

CD1d Is Involved in T Cell–Intestinal Epithelial Cell Interactions

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Summary

We assessed the role of the nonclassical class I molecule, CD1d, in the interaction between intestinal epithelial cells and T cells. In a mixed lymphocyte reaction (MLR) system where the stimulator cells were irradiated normal intestinal cells, the anti-CD1d monoclonal antibody (mAb) 3C11 inhibited T cell proliferation. In contrast, no inhibition was seen when mAb 3C11 was added to conventional MLR cultures (non T cell stimulators). Furthermore, no inhibition was seen when either airway epithelial cells were used as stimulator cells or lamina propria lymphocytes were used as responder cells. These latter two conditions along with a conventional MLR favor CD4⁺ T cell proliferation. However, we have previously shown that normal intestinal epithelial cells stimulate CD8⁺ T cells under similar culture conditions. Thus, CD1d expressed on intestinal epithelial cells may be an important ligand in CD8⁺ T cell–epithelial cell interactions.

Immune responses at mucosal sites are, by nature of the environment, subject to unique rules relating to recognition and effector functions. Over the past several years, it has become clear that many of the dogmas set for peripheral immune responses have not held for mucosal immunity (1–3). Many of these may relate to how antigen is handled in the gastrointestinal (GI) tract. Proteins delivered via the stomach and small intestine are preprocessed by intestinal enzymes (4). The resultant small peptides can be absorbed by the intestinal epithelium and could be delivered to the blood stream in a nonimmunogenic form. However, several groups have recently demonstrated that intestinal epithelial cells (IEC) are capable of a more proactive role in Ag handling. First, as has been reported for a number of epithelia in vitro, IEC can express class II Ags and activate T cells (5–9). However, in contrast to the typical “non-professional” APC, IEC, especially in the small intestine, constitutively express class II molecules in vivo (10, 11). This finding, along with their ability to take up large peptides and present them to primed T cells in vitro, suggest that IEC may play an important immunoregulatory role in vivo. This regulatory role may relate to the general suppressed tone of the GI tract. In fact, reports from several groups have demonstrated that IEC selectively activate suppressor T cells that may be Ag nonspecific even when activated in Ag-specific interactions (5, 6). The mechanism(s) resulting in the generation of these suppressor cells has not been clearly established. Our laboratory has demonstrated that the CD8 molecule itself plays a role in the suppressor T cell activation seen (12). However, the conventional

ligand for CD8, class I, has no effect in this system. Therefore, additional molecules were sought that would potentially interact with CD8. One such candidate is CD1d, a class I-like molecule recently reported to be expressed on murine and human IEC (13, 14). Whereas the functional relevance of CD1d has not been defined, in this study we provide evidence that this molecule is an important component in epithelial cell–T cell interactions.

Materials and Methods

Cell Isolation and Culture. PBMC were isolated from leukocyte concentrate packs as previously described (15). Further separation to T and non T cells was performed by rosetting and density gradient separation on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) (15). Lamina propria lymphocytes (LPL) were isolated from surgically resected intestinal specimens using enzymatic techniques as previously described (16). Briefly, resected intestine was rinsed in cold calcium magnesium free (CMF)-HBSS (Gibco BRL, Gaithersburg, MD), containing gentamicin (50 µg/ml) (Sigma Chemical Co., St. Louis, MO), penicillin/streptomycin (1% vol/vol) (Gibco BRL), and amphotericin B (1% vol/vol, Gibco BRL) and blotted free of mucus and debris on sterile gauze. The mucosa was stripped from the underlying submucosa by blunt dissection, cut into small 2-mm pieces, and incubated in dithiothreitol (2 mM, Sigma Chemical Co.) for 5 min. After this incubation, the mucosal pieces were washed three times in RPMI 1640 (Gibco BRL) and incubated three to four times in CMF-HBSS containing EDTA (0.75 mM, Sigma Chemical Co.). This suspension was rotated on a Junior Orbital Shaker (Fisher, Springfield, NJ) at 40 RPM for 45 min each at 37°C. Liberated epithelial cells were removed by pas-

sage through Nytex (Spectrum, Los Angeles, CA) and mucosal pieces were then incubated overnight in RPMI 1640, 1% FCS (Flow Laboratories, Maclean, VA), collagenase (80 ng/ml, Boehringer Mannheim, Indianapolis, IN), DNase (0.1 μ g/ml, Sigma Chemical Co.), and soybean trypsin inhibitor (0.1 μ g/ml, Sigma Chemical Co.). After incubation for 18 h, the liberated cells were collected by centrifugation and additional cells liberated by passage of tissue through a wire mesh. The resultant cell suspension was enriched for lymphocytes by passage over a Percoll density gradient, as previously described (16), collecting cells at the interface of 40–60% percoll. LPL were typically >95% HLe-1⁺ (T200) with 2–5% contaminating epithelial cells.

IEC were isolated as previously described (5). Mucosal pieces (pre-EDTA) were incubated at 37°C in 50 ml RPMI containing Dispase (3 mg/ml, Boehringer Mannheim) for 30 min. The suspension was vortexed every 5–10 min liberating epithelial cells. These cells could be freed from the mucosal pieces by passage through a Nytex filter. Epithelial cells were pelleted, washed three times in RPMI 1640, and irradiated 3,000 rad (cesium source) before use.

Epithelial cells were free of contaminating B cells and monocytes/macrophages assessed by the absence of CD19/20 and CD14 staining. T cell contamination of about 2–5% was typically seen which could be reduced by subjecting epithelial cells to Percoll density gradient centrifugation (5).

Isolation of airway epithelial cells (AEC) was performed from surgically resected specimens or bronchoalveolar lavage specimens (17). Isolation from resections was performed using Dispase and agitation as described for IEC. AEC were then cultured in DMEM/F12 (Gibco BRL) in the absence of serum, but in the presence of epidermal growth factor, insulin, transferrin, and hydrocortisone. After 5 d in culture, no viable monocytes were detectable and ciliary beating could still be seen on adherent epithelial cells. Plate-bound epithelium were irradiated 3,000 rad before coculture with T cells.

Allogeneic Mixed Cell Cultures. T cell–epithelial cell or non T cell cultures were incubated in RPMI 1640, 5% agammaglobulinemic serum, 1% penicillin/streptomycin, and 2 mM L-glutamine (Gibco BRL) (culture medium [CM]) for 5 d at 37°C in a 5% CO₂ water-jacketed incubator. During the last 18 h of the 5-d culture, 1 μ Ci [³H]thymidine (aqueous methyl sp act, 1.9 mCi; ICN, Irvine, CA) was added and cells harvested for counting as described (5).

Allogeneic mixed cell cultures were established as follows: 10⁵ T cells or LPL were cultured in 100 μ l CM in triplicate cultures in the presence or absence of varying concentrations of irradiated (3,000 rad) stimulator cells (2 \times 10⁵, 10⁵, 5 \times 10⁴ IEC, AEC, or non T cells). Background, unstimulated T cell proliferation was measured in cultures of T cells alone whereas a positive control of T cells stimulated with PHA (1 μ g/ml, Gibco BRL) was included in each experiment. In no experiment did T cells fail to proliferate in response to PHA.

Antibodies. mAbs 3C11 and 1H1 are rat IgM anti–mouse CD1d Abs that crossreact with human CD1d as described (13, 14). 1H1 appears to recognize an epitope or form of CD1d distinct from that recognized by 3C11. Abs were used as culture supernatant but adjusted to an Ab concentration of 10 μ g/ml stock solution.

In most experiments, mAb were added directly to the culture wells for the 5 d of culture. Given the lack of inhibition by 1H1 described in this paper, this mAb served as the isotype control for 3C11. Murine IgG1 anti-DNP served as the isotype control for VG2.

In some experiments, epithelial or T cells were preincubated with mAbs for 30 min at 4°C followed by washing with PBS, irradiation, and coculture with T cells.

Results

Anti-CD1d mAb 3C11 Inhibits Epithelial Cell but Not Non T Cell–driven Allogeneic Mixed Cell Cultures. Our previous studies (12) had shown that T cell–epithelial cell allogeneic mixed cell cultures could be inhibited by mAbs to CD8 but not its conventional ligand, class I MHC. These data suggested that CD8 was not only important in the process of T cell–IEC interaction, but also that this molecule might be activated through an alternate ligand. The finding that CD1d, a class I-like molecule, is expressed on human IEC (14) made it a candidate for such an interaction. Anti-CD1d mAb 3C11 was added at varying concentrations (1–5 μ g/ml) to normal epithelial cell–T cell allogeneic mixed cell cultures. Inhibition (50–80%) of T cell proliferation was seen in 8 of 12 experiments (Fig. 1 A, representative of eight experiments). This inhibition was evident at varying epithelial cell concentrations as well (Fig. 1 B). Anti-CD1d mAb 1H1 which recognizes a different epitope or form of CD1d on epithelial cells was unable to mediate inhibition in parallel cultures (Fig. 1 C). Inhibition by 3C11 was mediated via binding to epithelial cells as pretreatment of epithelial cells, but not T cells before allogeneic mixed cell culture was as effective in inhibiting T cell proliferation as when Abs were left in culture throughout the allogeneic mixed cell culture incubatory period (data not shown). The inhibition by CD1d mAb 3C11 is comparable to the inhibitory effect of an anti-CD8 mAb in this system. The effect of anti-CD8 appears to be specific since it fails to inhibit the proliferation of PHA-stimulated T cells (Fig. 1 D).

In contrast, neither 3C11, 1H1, nor anti-CD8 were able to inhibit conventional (T cell–non T cell) allogeneic MLRs (Fig. 2 A) suggesting that the inhibition seen in Fig. 1 was epithelial cell specific and not related to nonspecific inhibition of T cell activation. However, since the stimulated cell in epithelial cell–T cell allogeneic mixed cell cultures is the CD8⁺ T cell, the possibility exists that anti-CD1d mAbs selectively inhibit CD8⁺ T cell activation. Therefore, we isolated CD8⁺ T cells from peripheral blood and cocultured them with allogeneic non T cells. As seen in Fig. 2 B, 3C11 failed to inhibit CD8⁺ T cell proliferation induced by conventional stimulator cells, suggesting that the effect of anti-CD1d might be specific for IEC–T cell allogeneic mixed cell cultures.

mAb 3C11 Fails to Inhibit Peripheral Blood T–AEC Allogeneic Mixed Cell Cultures. AEC may serve similar barrier function to IEC, yet the Ag loaded in the lung is significantly less than that in the gut. This difference may underscore the functional differences that have been reported between these two cell types. Whereas both epithelia constitutively express class II molecules, only AEC stimulate CD4⁺ T cells (17). If, in fact, CD1d is important in CD8⁺ T cell activation by IEC, then mAb 3C11 should not inhibit peripheral blood T–AEC allogeneic mixed cell cultures. This indeed was the case. As seen in Fig. 3, mAb 3C11 failed to inhibit peripheral blood T–AEC cultures but was capable of inhibiting IEC stimulation of the same T cell preparation. Thus, consistent with the results seen with non T cells, cultures where CD4⁺ T

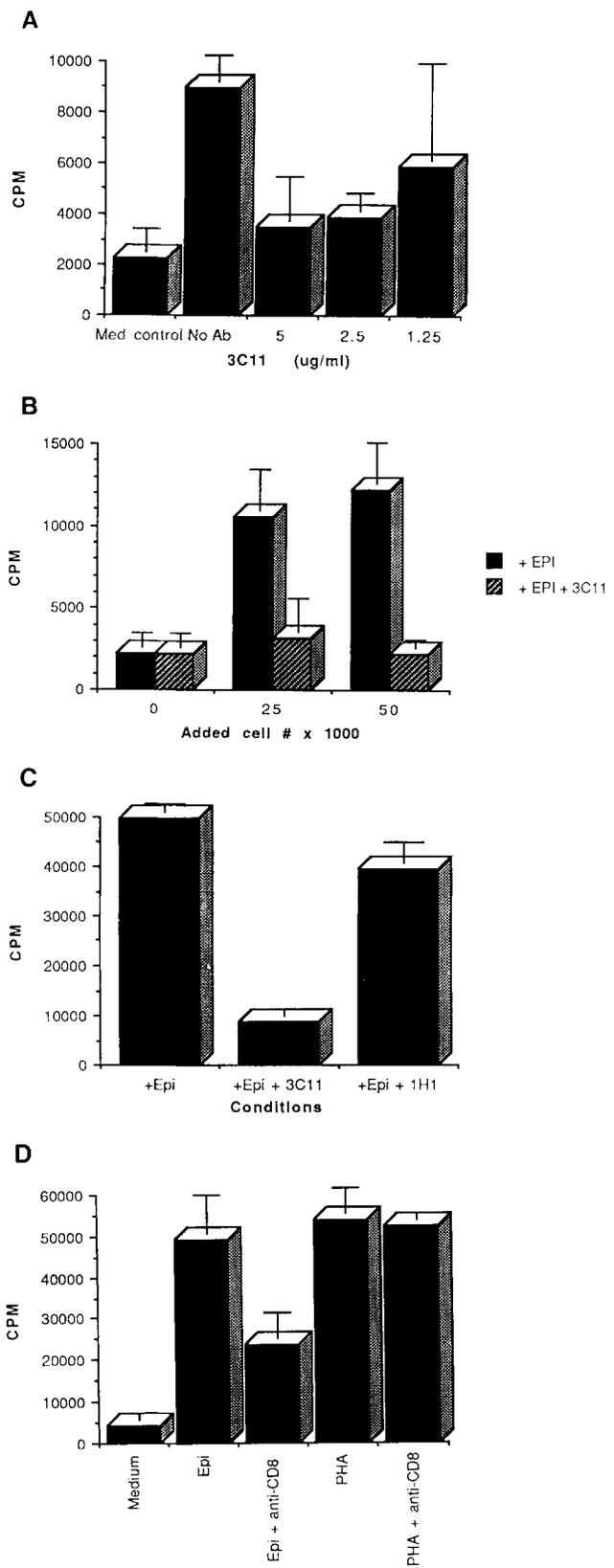


Figure 1. (A) 10^5 peripheral blood (PB) T cells were cocultured with an equal number of irradiated (3,000 rad) freshly isolated IEC for 5 d in the presence or absence of varying concentrations of anti-CD1d mAb 3C11. The isotype control mAb showed no inhibition (see Fig. 1 C). This figure

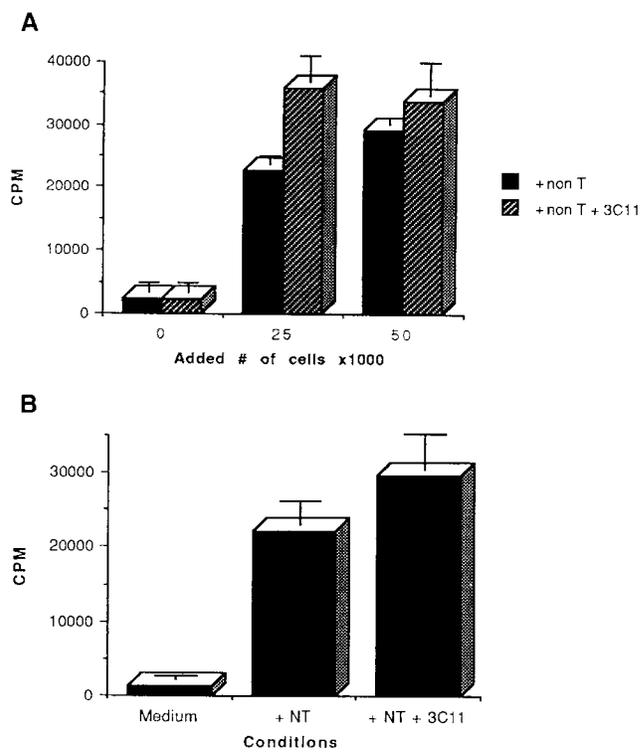


Figure 2. (A) 3C11 fails to inhibit a conventional MLR. Similar to the experimental design of Fig. 1 B, varying concentrations of non T cells were used to stimulate PB T cells in the presence (hatched bars) or absence (solid bars) of mAb 3C11 (5 μ g/ml). No inhibition is seen. This figure is representative of six experiments. (B) $CD8^+$ T cells were isolated by sorting and cocultured with non T cells in a conventional MLR culture in the presence or absence of anti-CD1d mAb 3C11. Similar to the findings in (A), no inhibition was seen.

cell proliferation predominates are not inhibitable with the anti-CD1d mAb.

mAb 3C11 Does Not Inhibit LPL-Normal Epithelial Cell Allogeneic Mixed Cell Cultures. In contrast to peripheral T cell-IEC cultures where proliferation of $CD8^+$ T cells predominate, LPL-epithelial cell cultures result in the proliferation of both $CD4$ and $CD8^+$ T cells (Panja, A., A. Barone, and L. Mayer, manuscript submitted for publication). Addition of mAb 3C11 to these allogeneic mixed cell cultures failed to produce any inhibition (Fig. 4; representative of six experiments). Thus the nature of the responder cell population is also critical in documenting the effects of 3C11. In experi-

is representative of eight experiments. (B) As reported in (A), 10^5 PB T cells were cocultured with either 2.5×10^4 or 5×10^4 irradiated (3,000 rad) freshly isolated IEC for 5 d in the presence (hatched bars) or absence (solid bars) of anti-CD1d mAb 3C11 (5 μ g/ml). The isotype control mAb showed no inhibition (see Fig. 1 C). (C) Comparison of anti-CD1d mAbs 3C11 vs. 1H1. Whereas both stain intestinal epithelium in tissue section, only 3C11 was capable of mediating inhibition. (D) Ability of anti-CD8 mAb to inhibit epidermal cell-driven T cell proliferation. T cells were untreated or preincubated with the anti-CD8 mAb, OKT8, for 30 min at 4°C . After removal of excess mAb by washing, T cells were cocultured with either an equal number of irradiated (3,000 rad) freshly isolated IEC for 5 d or PHA (1 μ g/ml) for 3 d. Anti-CD8 inhibited IEC but not PHA stimulation of T cells.

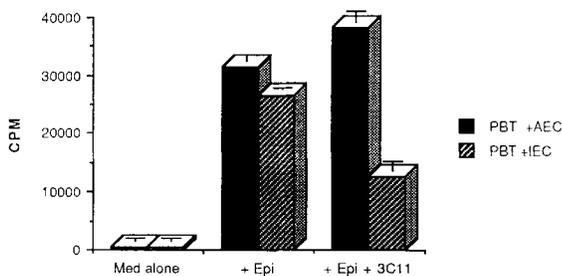


Figure 3. 3C11 fails to inhibit an AEC-stimulated MLR. 10^5 PB T cells were cocultured with either an equal number of irradiated (3,000 rad) freshly isolated IEC (hatched bars) or AEC (solid bars) for 5 d in the presence or absence of varying concentrations of anti-CD1d mAbs 3C11 ($5 \mu\text{g}/\text{ml}$). This figure is representative of two experiments.

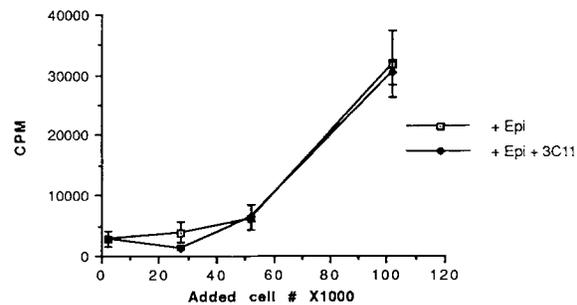


Figure 4. 3C11 fails to inhibit an LPL-epithelial cell MLR. 10^5 LP T cells were cocultured with varying numbers of irradiated (3,000 rad) freshly isolated IEC for 5 d in the presence (\diamond) or absence (\square) of varying concentrations of anti-CD1d mAb 3C11. This figure is representative of six experiments.

ments where epithelial cells from one donor were used to stimulate either allogeneic peripheral blood T cells or LPL from another donor, inhibition was seen only in peripheral blood cultures (data not shown). These data add further support for the role of CD1d in the activation of CD8^+ peripheral blood T cells.

Discussion

CD1d is a distinct member of the CD1 gene family which are nonpolymorphic MHC class I-like molecules. In contrast to CD1a-c which are primarily expressed thymically and to a limited extent extrathymically, CD1d is primarily an extrathymic molecule expressed on epithelial cells of a wide variety of tissues including the intestine (14, and Balk, S., and R. Blumberg, manuscript submitted for publication). Its function in immune regulation has not been clearly determined although there is some evidence that it can be recognized by cytolytic intraepithelial lymphocytes (18) either as a target or as an accessory molecule. Clearly its presence on epithelium may serve as a regulator of immune responses.

The nature of the immune response in the intestine is mixed. Whereas positive immune responses (IgA secretion) to specific Ags do occur (19, 20), the majority of Ags in the GI tract evoke either a nonresponse or quite commonly, active suppression (21). This active suppression is clearly seen in the phenomenon of oral tolerance where oral immunization to a protein Ag suppresses the ability of the host to become systemically immunized (22-24). Although the mechanism of induction of oral tolerance is not understood, recent studies have demonstrated that Ag presentation via IEC results in the activation of CD8^+ CD28^- suppressor T cells (5, 6). Furthermore, in humans, it appears that the CD8 molecule itself is important in this process as suppressor cell generation is inhibited by anti-CD8 but not anti-CD4 mAbs (12). The restriction elements regulating the activation of these cells have also been questioned as neither mAbs to class I (conventional CD8 ligand and restriction element) nor class II (pretreatment of epithelial cells only) Ags inhibit IEC-stimulated T cell activation in allogeneic mixed cell culture (12). One plausible scenario is that there is a novel ligand for CD8 that may act as a costimulatory signal for CD8^+

T cells. This may exist solely as a ligand for CD8 or, like class I, serving as a ligand as well as a restriction element. A role for CD8 in this system has been recently strengthened by the findings that normal epithelial cells activate the src-like tyrosine kinase p56lck, associated with the α chain of CD8 (25, and Li, Y., and L. Mayer, manuscript in preparation) in peripheral blood T cells. Furthermore, this activation appears to be a critical event in epithelial cell-driven CD8^+ T cell proliferation, since inhibition of tyrosine kinase by pretreatment of responder T cells with genestein blocked T cell proliferation in epithelial cell-T cell allogeneic mixed cell cultures.

Obvious candidates for novel CD8 ligands would be class I-like molecules that would serve the purposes described above. Clearly CD1d could fall into this group despite the current lack of direct evidence that any of the CD1 family binds to CD8. Furthermore, there is no evidence that CD1d can bind peptides allowing it to serve as a potential restricting element for the TCR or TCR-like molecules on T cells.

In this study, we provide evidence for the functional importance of CD1d. Besides CD8, it is the only known molecule that has been shown to be important in T cell stimulation by normal IEC. These studies strongly suggest that CD1d is important in CD8^+ T cell activation by IEC since non T cell- and AEC-stimulated allogeneic mixed cell cultures are not inhibited by a mAb to CD1d. Both of these stimulator cells preferentially activate CD4^+ T cells and the results from these experiments support the model that 3C11 inhibits cultures where CD8^+ T cell activation occurs. Furthermore, these latter experiments confirmed the fact that the anti-CD1d mAb is not mediating nonspecific inhibition. The findings in non T cell/AEC-stimulated T cell cultures were strengthened by the results seen in LPL-epithelial cell cultures. Here again, in non-CD8 stimulatory cultures, no suppression by mAb 3C11 was seen. Thus CD1d may be a novel CD8 ligand. These data suggest that it is associated with T cell activation and proliferation. It remains to be determined whether this is a cognitive or noncognitive event. This issue can be scrutinized more rigorously by the use of CD1d transfectants where other epithelial cell surface Ags would not be available to alter responses.

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