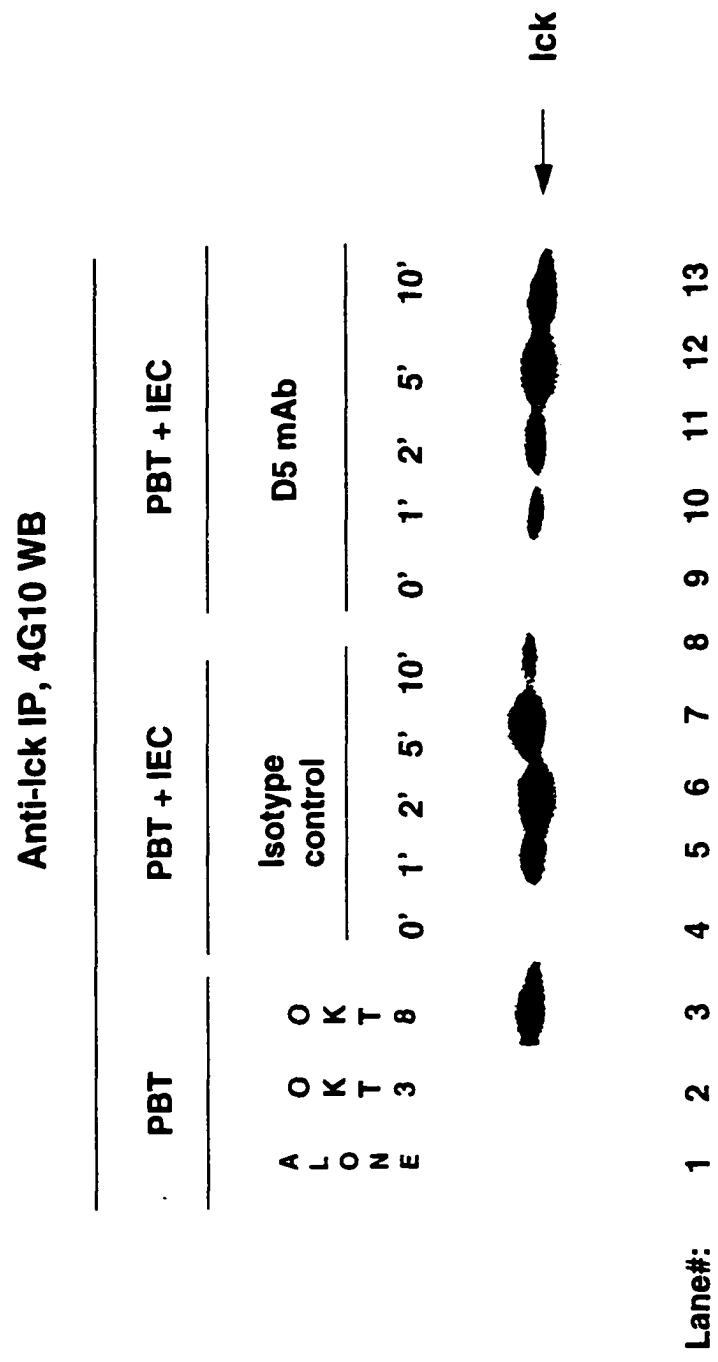

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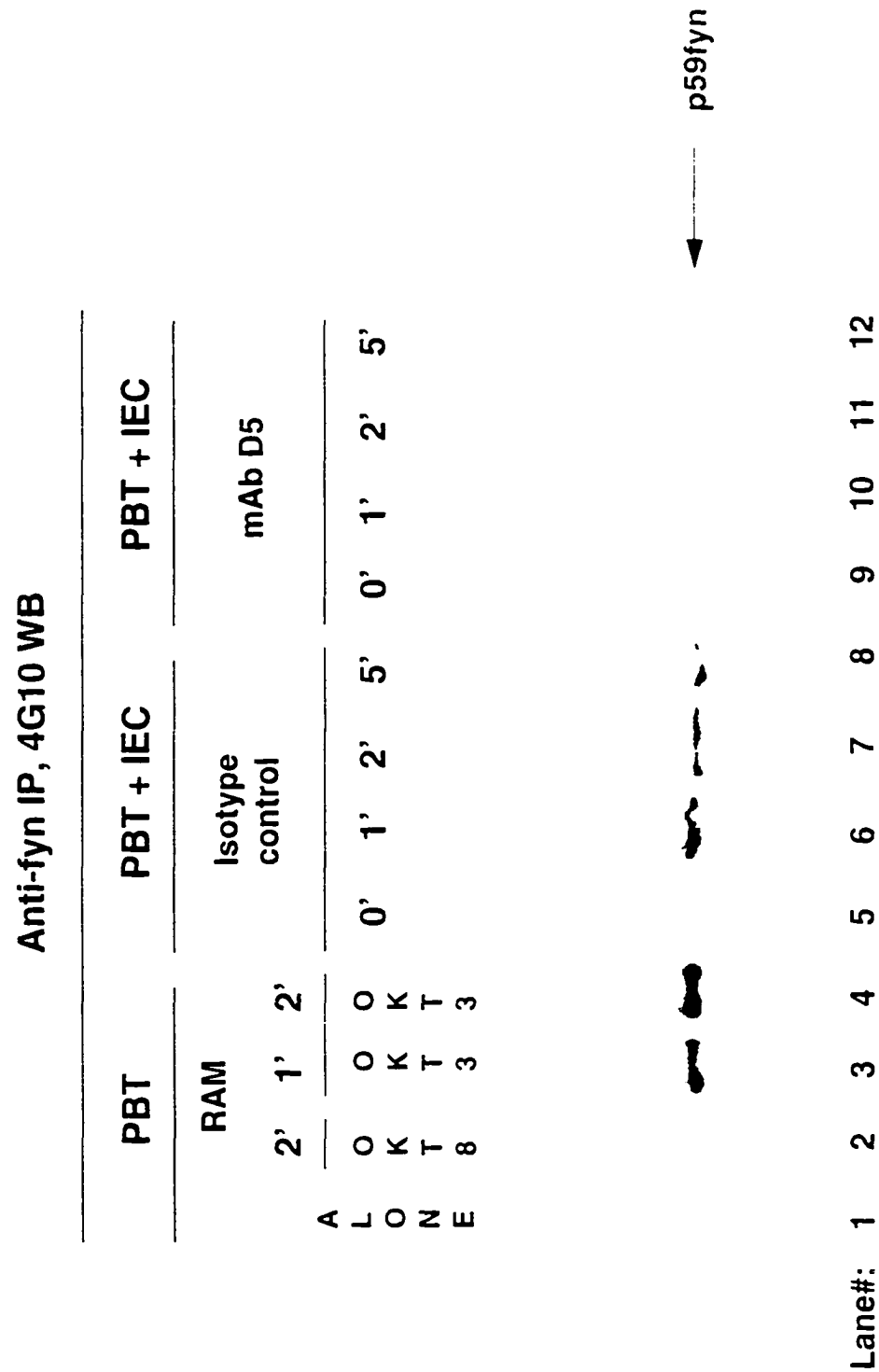
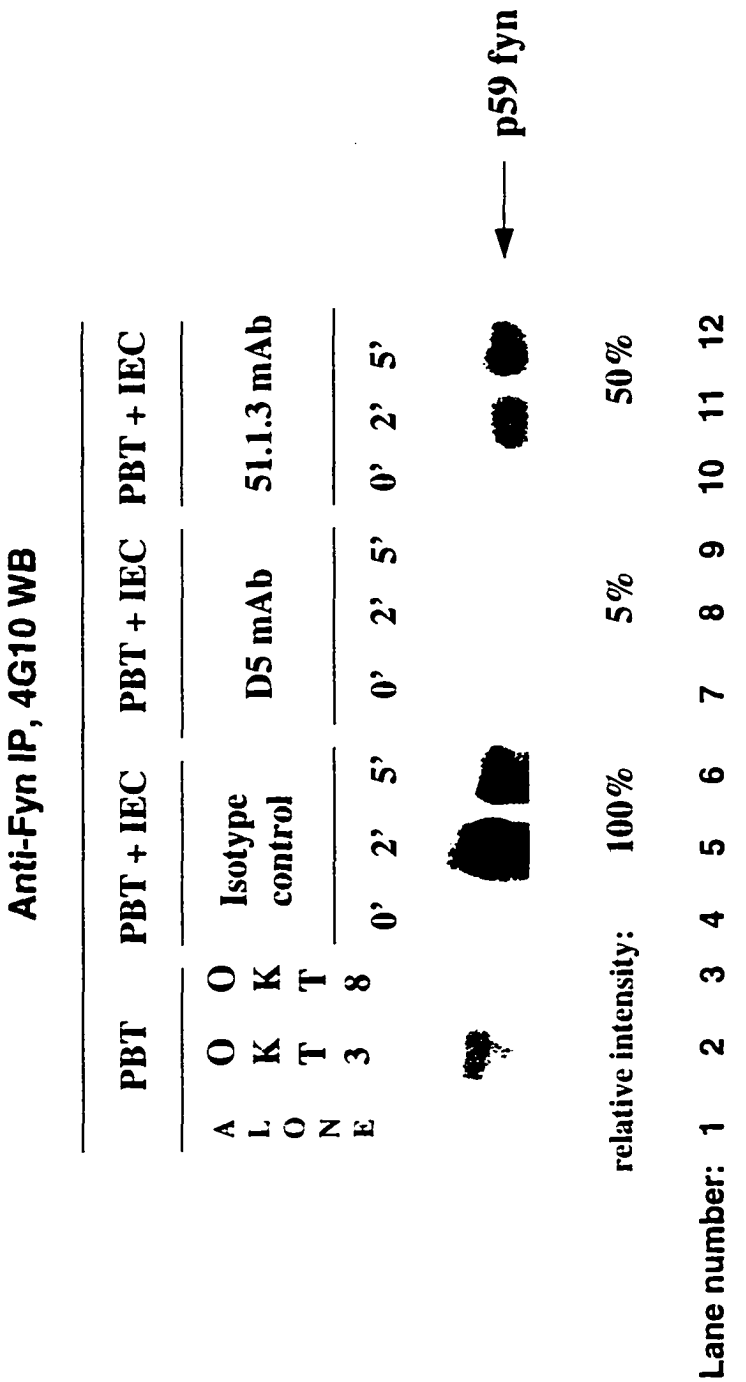


Figure 5



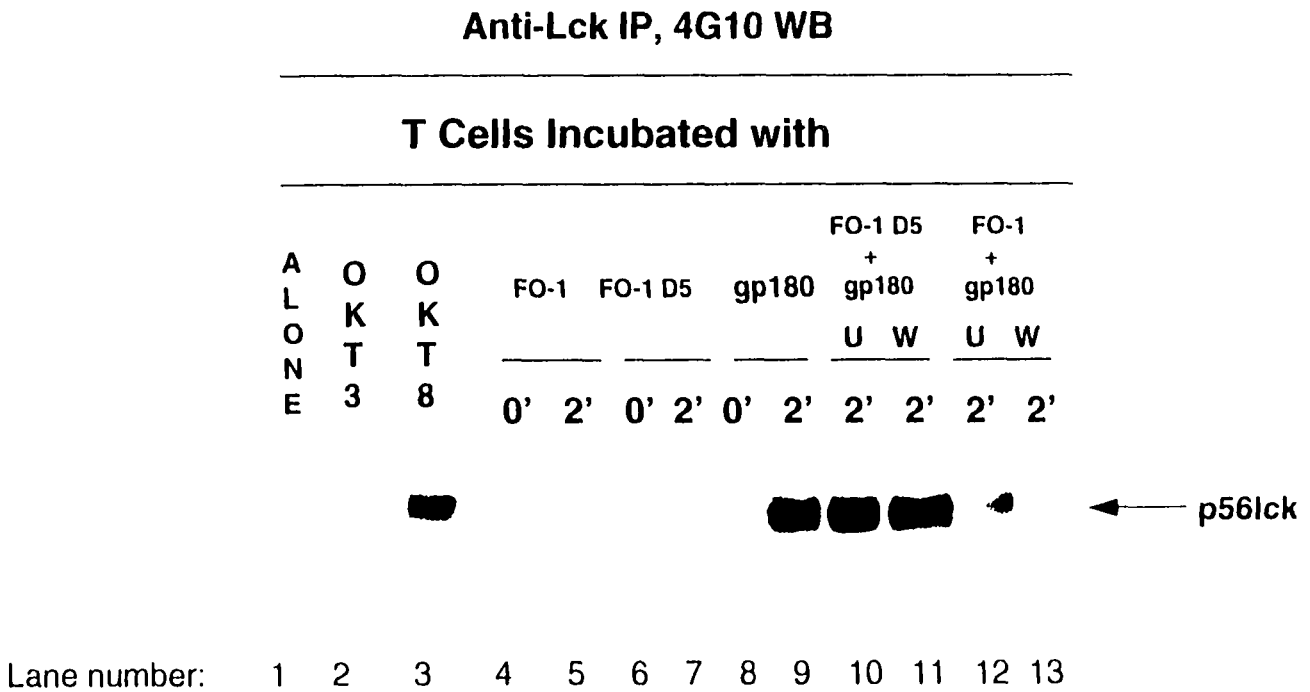


Figure 6

SECTION III

Distinct signaling cascades in IEC-stimulated versus cytotoxic T cells

ABSTRACT

Previous studies have shown that IECs are capable of selectively activating $CD8^+$ suppressor T cells. This activation is mediated by a novel complex which is recognized by the TCR:CD8 co-receptor complex on suppressor T cells and consists of the class Ib molecule CD1d and an associated epithelial cell surface glycoprotein, gp180. Since gp180 binds to any CD8 molecule, we wanted to determine whether IECs were targeting a selected subpopulation of cells or whether the CD1d:gp180 complex was transducing a specific signal that promoted cell activation from uncommitted $CD8^+$ T cells. A three-fold approach was taken: initially, the ability of IECs to activate $CD28^+$ versus $CD28^-$ $CD8^+$ T cells was determined; the signaling cascade in IEC-stimulated T cells or CTL was then compared; and the ability of gp180 to bind to the CD8 molecule and interfere with CTL effector function was investigated. IECs activate $p56^{lck}$ only in $CD8^+CD28^-$ T cells and not in $CD4^+$ or $CD8^+CD28^+$ T cells, although soluble gp180 is capable of activating $p56^{lck}$ in both $CD8^+$ T cell subsets. This suggested that a selected subpopulation was involved. Both IEC-stimulated and cytolytic $CD8^+$ T cells were activated via the MAP kinase cascade. However, the activation of this MAP kinase pathway in IEC activated T cells was

accomplished via the nucleotide exchange factor vav rather than the PLC-γ1 pathway observed in CTL. This suggests that these subsets of $CD8^+$ T cells signal through distinct pathways. In T cells activated by IECS, it was found that ZAP70 and vav were phosphorylated and recruited to ζ, $p59^{fyn}$ and $p56^{lck}$. Finally, while soluble gp180 bound to CD8 on CTLs, it was not able to inhibit CTL killing when compared to anti-class I MHC and anti-CD8 mAbs. These data suggest that the complex of CD1d and gp180 on IECs helps to target and signal a specific subpopulation of $CD8^+$ T cells which may be functionally distinct from classical class I restricted $CD8^+$ T cells.

INTRODUCTION

The fundamental concept of antigen recognition by T cells is that any one T cell is restricted to recognizing a peptide antigen only when it is complexed to a single allelic form of an MHC molecule. All individuals contain essentially the same complement of TCR genes in their genome. After distinct receptors are expressed on the surface of different T cells, the repertoire is modified by two related selection processes (1). Positive selection is the process by which the T cell repertoire becomes self MHC-restricted. Negative selection eliminates any potentially autoreactive clones (high affinitiy interactions with MHC) insuring that the mature T cell population is self tolerant. This phenomenon results in the selection of a mature repertoire of antigen-specific, self MHC-restricted T cells in the thymus from the larger set of possible specificities encoded in the germline (2). The extent to which non-classical restriction elements are able to influence the T cell repertoire is not clearly understood.

It has never been clearly determined if the non-classical class I molecule CD1d plays a role in this process, however, non-classical MHC molecules are expressed in the thymus. Previous studies have suggested that T cells restricted by class Ib molecules exist. In the gut, such cells appear to be more common. IEL killing of IEC lines can be CD1d restricted (3) and γ/δ T cells recognizing heat shock proteins may be restricted to CD1 or TL molecules as well.

CD1d is a nonpolymorphic isoform of the CD1 family which is expressed predominantly by IECs and is capable of binding peptides that are generally larger than those bound by classical restriction

elements (4). Other CD1 family members have been shown to bind highly hydrophobic lipids such as those derived from mycobacterium. The CD1 family of class I-like proteins is encoded outside the MHC and most members have limited homology in comparison to classical class I molecules. It is, therefore, suspected that these molecules may have a unique interaction with specialized populations of T cells.

We have recently demonstrated that CD1d associates with the novel CD8 ligand gp180, on the surface of IECs. We have shown that purified gp180 binds to CD8 molecules and activates the CD8α chain associated protein tyrosine kinase $p56^{lck}$ but not the TCR-associated protein tyrosine kinase $p59^{fyn}$ (5). When T cells are stimulated with intact IECs, both $p56^{lck}$ and $p59^{fyn}$ are phosphorylated. Interestingly, it was shown that CD1d is not responsible for the activation of the CD8-associated kinase $p56^{lck}$ but is responsible for the activation of the TCR-associated kinase $p59^{fyn}$ (6). This is consistent with previous studies which failed to show an association of CD1d with CD8 molecules.

Suspecting that IECs induce the activation of $CD8^+$ T cells in the gastrointestinal tract, we were interested in determining whether IECs were targeting a selected subpopulation of pre-committed cells that were CD1d restricted or whether the CD1d:gp180 complex was transducing a specific signal that promoted the generation of suppressor-like cells from uncommitted $CD8^+$ T cells.

The activation of the signaling cascade in T cells is a highly complex process which is not fully understood. Members of two

distinct classes of protein tyrosine kinases (PTKs), the Src family ($p56^{lck}$ and $p59^{fyn}$) and the Syk/ZAP70 family, have been implicated in TCR signal transduction (7). The activation of the CD8-associated PTK $p56^{lck}$ and the TCR-associated PTK $p59^{fyn}$ results in the recruitment of ZAP70, via its SH2 domain, to the membrane receptor complex (8). This triggers downstream events which include the activation of the phosphatidylinositol pathway, activation of Ras and the activation of several serine/threonine protein kinases (such as Raf-1) which trigger a kinase cascade involving mitogen-activated protein (MAP) kinase (9). The activation of Ras following TCR stimulation is the result of both protein kinase C (PKC) dependent and independent mechanisms.

Ras activity is regulated in a PKC-independent manner through its interactions with guanine nucleotide exchange proteins, such as vav (10). The exchange protein vav is tyrosine phosphorylated (11) and actually appears to have increased guanine nucleotide exchange activity with Ras following TCR stimulation (12). The PKC-dependent pathway also appears to be involved in the activation of $CD8^+$ T cells. Stimulation of the TCR/CD8 molecules in $CD8^+$ cytolytic T cells has been shown to activate the PLC-γ1 pathway (13). The mechanism by which PLC-γ1 is phosphorylated is not clear, however it has been shown that $p56^{lck}$ co-immunoprecipitates with PLC-γ1 through an interaction of the PLC-γ1 SH2 domain and $p56^{lck}$ (14).

In the present study, we investigated the role of IECs in the activation of $CD8^+$ T cells as well as the signaling pathway involved. The data suggest that IECs target the activation of a subpopulation

of $CD8^+$ T cells, which we have previously indicated are CD1d restricted.

MATERIALS AND METHODS

Monoclonal antibodies

Murine hybridomas, OKT3 and OKT8, were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and produce mAbs against CD3 and CD8 molecules, respectively. These hybridomas were cultured in RPMI 1640 with 10% fetal calf serum, 2 mM L-glutamine and 1% penicillin/streptomycin (Gibco BRL, Grand Island, NY) in a 37°C, 5% CO_2, humidified incubator.

B9 is a murine IgG1 anti-human IEC mAb which recognizes a human IEC membrane antigen, gp180. Ascites was generated at a concentration of 1 mg/ml and used at 10 μg/ml for blocking studies. An irrelevant murine IgG1 antibody was used as a negative control whenever necessary.

D5 is a murine IgG2b anti-human CD1d mAb generated against a glutathione-S-transferase CD1d fusion protein (15). The D5 antibody was used at a concentration of 5 μg/ml and an irrelevant murine IgG2b antibody was used as a negative control whenever necessary.

4G10 is a murine anti-human phosphotyrosine mAb obtained from Upstate Biotechnology Incorporated (Upstate Biotechnology Inc., Lake Placid, NY). This antibody was used for western blots at a concentration of 1 μg/ml. An HRP-conjugated goat anti-mouse Ig (Cappel, Durham, NC) antibody was used as the detection reagent in these western blots.

Cell Isolations

Peripheral blood mononuclear cells (PBMC) were isolated from leukocyte concentrate packs and separated into T and non-T cells using rosetting and density gradient centrifugation as previously described (16). Briefly, heparinized venous blood was collected from normal donors and separated by Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradient centrifugation. T cells and non-T cells were isolated from PBMCs by a rosetting method using neuraminidase-treated sheep red blood cells followed by Ficoll-Paque density gradient centrifugation.

Enterocytes were isolated by a method described previously (16, 17). Surgical specimens were obtained from the operating room. Specimens were washed extensively with PBS containing 1% penicillin/streptomycin and 1% fungizone (Flow Laboratory Inc., McLean, VA). The mucosa was stripped from the submucosa, cut into small pieces and placed in 1 mM dithiothreitol (Sigma) for 5 minutes at room temperature to remove the mucus. The pieces were then washed in PBS and incubated in dispase (3 mg/ml in RPMI 1640, Boehringer Mannheim, GmbH, Germany) for 30 minutes in a 37°C shaking incubator. This was repeated four times. The tissue pieces were removed and the cell suspension collected, pooled and centrifuged on a Percoll density gradient (Pharmacia, Piscataway, NJ). Enterocytes located at the 0-30% interface were washed three times with PBS and resuspended in RPMI with 0.1% BSA for co-culture experiments. Preparations of purified enterocytes were >90% viable, free of macrophages and B cell contamination as determined by staining with anti-CD14 and anti-CD20 mAbs (Coulter

Corp., Hialeah, FL) and contaminated with only 2-4% intraepithelial lymphocytes ($CD3^+$ cells).

Isolation of purified gp180.

Ten to twenty million isolated enterocytes or T84 cells harvested by non-enzyme cell dissociation solution (Sigma Chemical Co.), were washed three times with PBS and resuspended in 1 ml RPMI. 1 unit PIPLC (Sigma) was added into the cell suspension, mixed and incubated at 37°C for 45 min. At the end of this incubation, the cell suspension was centrifuged at 500g for 10 min and the supernatant was collected for further studies. The cell pellet was stained by various mAbs and analyzed by an Epics Profile III flow cytometer to confirm the removal of GPI-anchored molecules. To further purify gp180, the PIPLC treated supernatant was passed over an anti-gp180 (B9) affinity column. After elution, the resultant gp180 was resolved by SDS-PAGE, transferred to nitrocellulose paper and subjected to an mouse IgG1 anti-human gp180 western blot to confirm successful purification.

Co-culture and Immunoprecipitation Assays

T cells and IECs were resuspended to achieve a concentration of $1x10^7$ cells/ml in 0.1% BSA-RPMI 1640. Cells were prewarmed in a 37°C water bath. For each reaction, one million T cells were placed in a 1.5 ml eppendorf tube and microfuged at 13,000 rpm for 5 seconds. The supernatant was removed and the pellet loosened. One million IECs were mixed with the T cells, spun quickly for 20 seconds and placed in a 37°C water bath. After 0.5, 1, 2, 5 or 10

minutes, 1 ml of ice cold stop buffer (PBS with 10 mM sodium orthovanadate, Sigma, St. Louis, MO) was added. The pellet was then resuspended in 100 µl lysis buffer (20% PBS, 80% dH_2O, 100 mM Na_2VO_3, 1 mM PMSF, 5 mM iodoacetamide, 20 µg/ml leupeptin and 20 µg/ml aprotinin, Sigma) and racked 20x at the start and end of a 30 minute incubation on ice. As a positive control, one million T cells were incubated with 10 µg/ml anti-CD3 or anti-CD8 antibody (OKT3 and OKT8, respectively) for 30 minutes at 4°C and crosslinked with 10 µg/ml rabbit IgG anti-mouse Ig antibody for 2 minutes at 37°C. Time zero tubes were prepared by adding stop buffer to individual tubes containing T cells or IECs. The lysate from these two tubes were then combined into one tube to account for all constitutively active kinases and substrates in both cell types. 25 µl reducing buffer (50 mM Tris-HCl pH6.8, 5% 2-ME, 10% glycerol, 1% SDS) was added to all reactions prior to boiling for 5 minutes and the resulting samples were run on a 10% SDS-PAGE gel for western blot.

In some experiments, the lysate from these co-culture conditions were immunoprecipitated with a rabbit anti-human $p56^{lck}$ or rabbit anti-human $p59^{fyn}$ antibody bound to sepharose beads. These conjugated antibodies were rotated with lysates which had been pre-cleared with rabbit Ig coated sepharose 4B beads, for 1 hour at 4°C. The beads were washed four times with PBS and 75 µl reducing buffer (50 mM Tris-HCl pH 6.8, 5% 2-ME, 10% glycerol, 1% SDS) was added prior to boiling for 5 minutes. The resulting samples were then resolved by 10% SDS-PAGE and transferred to nitrocellulose paper (Schleicher & Schuell Inc, Keene, NH). A western blot was then performed.

Western blots

A 10% SDS-PAGE gel was prepared and one million cells were loaded per lane. The protein was transferred from the gel to nitrocellulose paper and blocked with 5% milk for 1 hour at RT. The membrane was then incubated in 1 μg/ml of primary antibody (anti-phosphotyrosine 4G10, anti-ZAP70, anti-vav or anti-PLC-γ1, Upstate Biotechnology, Lake Placid, NY) overnight at 4°C. The membrane was washed with PBS several times and incubated in 1 μg/ml of HRP-conjugated goat anti-mouse Ig (Cappel, Durham, NC) antibody for 1 hour at RT. The membrane was developed using an enzyme linked chemiluminescence (Dupont NEN, Boston, MA) reagent.

Cytotoxicity assay in the presence of blocking antibodies and substrates

To make effector cytolytic T cells (CTLs), fresh T cells were obtained using the procedure previously described in this thesis. Cultures were performed in 25 cm^2 flasks, with responders and stimulators at a 1:1 ratio at a concentration of $1x10^6$/ml. These cells were cultured for 6 days in RPMI with 1% penicillin, 1% L-glutamine and 10% FCS. On the same day, $5x10^6$ target cells were set up at a concentration of $1x10^6$ cells/ml and stimulated with 10 ml/ml PHA. On day 3, these same target cells were spun down, counted and resuspended at a concentration of $2x10^5$ cells/ml in RPMI constituted with 10% FCS and 5% biotest (Biotest Diagnostics Corp., Dreieich, Germany). On day 6, the targets are removed from the flasks and pooled in 50 ml centrifuge tubes. The cells are spun at 1500 rpm for 5 minutes and the supernatant is removed. The

cells were resuspended in 150 μCi ^{51}Cr and incubated at 37°C for 90 minutes. These cells were then washed extremely carefully to prevent chromium release. For some conditions, the target cells were incubated in varying concentrations of the anti-class I mAb for 30 minutes at RT. At the same time, the effector cells were washed and set up in 96-well round bottom plates at a concentration of 10^5 cell/100 μl. In some wells, the effector cells were incubated with varying concentrations of anti-CD8 mAb or gp180 for 45 minutes at RT. The cells were washed before target cells were added to the plate. In all cases, the target cells were diluted to achieve a 100:1 E:T ratio in 100 μl RPMI. The total volume in each well was always 200 μl. In some wells, the targets were incubated only in RPMI to determine the spontaneous release of chromium while others were incubated in 1% NP-40 to determine the maximal release of chromium. The target:effector reactions were incubated for 4 hours at 37°C at which time 100 μl was transferred to another plate and dried overnight. The counts were then measured using a Beckman Gamma 5500B counter.

RESULTS

Intestinal epithelial cells trigger the activation of CD8-associated $p56^{lck}$ in a distinct subset of $CD8^+$ T cells.

Previous studies have demonstrated that IECs can act as antigen presenting cells (APC) capable of stimulating primed T cells (17-20). Interestingly, despite the constitutive expression of class II MHC molecules on these cells, the T cells proliferating in these co-cultures are $CD8^+$(16, 18). The subset of $CD8^+$ T cells that proliferate when co-cultured with IECs are $CD8^+CD28^-$ and inhibit primary, secondary and unrelated mixed lymphocyte reactions, as well as B cell responses *in vitro* in an antigen non-specific manner (21). These data were generated by assaying the proliferating T cell population by flow cytometry. In order to confirm that this was the subpopulation of T cells responding to IECs, and more specifically gp180, we separated PBTs into the various subsets and cultured these cells with either intact IECs or purified gp180.

Five groups of T cells were generated: unseparated PBTs, $CD4^+$ T cells (negative selection), $CD8^+$ T cells (negative selection), $CD8^+CD28^+$ T cells and $CD8^+CD28^-$ T cells (negative selection). To generate $CD4^+$ and $CD8^+$ T cells, T cells were negatively selected with anti-CD4, leaving a $CD8^+$ population, or with anti-CD8, leaving a $CD4^+$ population. This was accomplished using DynaBeads (DYNAL, Lake Success, NY) conjugated with either anti-CD4 or CD8 mAb. All cell populations were analyzed for purity by flow cytometry prior to experimental use. The percentage of purity for each population was as follows: PBT (61% $CD4^+$, 39% $CD8^+$), $CD4^+$ (92%), $CD8^+$ (95%), $CD8^+CD28^+$ (95%), $CD8^+CD28^-$ (97%).

The cells were then co-cultured with freshly isolated IECs or purified gp180 for 0, 2, or 5 minutes. The T cell lysates were immunoprecipitated with an anti-$p56^{lck}$ mAb, run on a 10% SDS-PAGE gel, transferred to nitrocellulose and subjected to an anti-phosphotyrosine western blot. As seen in Figure 1a, $p56^{lck}$ phosphorylation was induced by co-culture with intact IEC in unseparated PBTs, unseparated $CD8^+$ T cells and unseparated $CD8^+CD28^-$ T cells. The intensity of the band was greatest in the $CD8^+CD28^-$ population. In contrast, there was no lck phosphorylation with the $CD8^+CD28^+$ population. All populations were capable of exhibiting $p56^{lck}$ phosphorylation after crosslinking with an anti-CD8 mAb. Given the possibility that the positively selected $CD28^+$ T cell population may have been affected by CD28 crosslinking (in the selection process), we co-cultured these same populations with purified gp180. In contrast to the results seen in Figure 1a, all populations demonstrated $p56^{lck}$ activation following co-culture with gp180 (Figure 1b). Thus, the selection process did not alter the ability of the $CD28^+$ T cells to become activated through the CD8 molecule. The interaction of IEC with $CD8^+CD28^+$ T cells may induce a negative (phosphatase or inhibitory) signal.

The MAP kinase cascade is activated in IEC-stimulated $CD8^+$ suppressor T cells.

Our laboratory has previously shown that IECs induce the selective proliferation (22) and activation (5) of $CD8^+$ suppressor T cells. We were interested in determining the downstream signal cascade involved in this activation process in comparison to other

$CD8^+$ T cells (cytolytic T cells). To begin our investigation into the signaling pathways triggered in IEC-stimulated T cells, we initially analyzed the MAPK cascade since this pathway is activated in $CD8^+$ T cells. Freshly isolated IECs were cultured with T cells for 0, 0.5, 1, 2, 5 or 10 minutes and the reactions stopped with 1 ml ice cold stop buffer. The T cells were lysed and immunoprecipitated with a rabbit anti-human MAP kinase antibody. The proteins were then resolved by SDS-PAGE, transferred to nitrocellulose and subjected to an anti-phosphotyrosine western blot. As seen in Figure 2a, it appears that MAPK phosphorylation is induced in T cells stimulated with IECs. The same experiment was performed with CTL:target cell co-cultures at 0, 1, 2, 5, and 10 minute time points. Although the kinetics of the response were different, the presence of phosphorylated MAPK protein in CTL was observed (Figure 2b). Since multiple intracellular signaling pathways can result in MAPK phosphorylation, the differences in the kinetics of the response between the two co-cultures might represent differences in upstream signaling.

The pathway involving PLC-γ1 activation does not appear to be critical in IEC-induced activation $CD8^+$ T cells.

Studies in other laboratories have indicated that the stimulation of the TCR/CD8 molecules in $CD8^+$ cytolytic T cells induces the activation of the MAP kinase cascade through the activation of PLC-γ1 (13). We were interested in determining if PLC-γ1 was playing a similar role in T cells activated by IEC..

Lysates from T cell:IEC co-cultures were immunoprecipitated with anti-human PLC-γ1 mAb. The resulting proteins were resolved by SDS-PAGE, transferred to nitrocellulose and subjected to an anti-phosphotyrosine (4G10) western blot. Interestingly, Figure 3 demonstrates that phosphorylation of PLC-γ1 was not observed in T cells stimulated by IECs. As a control, the same experiment was performed with CTL:target cells co-cultures at 0, 1, 2, and 5 minute time points. The presence of phosphorylated PLC-γ1 protein in CTL observed in Figure 3 confirms that CTL do utilize this pathway as previously reported by others.

PLC-γ1 is not associated with either $p56^{lck}$ or $p59^{fyn}$ in IEC-stimulated T cells.

The mechanism by which PLC-γ1 is phosphorylated is not clear, however it has been shown that $p56^{lck}$ co-immunoprecipitates with PLC-γ1 through an interaction of the PLC-γ1 SH2 domain and $p56^{lck}$ (14). To further examine whether or not the PLC-γ1 pathway is involved in the activation of suppressor T cells by IECs, the association of PLC-γ1 with both $p56^{lck}$ and $p59^{fyn}$ was studied.

Once again, freshly isolated IECs were co-cultured with T cells for varying times (0', 2' or 5'). The reactions were stopped with ice cold stop buffer and lysed for 30 minutes on ice. The lysate was then equally divided and rotated with either anti-$p56^{lck}$ or $p59^{fyn}$ mAb coated beads overnight at 4°C. The beads were washed several times, added to reducing buffer and boiled for 5 minutes prior to being resolved by SDS-PAGE gel. The protein was then transferred to nitrocellulose and an anti-PLC-γ1 western blot was

performed. Figure 4a clearly demonstrates that PLC-γ1 protein is not associated with either $p56^{lck}$ or $p59^{fyn}$ in T cells stimulated with IECs. However, PLC-γ1 in T cells stimulated in vitro with either anti-CD3 (OKT3) or anti-CD8 (OKT8) associates with $p59^{fyn}$ and $p56^{lck}$, respectively. This supports the findings in Figure 3 that, unlike CTLs (Figure 4b), IEC-stimulated T cells do not signal via the PLC-γ1 pathway.

ZAP70 and p97vav associate with the ζ chain of the TCR/CD3 complex.

Since the PLC-γ1 pathway does not appear to be critical in the IEC-induced activation of $CD8^+$ suppressor T cells, it was necessary to study alternate pathways that may be triggered in suppressor T cell signaling. Previous studies indicated that a CD1d:gp180 complex is expressed on freshly isolated IECs and that this complex is responsible for the activation of TCR-associated $p59^{fyn}$ and CD8-associated $p56^{lck}$, respectively (6). In conventional T cell signaling, ZAP70 contains an SH2 domain and becomes tightly associated with the cytoplasmic ITAM of the ζ chain only after TCR stimulation. ZAP kinase activity is dependent on this association and is thought to be responsible for the phosphorylation of PLC-γ1 and the consequent activation of the phosphinositide second messenger pathway (23-25). Along similar lines, PTK-mediated tyrosine phosphorylation of TCR subunits can also result in the recruitment of Grb2 and other adaptor proteins which, in turn, serve to link the activated receptor to guanine nucleotide exchange factors such as vav. This leads to the activation of Ras and downstream mitogen-activated protein

(MAP) kinase (26, 27). Since it appears that the PLC-dependent pathway is not activated in IEC-stimulated T cells, we wanted to determine whether or not ZAP-70 and vav were recruited to become associated with the ζ chain, $p59^{fyn}$ and/or $p56^{lck}$.

After varying incubation periods with IECs (0', 1', 2' or 5'), T cells were lysed. Pre-cleared lysate was then immunoprecipitated with a mouse anti-human ζ chain mAb. T cell lysate was equally divided, resolved by SDS-PAGE gel, transferred to nitrocellulose and subjected to either a mouse anti-human ZAP70 or a mouse anti-human vav western blot. Figure 5 (a and b, respectively) demonstrates that both ZAP70 and vav associate with the human ζ chain in IEC-stimulated T cells.

ZAP70 and p97vav associate with $p59^{fyn}$.

A similar experiment to that depicted in Figure 5 was conducted using an anti-fyn Ab to co-precipitate ZAP70 and vav. IECs were cultured with PBTs for varying time periods (0', 1', 2' or 5'), at which point, the T cells were lysed and immunoprecipitated with a rabbit anti-human $p59^{fyn}$ antibody. The lysate was resolved by SDS-PAGE gel, transferred to nitrocellulose and subjected to either a mouse anti-human ZAP70 or a mouse anti-human vav western blot. Figure 6 (a and b, respectively) demonstrates that both ZAP70 and vav associate with $p59^{fyn}$ in IEC stimulated T cells. Interestingly, vav exists in a 95 kd and 97 kd forms, and it appears that both forms are associated with $p59^{fyn}$ in IEC stimulated T cells. This is not true for ζ chain associated vav.

ZAP70 and p97vav associate with $p56^{lck}$.

From the data generated to this point, it appears that the class Ib molecule CD1d plays an important role in the activation of $CD8^+$ T cells by IECs. However, previous data clearly demonstrated that CD1d associates with the glycoprotein gp180, on the surface of IECs. More specifically, it was shown that CD1d is responsible for the activation of the TCR-associated kinase $p59^{fyn}$ while gp180 is responsible for the activation of the CD8-associated kinase $p56^{lck}$ (6) and that this complex may allow for maximal signaling. We, therefore, wanted to determine the role that gp180 played in the downstream signaling events by assessing the ability of ZAP70 and vav to be recruited to the CD8-associated kinase $p56^{lck}$ in IEC-stimulated T cells.

A similar experiment to that depicted in Figure 6 was conducted. IECs were co-cultured with T cells for varying incubation periods (0', 1', 2' or 5'), and the T cells were then lysed. The lysate was then immunoprecipitated with a rabbit anti-human $p56^{lck}$ antibody. The protein was resolved by SDS-PAGE gel and subjected to either a mouse anti-human ZAP70 or a mouse anti-human vav western blot. Figure 7a and 7b, respectively, demonstrate that both ZAP70 and vav associate with $p56^{lck}$ in IEC-stimulated T cells. Since ZAP70 and vav are recruited to both TCR and CD8 associated kinases and phosphorylated (data not shown) in IEC stimulated T cells, it appears that both CD1d and gp180 have a co-dependent role in the activation of $CD8^+$ T cells by IECs.

gp180 is unable to inhibit CTL killing

Thus far, it appears that IEC-stimulated T cells signal through a distinct pathway from that of classical class I restricted cytolytic T cells. The functional outcome of the activated CD8+ T cells (IEC-stimulated versus cytotoxic T cells) may either relate to our targeting a specific pre-committed subpopulation (ie. CD1d restricted T cells), the different maturation of cells from a common precursor pool, or differences in signaling pathways utilized in the generation of these cells. To address this question, a chromium release cytotoxicity assay was set up and the ability of soluble gp180 to inhibit killing was investigated.

Cytotoxic T lymphocytes were generated against specific target cells. For some conditions, the target cells were incubated with anti-class I mAb(W6/32) in two-fold dilutions from 25 μg/ml to 3.13 μg/ml for 30 minutes at RT. Effector cells were washed and set up in 96-well round bottom plates at a concentration of 10^5 cells/100 μl. In some wells, the effector cells were incubated with 25 μg/ml to 3.13 μg/ml (in two-fold dilutions) of anti-CD8 mAb (OKT8) or $1.5x10^6$ to $1.2x10^7$ cell equivalents (in two-fold dilutions) of gp180, or a glycoprotein control CEA, for 45 minutes at RT. The cells were washed before target cells were added to the plate. A chromium release cytotoxicity assay was then performed. The counts were then measured using a Beckman Gamma 5500B counter.

The results generated from this experiment are summarized in Figure 8. By blocking the CD8 molecule or class I MHC with specific antibodies, the ability of the CTL to kill the target cell was inhibited in a dose dependent manner. When compared to the

antibodies, gp180 was much less effective at inhibiting CTL activity. Although gp180 was able to slightly reduce the CTL activity by about 25%, this could not be increased by using higher concentrations of gp180. In addition, co-culture with the glycoprotein control CEA, which is a member of the family to which gp180 appears to belong and does not to bind CD8, resulted in some non-specific inhibition. Taking this into consideration, it appears that gp180 only affects the ability of CTL to kill by about 15%. The effect observed may be due to binding of gp180 to CD8 on CTL which may sterically hinder the interaction of class I MHC with the CD8:TCR complex. Alternatively, gp180 binds to some epitopes on the CD8 molecule which overlap with class I MHC binding, therefore, gp180 may bind to the CD8 molecule and partially occupy a class I MHC binding site. In conjunction with the data generated in Section I of this thesis where the gp180 and MHC class I binding sites of CD8 were investigated, the latter explanation may be more accurate.

DISCUSSION

The concept of suppressor cells regulating immune responses has been quite controversial. A number of reports reporting isolated cell populations, with unique restriction elements and phenotypic characteristics, failed to be confirmed in many laboratories. The absence of TCR rearrangement in these cells rendered them suspect as true regulatory T cells. Furthermore, it became increasingly apparent that there were multiple mechanisms that could be invoked to explain suppressor effector functions including suppressive cytokines, cytolytic activity, absorption of growth factors and negative signaling in cell:cell interactions. Both $CD4^+$ and $CD8^+$ T cells, as well as monocytes and macrophages, could be suppressor effector cells through one of these mechanisms. However, the difficulty remained in isolating such cells and maintaining them in long term culture or in generating cloned populations to allow for more careful study.

Over the past several years it has become clear that the mucosal immune system has many features which are distinct from those of the systemic immune system. As alluded to earlier, the nature of the antigenic load may account for this difference. Given the enormity of the exposure to antigen on a daily basis, the focus in the intestinal tract is to dampen immune responses rather than promote them. The phenomena of mucosal immunity such as oral tolerance and chronic controlled or physiologic inflammation are clear examples of this type of response. Thus suppression may be the normal response in the gut and the induction of suppressor inducer mechanisms might be best studied in this environment. Even

in the gut multiple pathways and cell types involved in suppressor cell generation may exist. Our laboratory has focused on one such model using the intestinal epithelial cell as an antigen presenting cell. In the initial studies, using an antigen specific system (tetanus toxoid), we were able to document that intestinal epithelial cells could process and present soluble protein to primed T cells. However, despite the express of MHC class II by these cells, the T cell proliferating in these cultures were $CD8^+$ and $CD28^-$. Furthermore, the functional characteristics of these cells were unique in that they exhibited potent antigen nonspecific suppressor activity. No cytolytic activity could be detected. The nature of the suppression has not been elucidated but it is clear that it is mediated by these $CD8^+$ T cells. Studies attempting to define the mechanism are currently in progress.

IECs express not only conventional restriction elements but also have been shown to express class Ib molecules as well. The role of these class Ib molecules in regulating T cell activation has only been recently addressed. Interestingly, CD1b and CD1c have been shown to present nonpeptide antigens to either $CD8^+$ or $CD4^-$ $CD8^-$ T cells. Thus novel restriction elements and novel antigens have been reported. In the gut, CD1d restricted cytolytic responses have been reported and the ability of anti-CD1d Abs to inhibit T cell proliferation induced by IECs has been noted.

In this paper we describe differences in the T cells as well as the signaling pathways when IEC are used as the APC. Since the $CD8^+$ T cells which proliferate in these co-cultures appear to be CD1d restricted, we may be targeting a subpopulation of $CD8^+$ T cells

in the peripheral blood that are pre-committed to a suppressor lineage. Such cells would not have easily been isolated using the conventional APCs systems used in the 1980s to define suppressor T cells. Furthermore, our inability to generate long term lines from these IEC activated cells may reflect a limited lifespan with the cells mediating their effector function and undergoing apoptotic cell death. They may be selected from a pre-committed pool of suppressor cells which differentiate and die. If they persisted they might continue to suppress in a global fashion resulting in an immunodeficient state.

It is not clear what subpopulation our system is targeting. Clearly there is activation of CD8-associated p56lck by IEC in $CD8^+CD28^-$ cells and not $CD8^+CD28^+$ cells. However there may still be a subpopulation of cells within this population. Clearly not all $CD8^+CD28^-$ cells will be CD1d restricted or suppressor T cells. Several groups have defined classical CTLs within this phenotypic subgroup. The nature of the Ag driving this response as well as further phenotypic characterization will help define the truly responsive subpopulation that exists in this group.

We have not completely ruled out the possibility that the $CD8^+CD28^-$ responding cells come from a $CD8^+CD28^+$ precursor pool or that our functional suppressor cells derive as a maturational outcome of another cell lineage. Since we do document that there is a difference in the signaling pathway used by classical CTLs and IEC activated T cells, it is still possible that there is a single pool of responsive T cells that mature into a distinct functional phenotype depending upon the conglomerate of cell surface interactions and

consequent intracellular signals. We have not even started to define the other potential cell:cell interactions involved which might more carefully define the type of effector cell that is generated. However, using this model system we can begin to characterize these cells more explicitly and hopefully come to a better understanding of the regulation and activation of suppressor T cells, at least in the mucosal associated lymphoid tissue.

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FIGURE LEGENDS

FIGURE 1: Intestinal epithelial cells selectively activate CD8+CD28- T cells which appear to be suppressive in nature.

PBTs were separated into $CD4^+$ (negative selection with anti-CD8 mAb), $CD8^+$ (negative selection with anti-CD4 mAb), $CD8^+CD28^+$ (negative selection with anti-CD4 mAb and positive selection with anti-CD28 mAb) or $CD8^+CD28^-$ (negative selection with anti-CD4 and anti-CD28 mAbs) subsets using DynaBeads (Dynal Inc.) and then co-cultured with freshly isolated IECs (Figure 1a) or purified gp180 (Figure 1b). The T cell lysates were immunoprecipitated with an anti-$p56^{lck}$ Ab, resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose and subjected to an anti-phosphotyrosine western blot. Lane 1: PBT cells alone. Lane 2: PBT incubated with anti-CD8 mAb for 45 minutes and crosslinked with RAM for 2 minutes. Lanes 3 to 5: PBTs co-cultured with freshly isolated IECs (a) or gp180 (b) for 0, 2 or 5 minutes. Lane 6: $CD8^+$ T cells alone. Lane 7: $CD8^+$ T cells incubated with anti-CD8 mAb for 45 minutes and crosslinked with RAM for 2 minutes. Lanes 8 to 10: $CD8^+$ T cells co-cultured with freshly isolated IECs (a) or gp180 (b) for 0, 2 or 5 minutes. Lane 11: $CD8^+CD28^+$ T cells alone. Lane 12: $CD8^+CD28^+$ T cells incubated with anti-CD8 mAb for 45 minutes and crosslinked with RAM for 2 minutes. Lanes 13 to 15: $CD8^+CD28^+$ T cells co-cultured with freshly isolated IECs (a) or gp180 (b) for 0, 2 or 5 minutes. Lane 16: $CD8^+CD28^-$ T cells alone. Lane 17: $CD8^+CD28^-$ T cells incubated with anti-CD8 mAb for 45 minutes and crosslinked with RAM for 2 minutes. Lanes 18 to 20: $CD8^+CD28^-$ T cells co-cultured with freshly isolated IECs (a) or gp180 (b) for 0, 2 or 5 minutes. Co-

culture of $CD4^+$ T cells with IECs did not result in the phosphorylation of $p56^{lck}$ (data not shown). This figure is representative of two separate experiments.

FIGURE 2: MAPK is activated in both suppressor and cytolytic T cells.

Figure 2a: Freshly isolated IECs cultured with T cells. Figure 2b: CTL cultured with target cells. T cells were lysed and immunoprecipitated with a rabbit anti-human MAP kinase antibody and subjected to an anti-phosphotyrosine western blot. Lane 1: negative control of T cells alone. Lane 2: positive control of T cells stimulated with anti-CD3 (OKT3) mAb and crosslinked with RAM for 2 minutes. Lane 3: positive control of T cells stimulated with anti-CD8 (OKT8) mAb and crosslinked with RAM for 2 minutes. Lanes 4 to 8: IECs (a) or CTL (b) co-cultured with T cells for 0, 0.5, 1, 2, 5 or 10 minutes. Compared to the positive controls, it appears that the MAPK cascade is activated in both suppressor (Figure 2a) and cytolytic (Figure 2b) $CD8^+$ T cells. Note that there is no 0.5 minute time point in Figure 2b. These experiments were repeated three times. The inset represents an anti-MAPK western blot to confirm equal loading of the lanes.

FIGURE 3: PLC-γ1 protein is phosphorylated in CTL:target co-cultures but not T cell:IEC co-cultures.

Freshly isolated IECs were cultured with T cells and CTL were cultured with target cells. The reaction times in both cases were 0, 1, 2, or 5 minutes. Descriptions of the lanes are the same in both blots. The cells were lysed, immunoprecipitated with an anti-PLC-γ1 mAb and an anti-phosphotyrosine (4G10-HRP) western blot was performed. Lane 1: negative control of T cells alone. Lane 2: positive control of T cells stimulated with anti-CD8 (OKT8) mAb and crosslinked with RAM for 2 minutes. Lanes 3 to 6: IECs co-cultured with T cells for 0, 1, 2, or 5 minutes. Lane 7: negative control of CTL alone. Lane 8: positive control of CTL stimulated with anti-CD8 (OKT8) mAb and crosslinked with RAM for 2 minutes. Lanes 9 to 12: target cells co-cultured with CTL for 0, 1, 2, or 5 minutes. Compared to CTL co-cultured with target cells, it does not appear that PLC-γ1 protein is phosphorylated in IEC-stimulated T cells. This experiment was repeated twice. The inset represents a PLC-γ1 western blot to document equivalent loading of the lanes.

FIGURE 4a: PLC-γ1 does not associate with either CD8-associated p56lck or TCR-associated p59fyn in suppressor T cells.

IECs were cultured with PBTs for varying time periods. The cells were lysed, immunoprecipitated with an anti-$p56^{lck}$ or anti-$p59^{fyn}$ Ab and an anti-PLC-γ1 western blot was performed. Lanes 1 to 6 represent the anti-$p59^{fyn}$ immunoprecipitates while lanes 7 to 12 represent the anti-$p56^{lck}$ immunoprecipitates. Lane 1: negative control of T cells alone. Lane 2: control of T cells stimulated with anti-CD3 (OKT3) mAb and crosslinked with RAM for 2 minutes. Lane 3: control of T cells stimulated with anti-CD8 (OKT8) mAb and crosslinked with RAM for 2 minutes. Lanes 4 to 6: IECs co-cultured with T cells for 0, 2 or 5 minutes. Lane 7: negative control of T cells alone. Lane 8: control of T cells stimulated with anti-CD8 (OKT8) mAb and crosslinked with RAM for 2 minutes. Lane 9: control of T cells stimulated with anti-CD3 (OKT3) mAb and crosslinked with RAM for 2 minutes. Lanes 10 to 12: IECs co-cultured with T cells for 0, 2 or 5 minutes. It appears that PLC-γ1 protein is only associated with $p59^{fyn}$ and $p56^{lck}$ where T cells were stimulated with either anti-CD3 and anti-CD8 antibodies, while no association was observed in IEC-stimulated T cells. This experiment was repeated three times. The insets represent anti-fyn and anti-lck western blots to document equivalent loading of the lanes.

FIGURE 4b: PLC-γ1 associates with $p56^{lck}$ and $p59^{fyn}$ in cytolytic T cells.

Target cells were cultured with CTL for varying time periods. The cells were lysed, immunoprecipitated with an anti-$p56^{lck}$ or anti-$p59^{fyn}$ Ab and an anti-PLC-γ1 western blot was performed. Lanes 1 to 5 represent the anti-$p59^{fyn}$ immunoprecipitates while lanes 6 to 10 represent the anti-$p56^{lck}$ immunoprecipitates. Lane 1: negative control of CTL alone. Lane 2: CTL stimulated with anti-CD3 (OKT3) mAb and crosslinked with RAM for 2 minutes. Lanes 3 to 5: target cells cultured with CTL for 0, 2 or 5 minutes. Lane 6: negative control of CTL alone. Lane 7: CTL stimulated with anti-CD8 (OKT8) mAb and crosslinked with RAM for 2 minutes. Lanes 8 to 10: target cells cultured with CTL cells for 0, 2 or 5 minutes. It appears that PLC-γ1 protein is associated with $p59^{fyn}$ and $p56^{lck}$ in $CD8^+$ cytotoxic T cells. The inset represents anti-fyn and anti-lck western blots of the lysates to document equivalent loading.

FIGURE 5: ZAP70 and p97vav associate with the ζ chain of the TCR/CD3 complex.

IECs were co-cultured with T cells for 0', 1', 2' or 5'. The T cells were lysed, immunoprecipitated with a mouse anti-human ζ chain mAb, and subjected to a either a mouse anti-ZAP70 mAb (Figure 5a) or a mouse anti-vav mAb (Figure 5b) western blot. Lane 1: negative control of T cells alone. Lane 2: positive control of T cells stimulated with anti-CD3 (OKT3) mAb and crosslinked with RAM for 2 minutes. Lanes 3 to 6: IECs were co-cultured with T cells for 0, 1, 2 or 5 minutes. Both ZAP70 and vav appear to associate with ζ

chain in IEC-stimulated T cells. This experiment was repeated three times. The inset represents anti-zeta western blot of the IP documenting equivalent loading.

FIGURE 6: ZAP70 and vav associate with p59fyn.

Culture conditions are similar to those described in Figure 5. IECs were co-cultured with T cells for 0', 1', 2' or 5'. The T cells were lysed, immunoprecipitated with a rabbit anti-human $p59^{fyn}$ antibody, and subjected to a either a mouse anti-ZAP70 mAb (Figure 6a) or a mouse anti-vav mAb (Figure 6b) western blot. Lane 1: negative control of T cells alone. Lane 2: positive control of T cells stimulated with anti-CD3 (OKT3) mAb and crosslinked with RAM for 2 minutes. Lanes 3 to 6: IECs were co-cultured with T cells for 0, 1, 2 or 5 minutes. Both ZAP70 and vav appear to associate with $p59^{fyn}$ in IEC-stimulated T cells. This experiment was repeated three times. The inset represents an anti-fyn western blot of the lysates to document equivalent loading.

FIGURE 7: ZAP70 associates with p56lck.

Similar to Figure 6, but here the lysates were immunoprecipitated with a mouse anti-human $p56^{lck}$ mAb, and subjected to a either a mouse anti-ZAP70 mAb (Figure 7a) or a mouse anti-vav mAb (Figure 7b) western blot. Lane 1: negative control of T cells alone. Lane 2: positive control of T cells stimulated with anti-CD8 (OKT8) mAb and crosslinked with RAM for 2 minutes. Lanes 3 to 6: IECs co-cultured with T cells for 0, 1, 2 or 5 minutes. Both ZAP70 and vav appear to associate with $p56^{lck}$ in IEC-stimulated T cells. This experiment

was repeated three times. The inset represents an anti-lck western blot of the lysates to document equivalent loading.

FIGURE 8: gp180 does not interfere with the ability of CTL to kill. This graph represents a chromium release cytotoxicity assay which was conducted to determine the effect of gp180 on the ability of CTL to kill their target. Black diamond (♦): a dose response curve (3.13 μg/ml to 25 μg/ml) of isotype control IgG1 added to target:CTL cultures. Light grey triangle (▲): a dose response curve ($1.5x10^6$ to $1.2x10^7$ cell equivalents) of CEA added to target:CTL cultures as a glycoprotein control. Dark grey square (■): a dose response curve ($1.5x10^6$ to $1.2x10^7$ cell equivalents) of gp180 added to target:CTL cultures. Light grey cross (×): a dose response curve (3.13 μg/ml to 25 μg/ml) of mouse anti-human class I MHC mAb added to target:CTL cultures. Dark star(*): a dose response curve (3.13 μg/ml to 25 μg/ml) of mouse anti-human CD8 mAb added to target:CTL cultures. The results are depicted as the percentage of the positive control (isotype control). This experiments was repeated twice.

Figure 1a

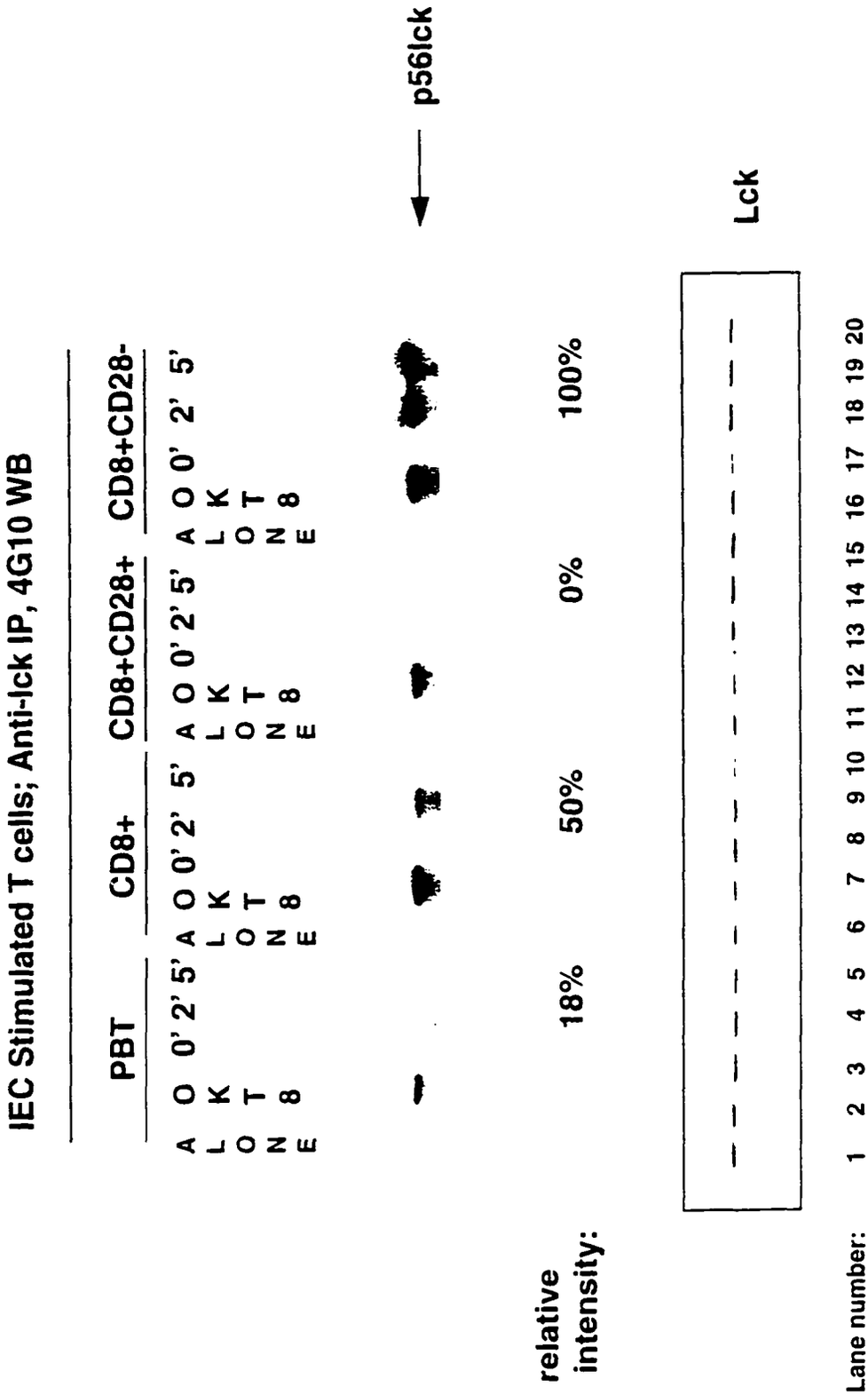


Figure 1b

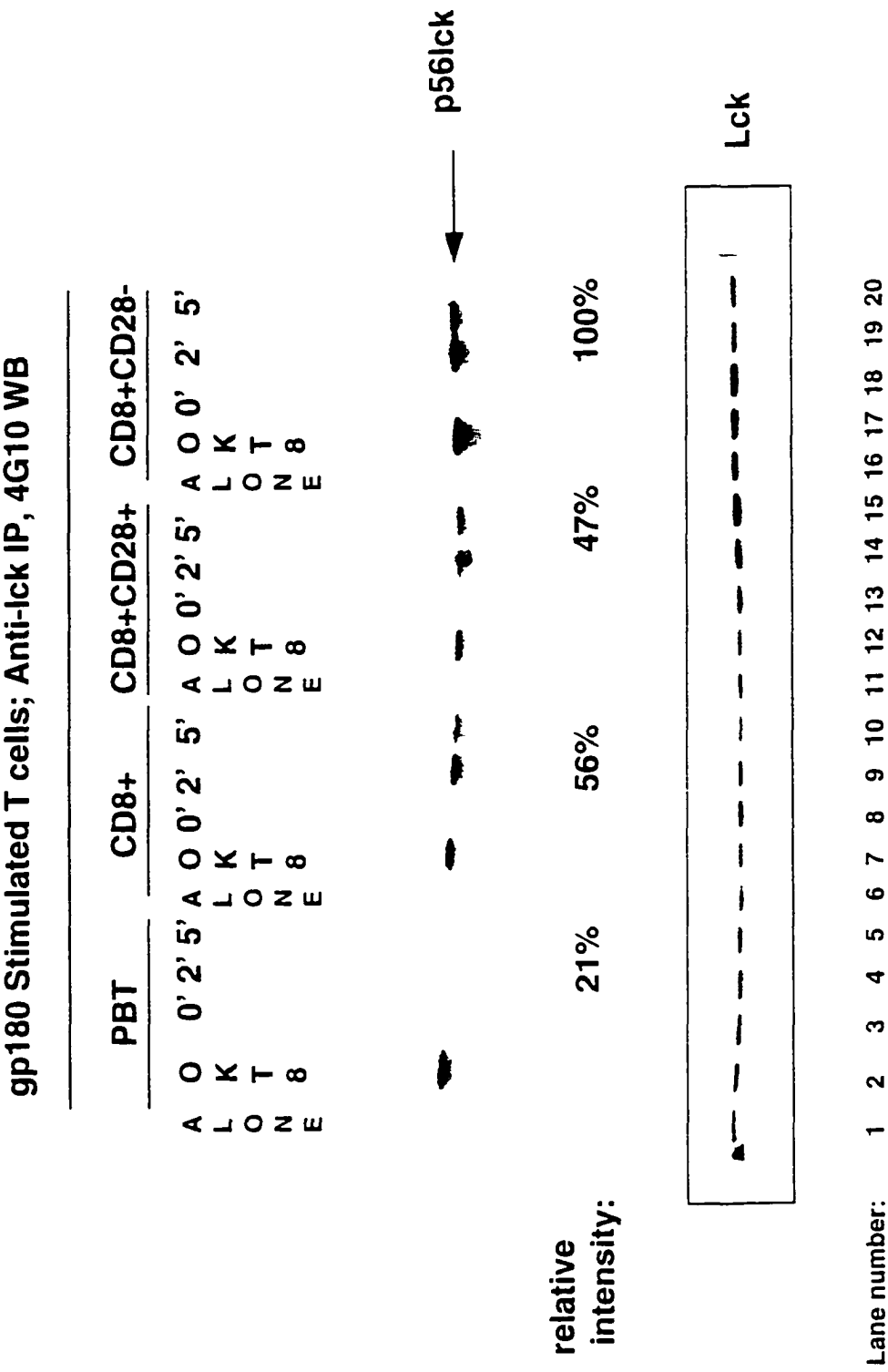
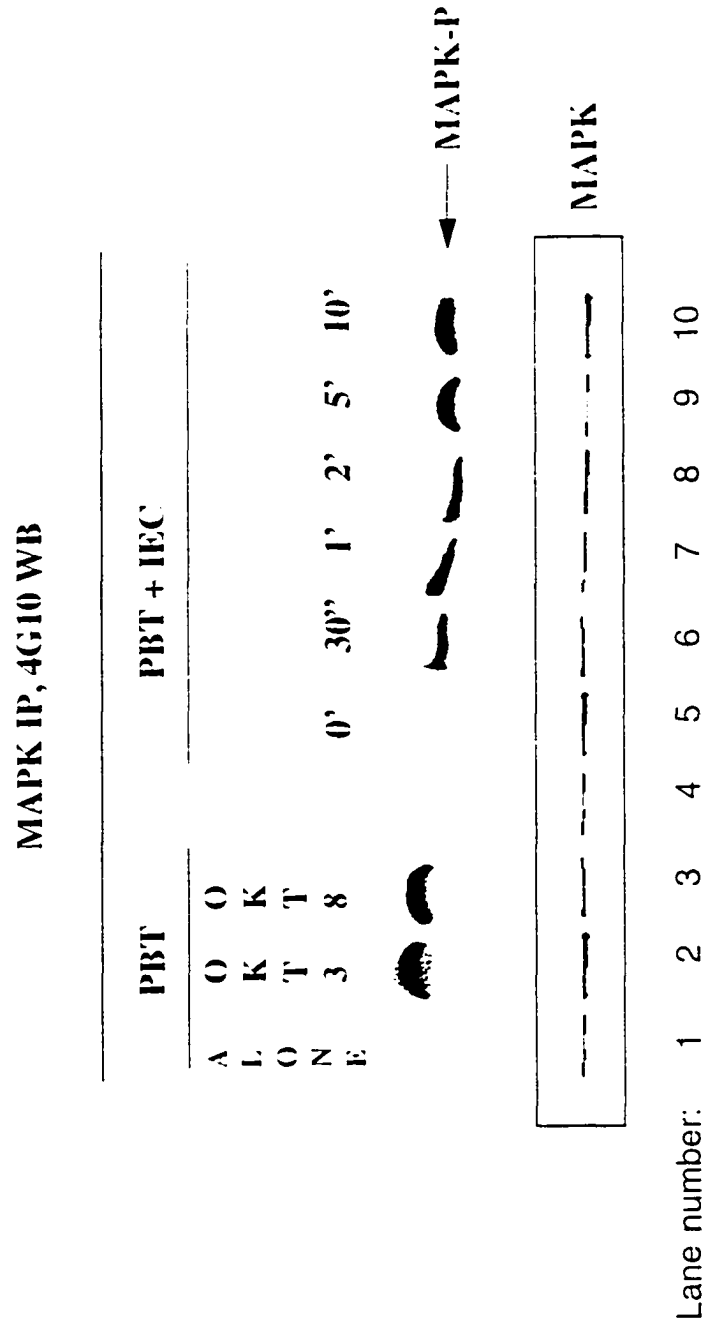


Figure 2a



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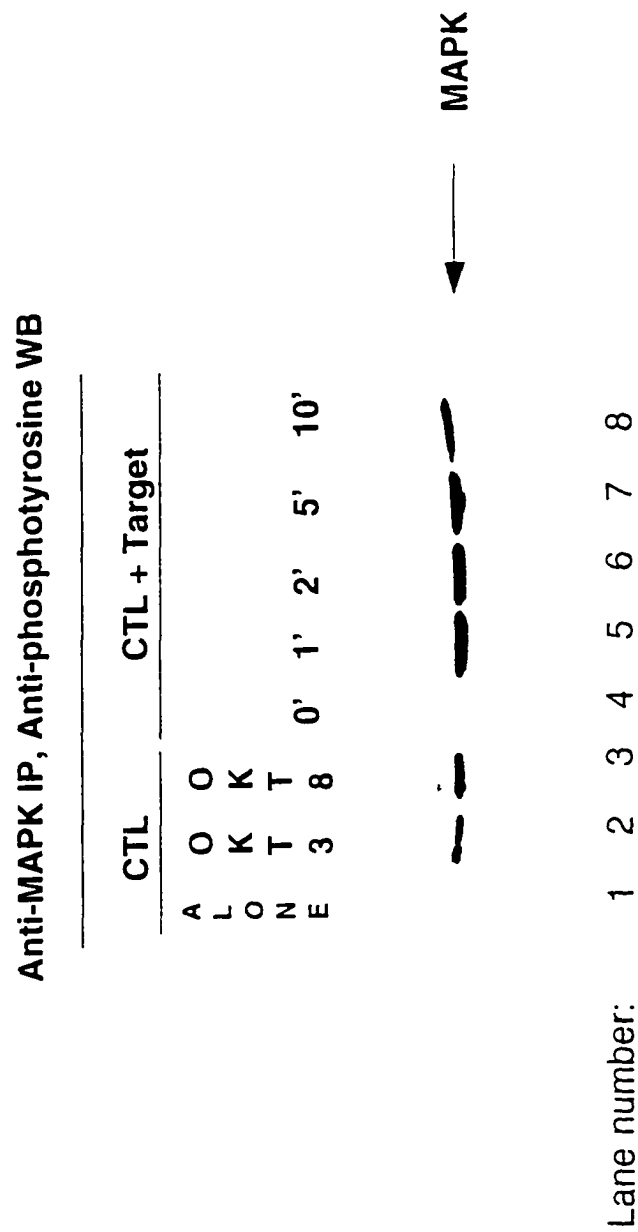
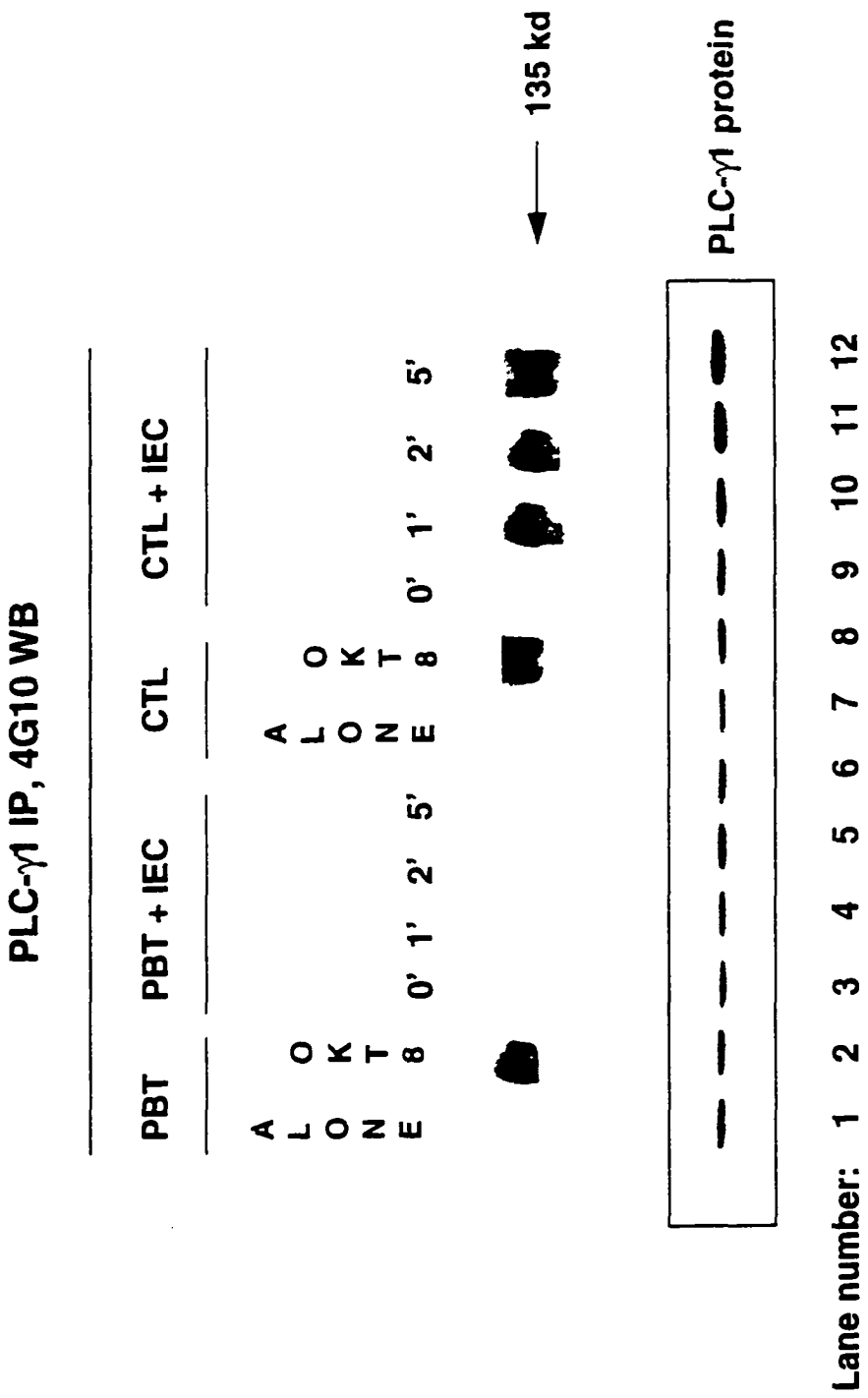


Figure 3



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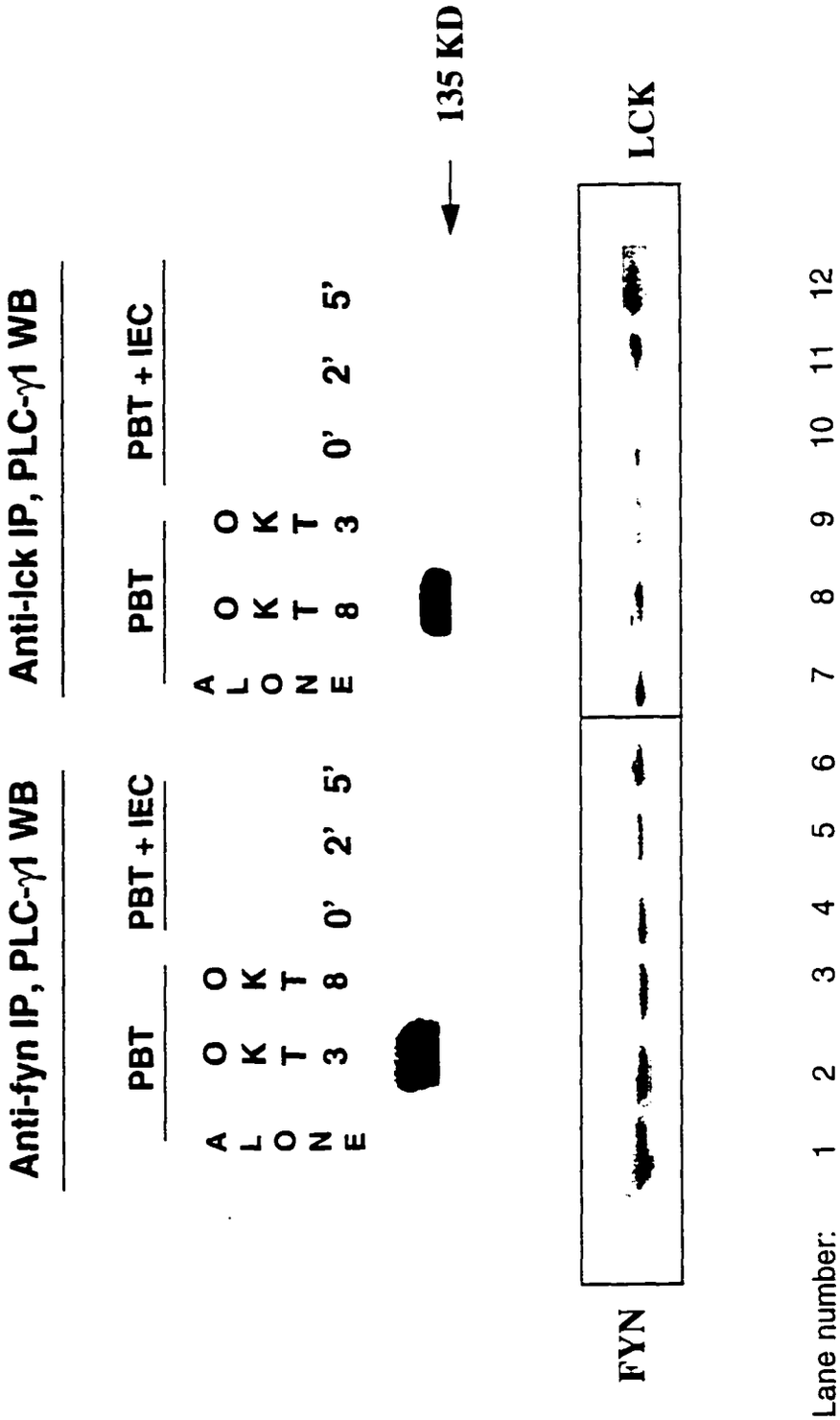

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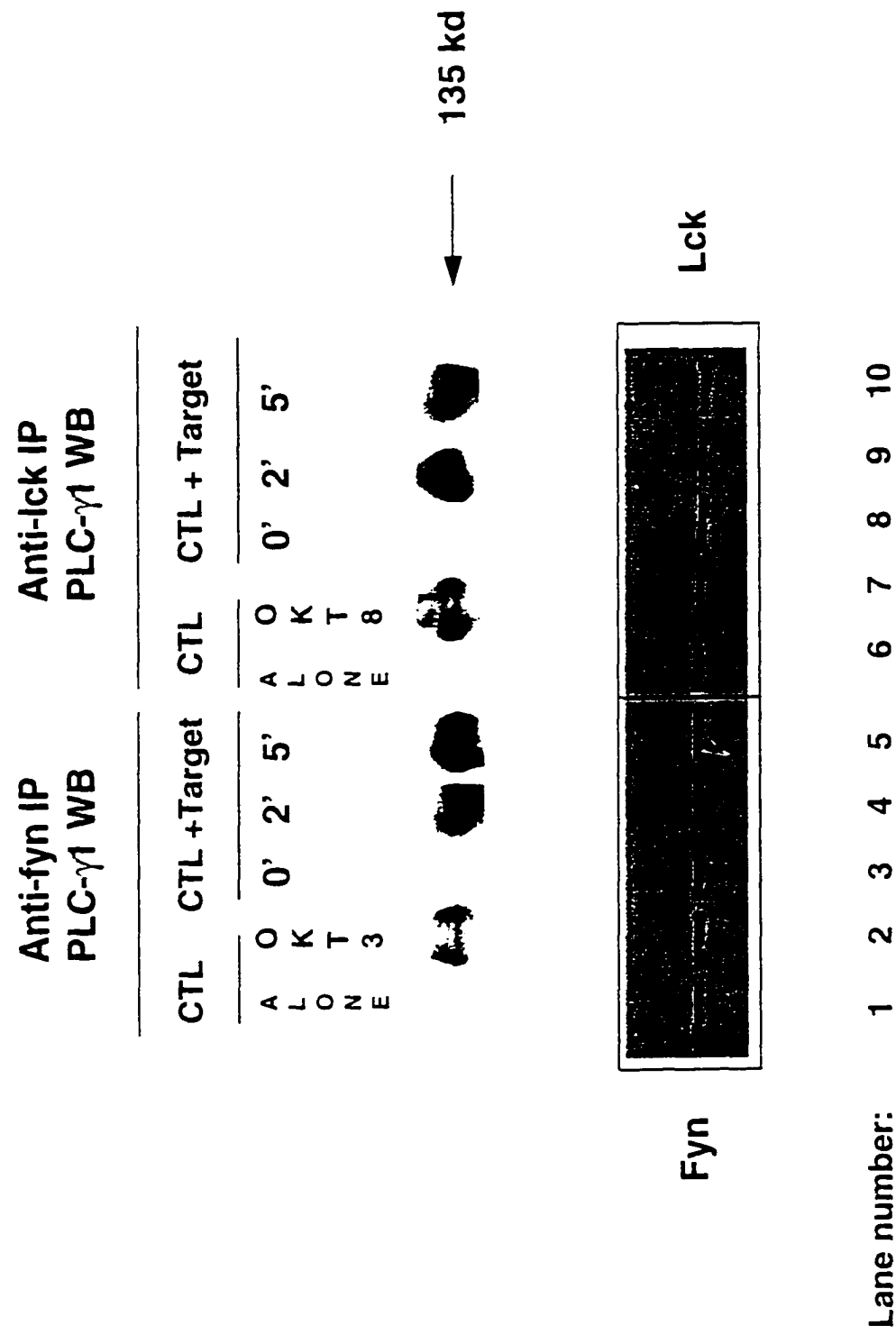

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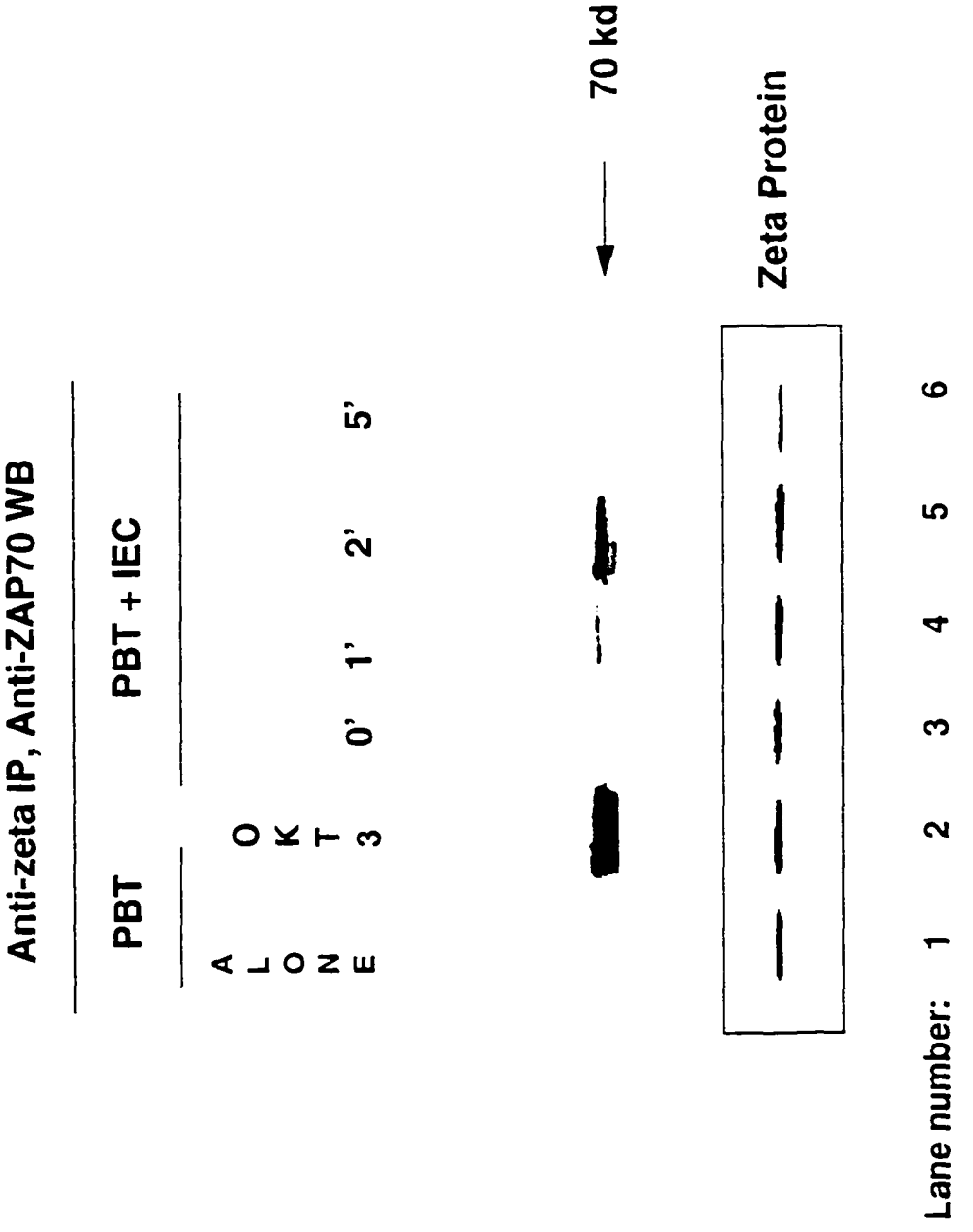

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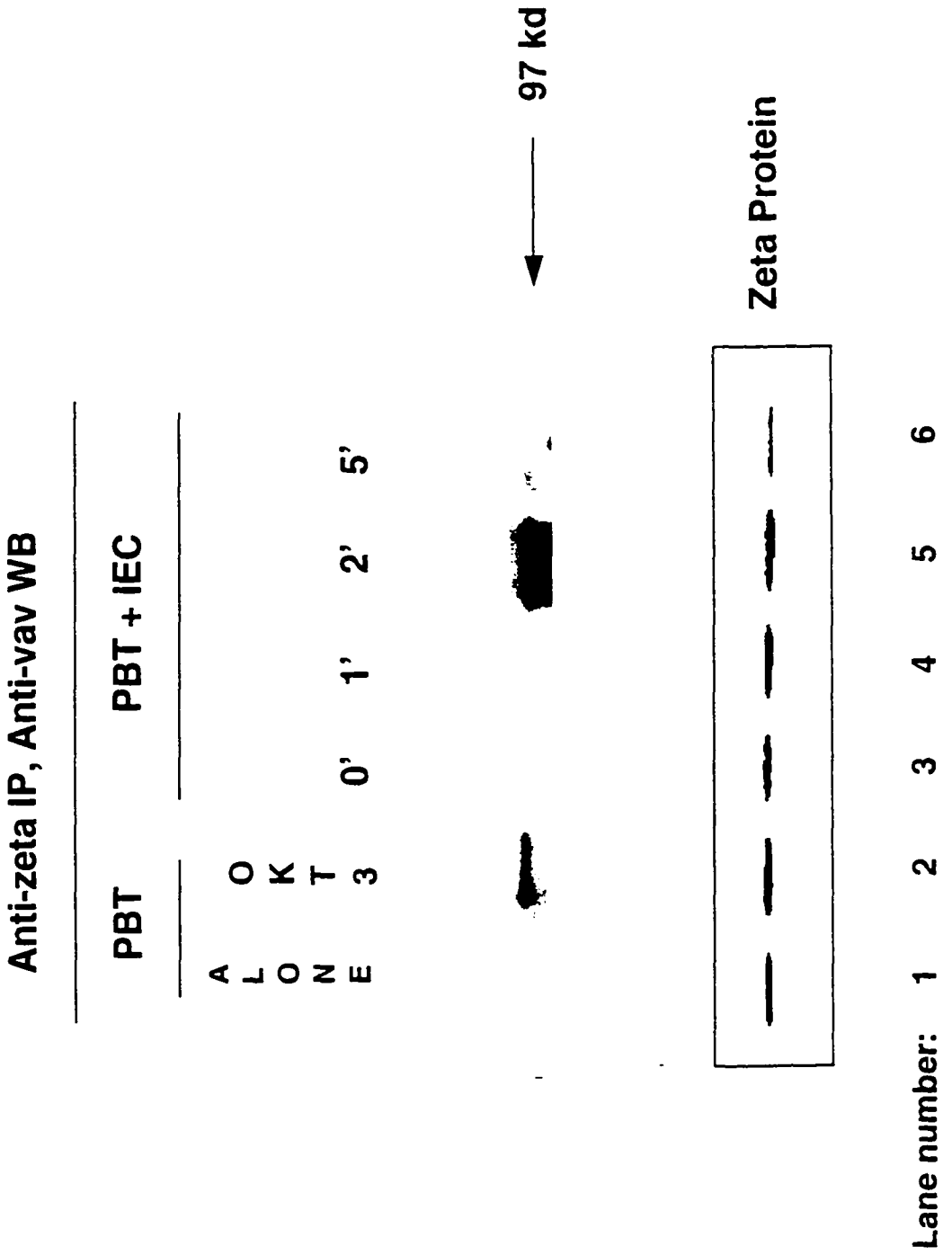


Figure 6a

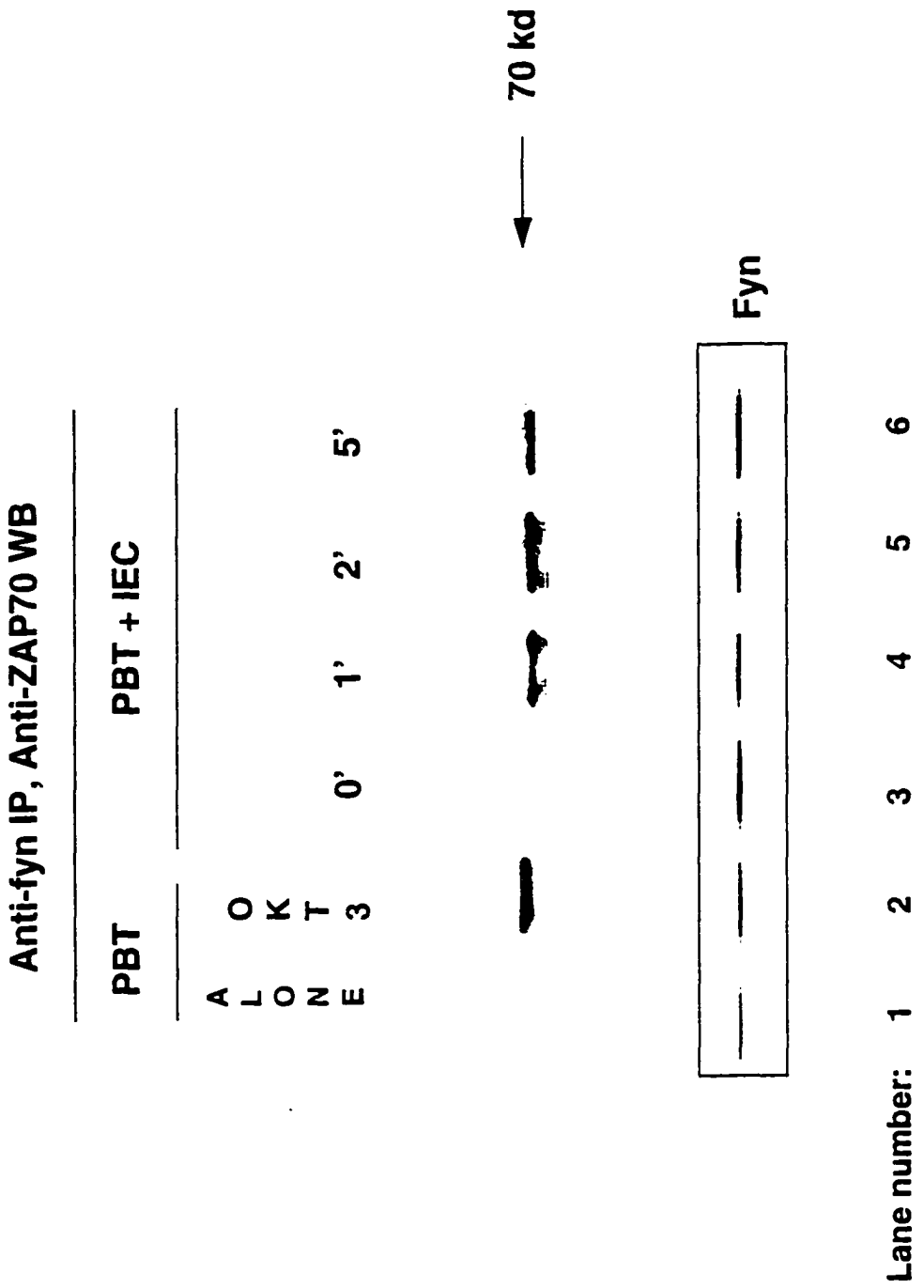


Figure 6b

Figure 7a

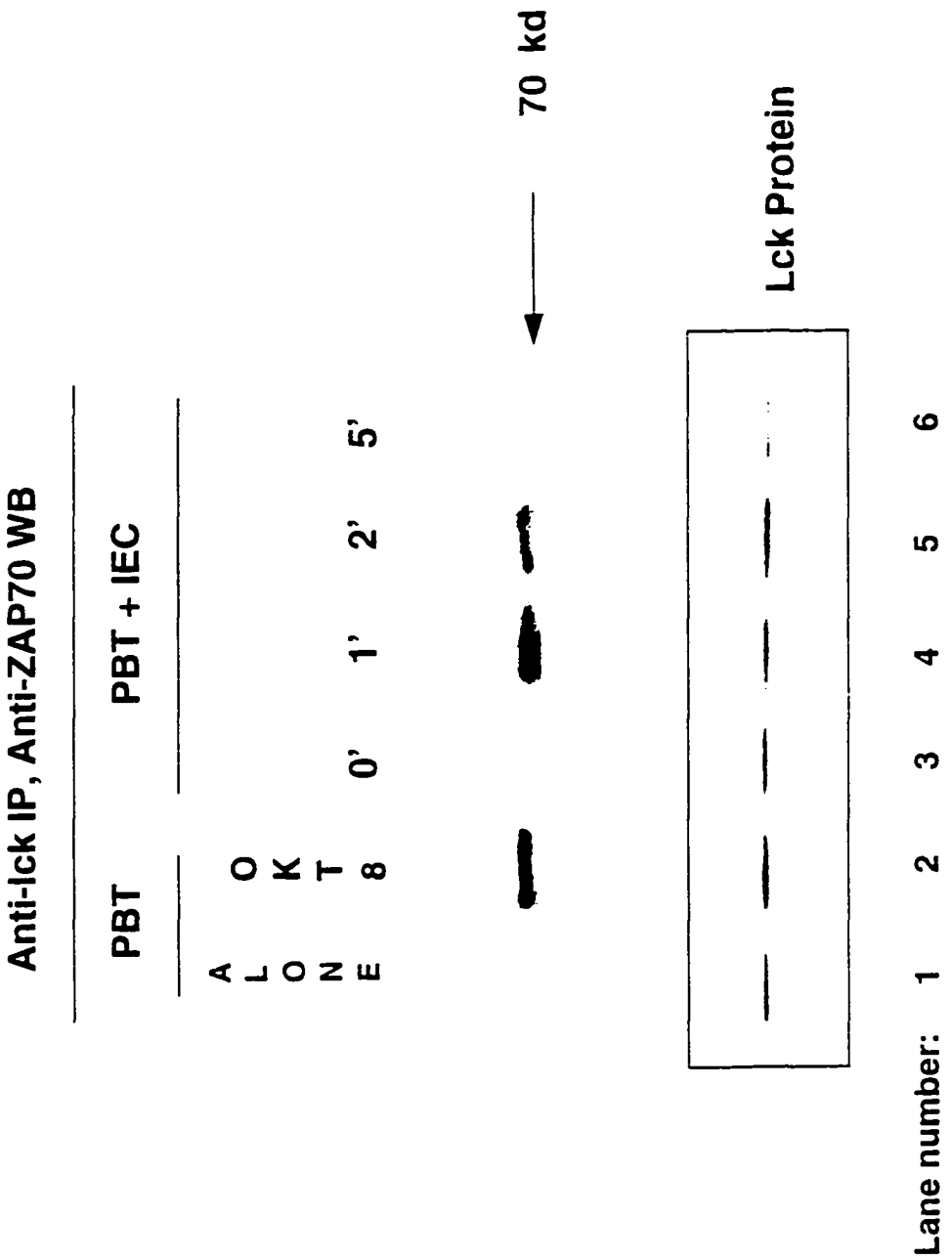


Figure 7b

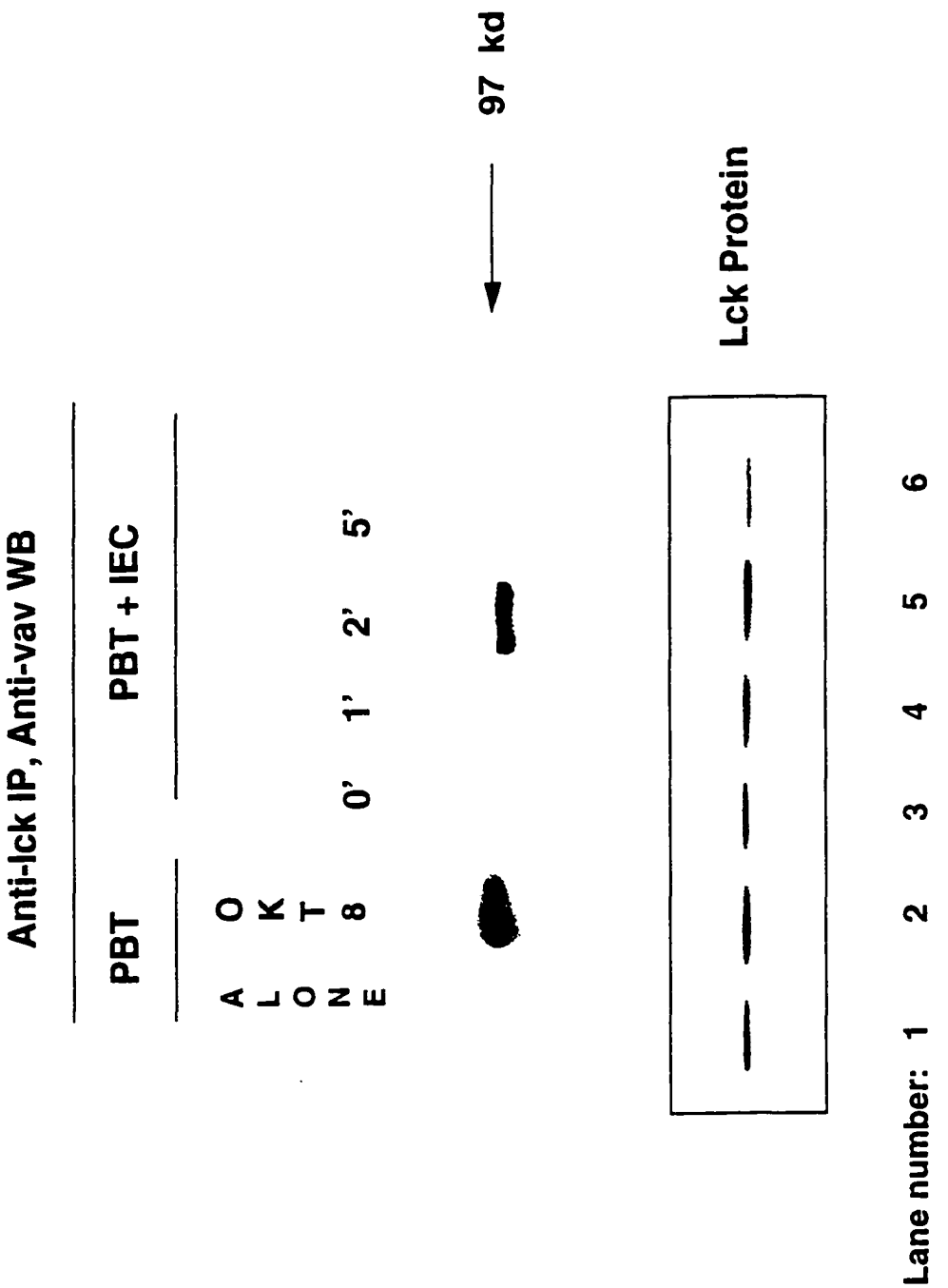
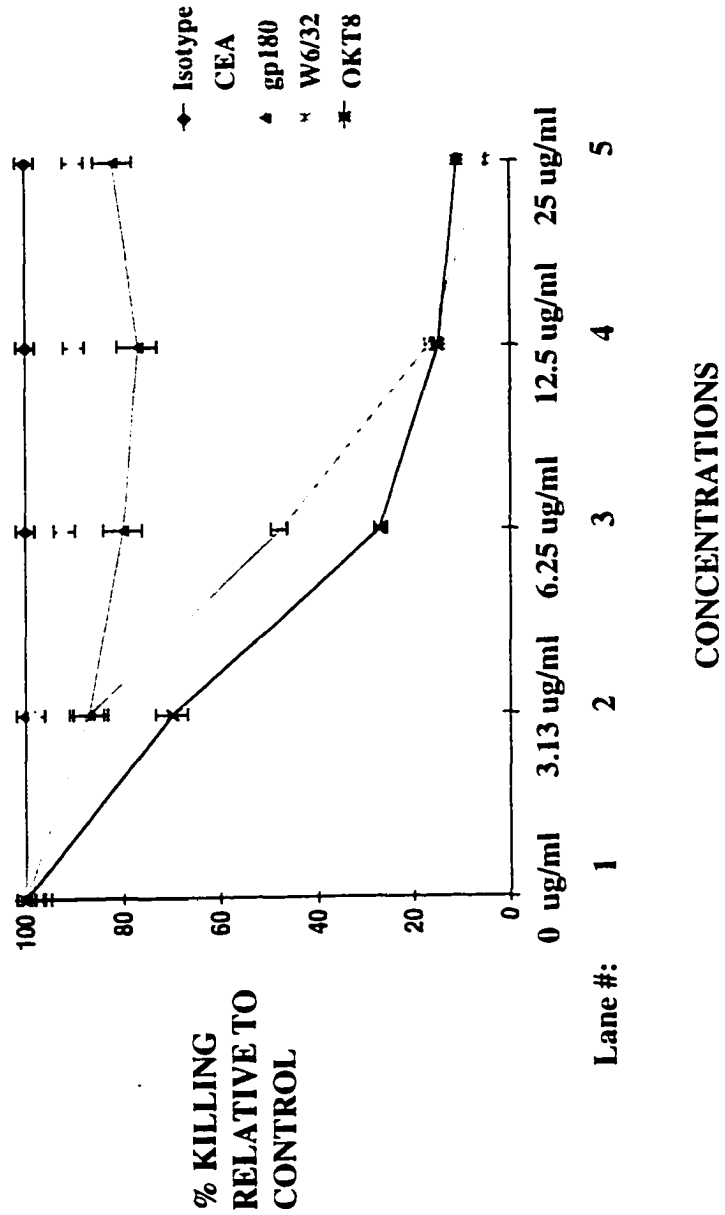


Figure 8



GENERAL DISCUSSION

Like the systemic immune system, the mucosal immune system is triggered by antigen. However, the response generated to antigen differs greatly. Oral antigen priming most often results in systemic immune hyporesponsiveness or tolerance while administering the same antigen systemically results in an active immune response (28). Oral tolerance may be mediated by several mechanisms including induction of anergy, activation of suppressor T cells and the secretion of suppressive cytokines. However, early studies indicate that $CD8^+$ suppressor T cells may play a critical role in the development of this tolerant state (9, 10, 16, 18). These early studies showed that the transfer of $CD8^+$ splenic T cells from an orally tolerized mouse to a naive mouse would effectively transfer this state of antigen specific systemic tolerance (20). The mechanism by which $CD8^+$ T cells were activated was not clearly defined.

Recently, IECs have been recognized as a participant in mucosal immune responses. Specifically, IECs have been suggested to be a key regulator of such responses by acting as an antigen presenting cell (2, 42). Normal IECs constitutively express class II MHC molecules and are capable of taking up, processing, and presenting exogenous antigens to primed T cells in the mouse, rat and man (2, 3, 26, 39, 41, 42, 77). Interestingly, antigen presentation in this system appears to be different from that of conventional antigen presenting systems in that, it is neither class I nor class II MHC restricted (42, 50) and the T cells proliferating in

these systems are $CD8^+$ rather than $CD4^+$ (50). In addition, further functional studies have indicated that these $CD8^+$ T cells are suppressive (antigen nonspecific) in nature.

Suppressor T cells, whose function is to inhibit the activation phase of immune responses, are a class of lymphocytes thought to be distinct from helper and cytolytic T lymphocytes. A major problem in the study of suppressor T cells has been that attempts to purify these cells in numbers sufficient for the establishment of stable cloned lines with specific suppressive activity, have been largely unsuccessful. As a result, even basic questions, such as the nature of the receptors expressed by suppressor cells, are unresolved. There is sufficient evidence to suggest that suppressor T cells do exist, the problem now is determining how these cells are activated and the mechanisms by which their suppressor function is mediated. One problem may be that previous attempts to clone suppressor T cells utilized conventional APCs and antigens. In the gastrointestinal tract where suppression is critical, alternative APCs and restriction elements may be employed.

Although the role of classical restriction elements in T cell development has been relatively clearly defined, the function of the structurally related class Ib proteins has not yet been thoroughly investigated. These molecules include the CD1 family of proteins. The human CD1 gene family is composed of five members, four of which (CD1a-d) are known to be expressed *in vivo*. The CD1 family of class I-like proteins is encoded outside the MHC and most members have limited homology with regard to classical class I molecules (53).

It has been found that CD1d is expressed predominantly by IECs (56) and hepatocytes. The predominant expression of CD1d by human IECs suggests that CD1d may be a ligand for T cells in the intestinal epithelium. Furthermore, recent data has indicated that CD1d is expressed in a polarized fashion on IECs (60). CD1d localization to the apical and lateral membrane domains places CD1d in an ideal location to serve as a luminal antigen sampling and presentation molecule to laterally positioned IEL (61). In addition, CD1d has been found to congregate just beneath the apical surface (60) suggesting that CD1d may be cycling between the apical and basolateral regions and essentially sampling antigen from the lumen and presenting it to IEL. The non-polymorphic nature and class I-like structure make CD1d a candidate ligand for TCR mediated activation of $CD8^+$ T cells in the epithelium.

Data generated in this thesis clearly indicate that the activation of a unique $CD8^+CD28^-$ suppressor T cell population in the gastrointestinal tract involves IECs. More specifically, it was shown that the novel CD8 ligand, gp180 and class Ib molecule, CD1d, associate on the surface of IECs and it is this complex which interacts with the CD8:TCR complex on suppressor T cells. In our model (Appendix Figure 1), each component of this complex has a unique function: gp180 binds to the CD8 molecule resulting in the activation of the CD8-associated kinase $p56^{lck}$ while CD1d interacts with the TCR causing the phosphorylation of the TCR-associated kinase $p59^{fyn}$.

The presence of a functioning class Ib molecule on the surface of IECs led us to investigate the possibility that, unlike class I MHC-restricted $CD8^+$ cytolytic T cells, $CD8^+$ suppressor T cells are CD1d-restricted. If these cells are actually CD1d restricted, it would help toexplain the difficulty in purifying $CD8^+$ suppressor T cells in numbers sufficient for the establishment of stable cloned lines. All previous attempts to do so involved the assumption that suppressor T cells were restricted in the same manner as cytolytic T cells. We now believe that this is not the case.

Using our co-culture system where IECs are cultured with T cells, it was determined that a different signaling cascade is utilized in suppressor T cells compared to that of CTL (Appendix Figure 2). The activation of $CD8^+$ T cells following TCR stimulation is the result of both protein kinase C (PKC) dependent and independent mechanisms. Suppressor T cell activity is regulated in a PKC-independent manner through Ras/MAPK interactions with guanine nucleotide exchange proteins, such as vav. The exchange protein vav is tyrosine phosphorylated and has increased guanine nucleotide exchange activity with Ras resulting in the activation of the MAPK cascade. The PKC-dependent pathway also appears to be involved in the activation of cytotoxic $CD8^+$ T cells. Stimulation of the TCR/CD8 co-receptor complex in $CD8^+$ cytolytic T cells, but not $CD8^+$ suppressor T cells, has been shown to activate the PLC-γ1 pathway.

This difference does not confirm whether these cells are derived from a general $CD8^+$ T cell pool, in which different signaling

pathways are triggered, resulting in either suppressor or cytolytic T cell differentiation. It is known that gp180 binds to any CD8 molecule, therefore, the effect of gp180 binding to CD8 on cytolytic T cells was investigated. If gp180 binding to CD8 inhibits the ability of CTL to kill, this may suggest that cytolytic and suppressor functions are generated by triggering different signaling cascades in cells from a common $CD8^+$ pool. If gp180 does not inhibit killing, it may suggest that these two CD8 T cell subsets are pre-committed by different restriction elements to have specific effector activity. Interestingly, it was determined that gp180 was not able to interfere with the ability of CTL to kill.

This finding may have a great impact on the understanding of mucosal immunity. Over the past few decades, several hypotheses on the existence of suppressor T cells have come and gone. These include the early studies done in mice indicating that the stimulation of suppressor T cells may be restricted by a region of the class II MHC called I-J (83). The I-J glycoprotein was thought to distinguish suppressor cells from all other cells and was thought to be located between the I-A and I-E loci based on analyses of various inbred strains (83). However, sequencing of the entire mouse class II MHC has demonstrated that there is no DNA coding for a unique I-J molecule at this site, and attempts to demonstrate an I-J encoded cell surface glycoprotein have generally failed. Many scientists have bluntly dismissed the existence of suppressor T cells, by assuming these observations were entirely artifact. It is still not clear whether molecules such as I-J exist, as the explanation for these assumed artifacts remains obscure.

These findings still leave many open questions. If, in fact, we are defining a population of committed suppressor cells, are these unique to our system or can one explain the difficulty in identifying suppressor T cells in the past based on their requirement for novel restriction elements and novel co-stimulatory pathways? CD1d has a limited tissue distribution so its role in generating suppressor T cells may be specific to the intestinal tract. Other nonclassical class I molecules may be involved in suppressor cell generation in other tissues. Along these lines we have noted that the gp180 expressed in the placenta is associated with the nonclassical class I molecule HLA-G (Campbell and Mayer unpublished). This would be a rational site for the induction of suppressor T cells that prevent immune responses against the fetus. The fact that the majority of our studies have been performed using T cells derived from the peripheral blood suggests that precommitted suppressor cells are present in many immunologic compartments but appear to require unique interactions to become activated. It would clearly be of interest to be able to identify this population with more specific phenotypic markers (i.e. within the $CD8^+$ $CD28^-$ population).

The original findings relating to the ability of IECs to act as APCs used T cell proliferation as a read out. Our current studies have focused on the signaling pathways involved in this activation process. The induction of proliferation was clearly antigen driven, either utilizing tetanus toxoid as the nominal antigen or alloantigen. In the systems described in this thesis, it is not clear how much of a requirement there is for antigen. gp180 by itself can activate CD8 associated $p56^{lck}$. CD1d expressed on a transfectant line activates

TCR associated $p59^{fyn}$. Based upon the current concepts of T cell activation we would assume that CD1d is presenting antigen to the suppressor T cells. It would be unlikely that just the interaction of CD1d with the TCR would result in fyn activation. If this were the case then such cells would be activated on an ongoing basis. An on switch should be required. In the case of the transfectants either Ags present in the serum or other internally processed antigens could be candidates. We have not defined the nature of the antigen driving this response. CD1 molecules have been shown to bind lipid, carbohydrate (possibly), and peptide Ags. Given the deep hydrophobic pocket seen in the resolved crystal structure of CD1 it is conceivable that lipoproteins might be found in the antigen binding groove. Is there a ubiquitous suppressor inducer Ag that is a by product of T cell activation by conventional means? Others have shown T cell proliferation to lipid and carbohydrate antigens but the effector function has been determined only in a small subset of these studies (e.g. cytolytic activity). No measurement of suppressor activity was reported.

Interestingly, CD1 is localized to the endosome which places it in a position to interact with Ags that have been taken up exogenously. Furthermore, whatever the antigen, it is clear that CD1 restricted T cell responses require processing of Ag. In the presence of chloroquine or cell fixation, T cell responses to CD1 presented Ags are inhibited. This might suggest that some peptide component is presented since the alterations in pH induced by chloroquine generally inhibit proteases. However, CD1 is nonpolymorphic, thus its repertoire of peptide binding may be

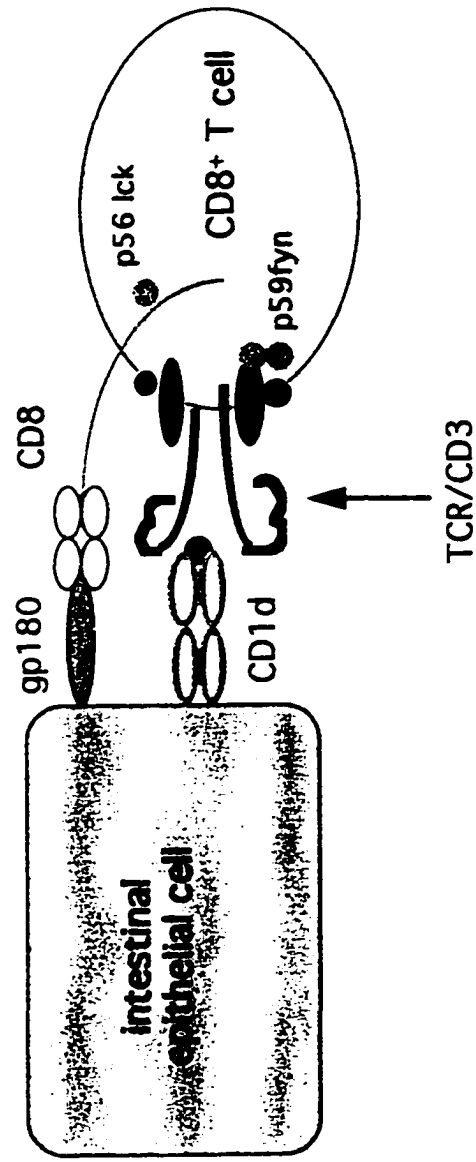
limited. Furthermore, unless CD1 is loaded with class I and class II peptides, it is not likely to generate an "allo" response. This would add more support to the concept that a common Ag, capable of activating T cells, is presented by this restriction element. If such T cells get activated they may mediate their suppressor function and subsequently undergo apoptosis as a regulatory event (i.e. they suppress and die analogous to the short life span of antibody producing plasma cells). If a limited set of peptides are presented by these restriction elements then one would expect a limited repertoire of T cells responding. Initial experiments performed in our lab have suggested that there is oligoclonality in these co-cultures but this is difficult to interpret given the background oligoclonality in the CD8+ T cell population. It would be important to demonstrate that the oligoclonality is consistent between different individuals (i.e. if a specific generic regulatory cell is activated).

Lastly, we have previously shown that the T cells activated by IEC are antigen specific in their induction but antigen nonspecific in their effector phase. This leaves the door open for multiple Ags driving the response of a limited number of effector T cell clones. The ability to clone out these cells will allow us to address these issues in the future.

In summary, the data generated in this thesis may account for several features observed in the gastrointestinal tract. The existence of a CD1d:gp180 complex would explain the presentation of exogenous antigen to $CD8^+$ T cells by IECs, the presence of predominantly $CD8^+$ T cells in the IEC compartment, the inability to

clone suppressor T cells, and the poor activation of mucosal lymphocytes by conventional antigen presenting cells. The necessity for the activation of these suppressor T cells in the gut is clear. Any inability to do so is costly, resulting in inflammation and the loss of functional integrity in the gastrointestinal tract. The use of distinct molecules and restriction elements provide further evidence for the differences between systemic and mucosal immunity. This dichotomy may evolve from the differences in antigen load and the requirement for controlled rather than active responses in the gastrointestinal tract.

APPENDIX: Figure 1



APPENDIX: Figure 2

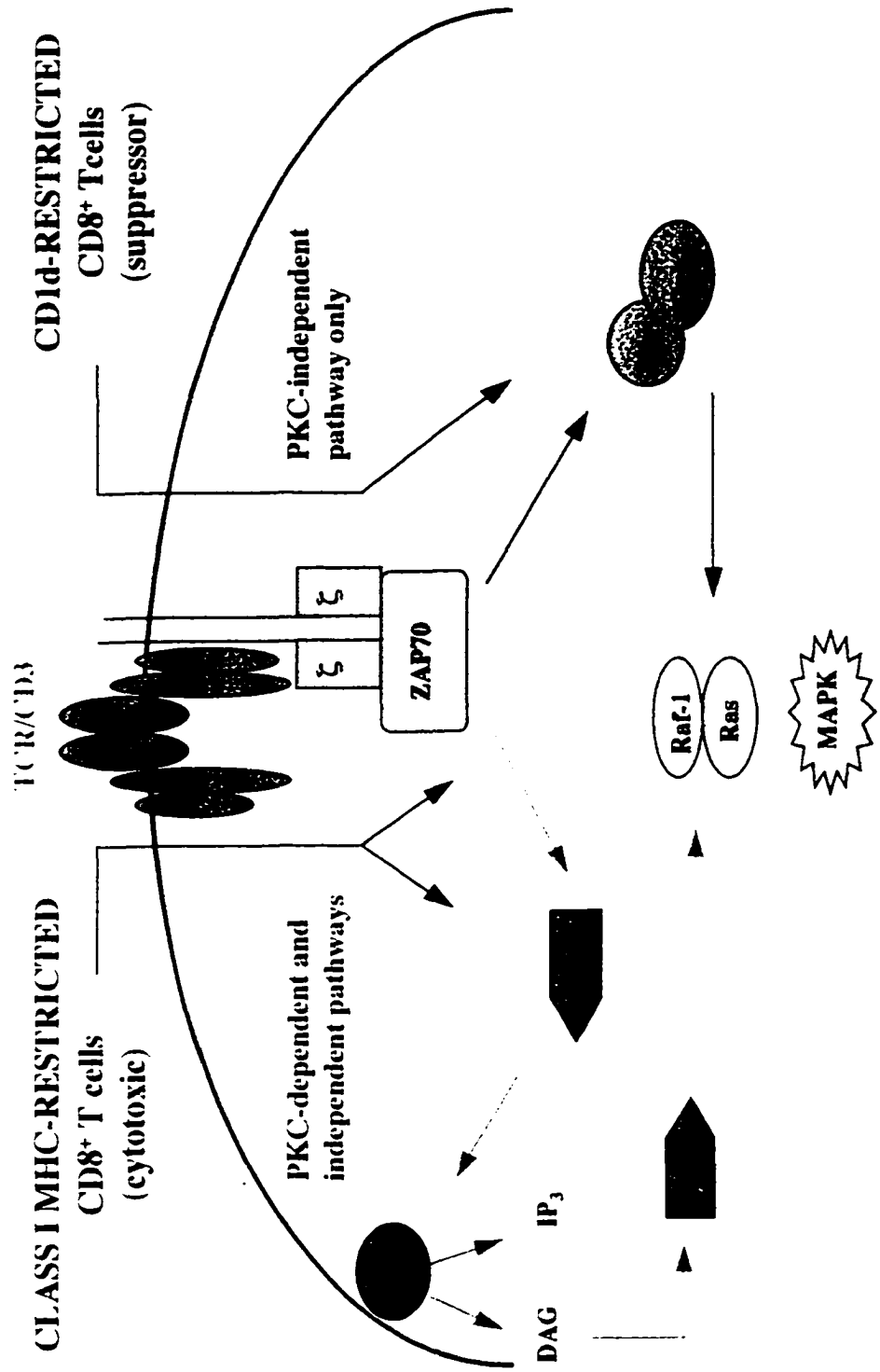


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IMAGE EVALUATION
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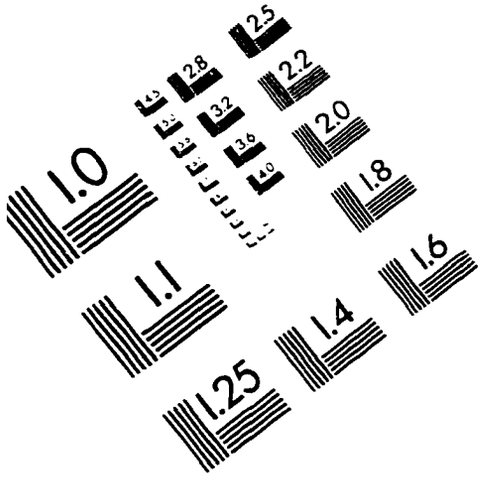

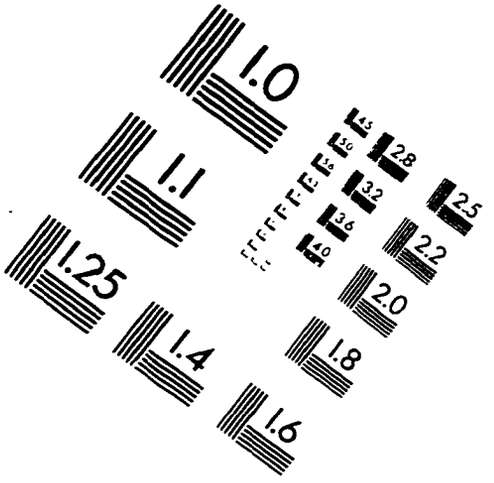

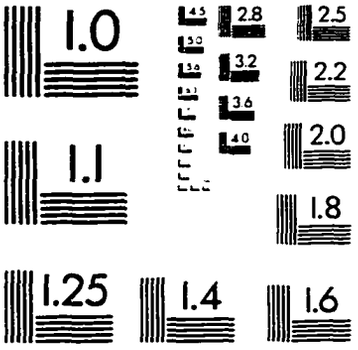

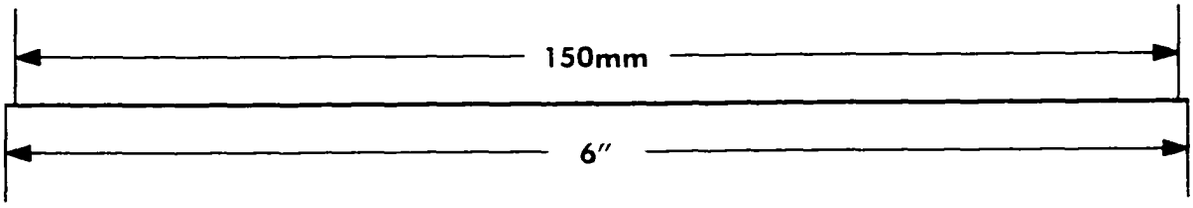

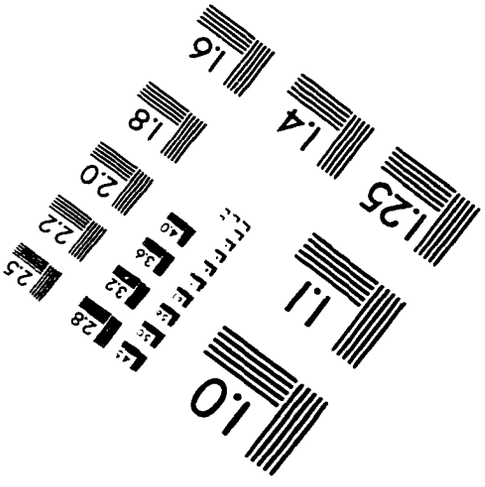

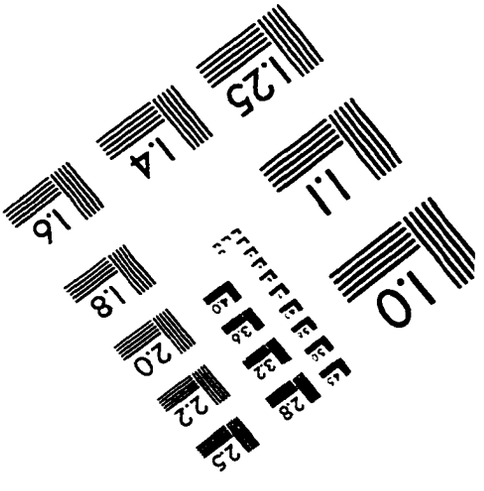

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