Colitis immunoregulation by CD8$$^+$$ T cell requires T cell cytotoxicity and B cell peptide antigen presentation

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McPherson M, Wei B, Turovskaya O, Fujiwara D, Brewer S, Braun J. Colitis immunoregulation by CD8$$^+$$ T cells requires T cell cytotoxicity and B cell peptide antigen presentation. Am J Physiol Gastrointest Liver Physiol 295: G485–G492, 2008. First published July 10, 2008; doi:10.1152/ajpgi.90221.2008.—Deficient immunoregulation by CD4$$^+$$ T cells is an important susceptibility trait for inflammatory bowel disease, but the role of other regulatory cell types is less understood. This study addresses the role and mechanistic interaction of B cells and CD8$$^+$$ T cells in controlling immunemediated colitis. The genetic requirements for B cells and CD8$$^+$$ T cells to confer protective immunoregulation were assessed by cotransfer with cotoligenic Gα2$$^-/-$$ T cells into immune-deficient mice. Disease activity in Gα2$$^-/-$$ T cell recipients was evaluated by CD4$$^+$$ T intestinal lymphocyte abundance, cytokine production levels, and large intestine histology. B cells deficient in B1/B7.2, CD40, major histocompatibility complex (MHC) II (Abb), or native B cell antigen receptor (MD4) were competent for colitis protection. However, transporter-1-deficient B cells failed to protect, indicating a requirement for peptide MHC I presentation to CD8$$^+$$ T cells. CD8$$^+$$ T cells deficient in native T cell receptor repertoire (OT-1) or cytolysis (perforin$$^-/-$$) also were nonprotective. These findings reveal an integrated role for antigen-specific perforin-dependent CD8$$^+$$ T cell cytotoxicity in colitis immunoregulatory and its efficient induction by a subset of mesenteric B lymphocytes.

CD8-positive T lymphocytes; B lymphocytes; immunoregulation; inflammatory bowel disease; cytolysis

INFLAMMATORY BOWEL DISEASE (IBD) results from a dysregulated immune response against intestinal antigens (8, 70), with tissue damage driven by TH2, TH1, or TH17-type CD4$$^+$$ T cells (65). IBD susceptibility traits modify the mucosal barrier function (2), T cell receptor (TCR), and antigen presentation cell (APC) interactions, and immunoregulatory cell formation (6). Among these, there has been a particular emphasis on the importance of regulatory CD4$$^+$$ T cells in immune-microbe homeostasis (34).

Previous work has uncovered a role for B cells in suppressing colitis associated with TCR-α$$^-/-$$ mice (46), through IL-10 and CD1d-dependent processes (47, 60). Similarly, in models with TH1 or TH17 colitis (CD4$$^+$$CD45RB$$^{hi}$$ or Gα2$$^-/-$$ T cells), B cells conferred protection (67) and also were shown to require IL-10 sufficiency (66). The exact cellular target of B cells in these regulatory responses to colitis remains uncertain. In TCR-α$$^-/-$$ mice, B cell protection was independent of B cell major histocompatibility complex (MHC) II, suggesting the absence of cognate interaction with CD4$$^+$$ T cells. In CD4$$^+$$CD45RB$$^{hi}$$ and Gα2$$^-/-$$ T cell settings, B cell protection required the presence of CD8$$^+$$ T cells. These findings point to a protective mechanism in colitis distinct from regulatory CD4$$^+$$ T cells, possibly involving recruitment of regulatory CD8$$^+$$ T cells. Human studies have identified regulatory CD8$$^+$$ T cells induced by intestinal epithelial cells and MHC I b molecules, and defects in this interaction are a feature of some patients with IBD (1, 9, 52).

In the present study, we used genetic methods to examine the requirements for B cell immunoregulation in the Gα2$$^-/-$$ T cell colitis. The results revealed that B cells function primarily as class I antigen presentation cells, to induce antigen-specific CD8$$^+$$ regulatory T cells that act via a cytotoxic mechanism. These observations highlight a novel cytotoxic regulatory function of CD8$$^+$$ T cells in the attenuation of a murine model of TH1/TH17 colitis.

MATERIALS AND METHODS

Mice. B7.1/B7.2$$^-/-$$ (7), CD40$$^-/-$$ (37), TAP1$$^-/-$$ (63) (tapasin 1; MHC I), perforin$$^-/-$$ (36), RAG 1$$^-/-$$ (49), and MD4 (22) transgenic mice were obtained from The Jackson Laboratory (Bar Harbor, ME) on a C57BL/6 background. Aβ(1-24) (H-2-Ab1; MHC II) mice on the C57BL/6 background and common γ-chain (γc$$^-/-$$)/Rag-2 double knockout (DKO) mice were obtained from Taconic (Hudson, NY). Gα2$$^-/-$$ (56) mice (B6/129Sv background) were bred at the University of California, Los Angeles (UCLA) Department of Laboratory and Animal Medicine. OT-1 (30) transgenic mice on C57BL/6 RAG$$^-/-$$ background were a gift from Dr. Carrie Miceli (UCLA), and C57BL/6 CD1d$$^-/-$$ (58) mice were a gift from Dr. Mitchell Kronenberg (La Jolla Institute for Allergy and Immunology).

Mice were housed in specific pathogen-free conditions with sterilized cages, bedding, rodent chow (egg-free), and water. All RAG$$^-/-$$ recipients and B cell donors were age-matched females 6–12 wk old. Gα2$$^-/-$$ T cell donors were either male or female and were at least 6 wk old when colitis was observed and cells harvested for transfer. All procedures involving animals were performed under approved protocols of the UCLA Animal Research Committee.

Cell isolation. Spleen cells were isolated by tissue grinding between two frosted slides, filtering, and erythrocyte lysis. To isolate small and large intestine superficial villous lymphocytes, Peyer’s patches were removed from intestinal tissues and the intestines tubes were washed and cut into 1-cm segments and then incubated with 1 mM dithiothreitol (Sigma, St. Louis, MO) in DMEM at 37°C for 40 min shaking at 220 rpm. Lymphocytes were enriched by use of a discontinuous 40%:70% Percoll gradient (MP Biomedicals, Solon, OH).

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Flow cytometry. Isolated cells (1 × 10⁶) were stained with antibodies against cell surface markers for 30 min and then washed and fixed in 200 µl PBS containing 2% paraformaldehyde. Intracellular cytokine staining was performed on cells stimulated with BD Biosciences Activation cocktail for 4–5 h according to the manufacturer’s instruction. Lymphocytes were stained with anti-mouse CD4, CD8α, CD3, etc. before the treatment with BD Biosciences Cytofix/Cytoperm reagent (BD Biosciences, San Diego, CA). Intracellular cytokine staining for several cytokines including IFN-γ, TNF-α, IL-17, IL-2, etc. was performed after fixation and permeabilization. Isotype and species matched control antibodies were used to assess nonspecific binding. Data acquisition was performed at the UCLA Jonsson Cancer Center Flow Cytometry Core Facility by use of BD FACSCalibur, and analysis was carried out via CellQuest (BD) or FCS Express 3 software (DeNovo Software, London, Ontario, Canada). Serum cytokines were measured in triplicate according to instructions for BD Bioscience’s Mouse Inflammation Cytometric Bead Array Kit.

Role of B cell costimulatory molecules and BCR repertoire. We first evaluated the requirement for B cell costimulation of T cells via B7 or CD40 in colitis immunoregulation. CD3⁺/Gai2⁻/⁻ T cells were transferred into RAG⁻/⁻ recipients, together with either WT B cells (positive control), B cells lacking both B7-1 (CD80) and B7-2 (CD86), or CD40. Recipients of only Gai2⁻/⁻ T cells served as the disease control group. When the disease control group developed clinical disease (1–2 mo after transfer), the other experimental groups were examined for intestinal T cell levels, proinflammatory cytokine production, and intestinal histology.

Flow cytometry of intestinal lymphocytes revealed expansion of large intestine CD4⁺ T cells and correspondingly low levels of the normal resident CD8⁺ cells in disease control mice (Fig. 1A, left). As expected, intestinal CD4⁺ T cells were reduced in mice cotransferred with WT B cells (Fig. 1A, right). However, CD19⁺ cells cotransferred from CD40⁻/⁻ or B7⁻/⁻ mice were equally effective in suppressing pathogenic CD4⁺ T cell expansion (Fig. 1A, middle; Table 1). Proinflammatory cytokine levels (IFN-γ, IL-6, and TNF-α, etc.) in serum were elevated in mice receiving T cells only but reduced levels in mice receiving WT or CD40⁻/⁻ B cells (Fig. 1B). Histological examination demonstrated severe inflammation in the large intestine of Gai2⁻/⁻ T cells only recipients but not in those cotransferred with either CD40⁻/⁻ or WT MLN B cells (Fig. 1C). Histological scores from mice receiving either WT, CD40⁻/⁻, or B7.1/B7.2⁻/⁻ B cells were statistically lower than T cell controls (P ≤ 0.05) (Fig. 1D, Table 2). These findings indicate that B cell immunoregulation did not require expression of costimulatory molecules (CD80 and CD86, or CD40) that are typically required for B cell interaction with CD4⁺ T cells.

The sufficiency of B cells to regulate disease without costimulatory molecules [such as CD40, which plays a central role in T cell-mediated B cell activation and antibody production (54)] suggested that B cells may not regulate colitis through production of cognate immunoglobulin. This is a notable negative, since secretory IgG and IgA production shapes the intestinal microbiota (18) and contributes to colitis resistance in some settings (47, 53, 60). An addition role for membrane immunoglobulin is critical for efficient B cell anti-
gen capture and antigen presentation (29). To address whether an antigen-specific B cell response was necessary for protection, we evaluated the protective proficiency of MLN B cells from BCR transgenic MD4 mice, which lack detectable native cognate B cell responses to endogenous antigens (10). CD4+ T cell levels (Table 1), histological scores (Table 2), and intestinal cytokine staining (data not shown) revealed that MD4 B cells were proficient in colitis protection. This finding indicates that humoral immunity and BCR antigen capture do not play a role in B cell immunoregulation.

Role of antigen presentation. Since BCR-mediated signals were not required to maintain protective function, we further delineated the role of antigen presentation as a B cell trait in colitis protection. On the one hand, we wondered whether B cell antigen presentation to CD4+ T cells might redirect their fate away from TH1 or TH17 differentiation or promote CD4+ regulatory T cell (Treg) formation. On the other hand, B cell antigen presentation to natural killer (NK) T (NKT) or CD8+ T cells might confer colitis protection, since both cell types selectively expanded in the context of B cell protection (67). To address these questions, B cells from mice deficient in different MHC genes (CD1−/−, TAP1−/−, ABB−/−) mice were cotransferred with Gα2−/− T cells (Table 1), production of proinflammatory cytokines (Fig. 2A), and large intestine histology (Table 2). Similarly, B cells deficient in both MHC I and MHC II (abb/β2M−/− mice) were also incompetent for colitis protection (66).

These results suggested that MHC I-mediated antigen presentation was an important protective trait on B cells. To further exclude the possible effects of cytolytic NK cells and other cell types in our cotransfer model, we performed Gα2−/− T cells and TAP−1−/− MLN B cells cotransfer into recipient common γ-chain (γc−/−)/Rag−2−/− DKO mice. These recipients lack T cells, B cells, and other immune cells because of the absence of functional receptors for cytokines including IL-2, IL-4, IL-7, IL-9, and IL-15 (12). As shown in Fig. 2C, TAP−1−/− MLN B cells failed to protect colitogenic T cell induced mucosal inflammation in common γ-chain and Rag-2 double knockout mice. Histological scoring results indicated significant difference between TAP−1−/− and WT MLN B cells in colitis protection (Fig. 2D). Taken together, these findings implicate peptide-restricted MHC I antigen presentation as a critical trait required for B cell colitis protection.

Role of TCR specificity and cytolytic competence of CD8+ T cells in colitis protection. The preceding findings indicated that conventional CD8+ T cells were a direct target of B cells in colitis protection. We therefore turned to the CD8+ T cell compartment to evaluate traits required for their role in immunoregulation. With regard to TCR specificity, peptide/MHC I B cell antigen presentation implied that protective regulation involved selection by B cells of CD8+ T cells through their native, cognate TCR. Accordingly, we tested CD8+ T cells from OT-1 mice, which are genetically constrained to a TCR for the irrelevant ovalbumin xenoantigen (Fig. 3). As expected, WT (B6) CD8+ T cells reduced histological inflammation (Fig. 3A), infiltrating CD4+ T cells (Fig. 3B), and proinflammatory CD4+ T cells (Fig. 3C) compared with the disease control (mice without donor B cells). However, OT-1 CD8+ T cells failed to induce colitis protection by these three criteria. These findings indicate that a native cognate TCR repertoire is indeed required for protective competence of CD8+ T cells.

Several effector traits might account for the protective function of CD8+ T cells: cytolsis of target host cells, suppression or death signaling via CD95 or other cell-cell interaction molecules, or competition with CD4+ T cells for growth/survival cytokines like IL-7 or IL-15. As a simple focal point, we evaluated Prf1−/− mice, which are deficient in cytolytic function, to test whether this trait was required for protective activity.

Compared with WT (B6) CD8+ T cells, Prf1−/− CD8+ T cells were indeed deficient in reducing large intestine histological inflammation (Fig. 3A), CD4+ T cell infiltration (Fig. 3B), and levels of proinflammatory CD4+ T cells (Fig. 3C). Thus cytolytic competence is a key trait of CD8+ T cells in colitis protection. However, it leaves open the possibility that other

Table 1. Summary of large intestine CD4+ T cells

<table>
<thead>
<tr>
<th>Molecule</th>
<th>CD3+ Gα2−/− T</th>
<th>CD3+ Gα2−/− T</th>
<th>CD3+ Gα2−/− T</th>
</tr>
</thead>
<tbody>
<tr>
<td>KO CD19+</td>
<td>KO CD19+</td>
<td>KO CD19+</td>
<td>KO CD19+</td>
</tr>
<tr>
<td>MLN B</td>
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<td>WT CD19+</td>
<td>WT CD19+</td>
</tr>
<tr>
<td>No B Cells</td>
<td>No B Cells</td>
<td>No B Cells</td>
<td>No B Cells</td>
</tr>
<tr>
<td>B7/1/B7.2</td>
<td>45.7±6.2*</td>
<td>61.3±4.0*</td>
<td>78.1±4.2*</td>
</tr>
<tr>
<td>CD40</td>
<td>27.7±3.0*</td>
<td>46.8±7.2*</td>
<td>71.7±4.1*</td>
</tr>
<tr>
<td>MHCI</td>
<td>77.6±1.9*</td>
<td>75.7±3.7*</td>
<td>85.8±2.8*</td>
</tr>
<tr>
<td>TAP-1</td>
<td>85.4±3.8</td>
<td>63.6±5.1*</td>
<td>90.1±1.8</td>
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<tr>
<td>CD1d</td>
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<td>65.6±2.3*</td>
<td>80.1±1.9</td>
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<tr>
<td>MD4</td>
<td>26.1±4.3*</td>
<td>33.6±6.5*</td>
<td>70.5±0.1</td>
</tr>
</tbody>
</table>

The percentage (mean ± SE) of large intestine mucosal CD4+ T cells was tabulated by using lymphocyte scatter gate and CD3+ gate. Each value was compiled from 2 independent experiments and 6–8 total animals in each group. *P ≤ 0.05 compared with disease-positive control (Gα2−/− T cells alone).

Table 2. Summary of large intestine inflammation

<table>
<thead>
<tr>
<th>Molecule</th>
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<th>CD3+ Gα2−/− T</th>
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<tbody>
<tr>
<td>KO CD19+</td>
<td>KO CD19+</td>
<td>KO CD19+</td>
<td>KO CD19+</td>
</tr>
<tr>
<td>MLN B</td>
<td>WT CD19+</td>
<td>WT CD19+</td>
<td>WT CD19+</td>
</tr>
<tr>
<td>No B Cells</td>
<td>No B Cells</td>
<td>No B Cells</td>
<td>No B Cells</td>
</tr>
<tr>
<td>B7/1/B7.2</td>
<td>1.6</td>
<td>1.4</td>
<td>6.4</td>
</tr>
<tr>
<td>CD40</td>
<td>0.66</td>
<td>1.4</td>
<td>6.4</td>
</tr>
<tr>
<td>MHCI</td>
<td>1.8</td>
<td>4.5</td>
<td>5.3</td>
</tr>
<tr>
<td>TAP-1</td>
<td>5.2</td>
<td>1.7</td>
<td>3.9</td>
</tr>
<tr>
<td>CD1</td>
<td>2.7</td>
<td>1.7</td>
<td>6.4</td>
</tr>
<tr>
<td>MD4</td>
<td>2.1</td>
<td>2.1</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Mean histological score for the large intestine was tabulated in all cotransfer groups evaluating B cell traits. Data were collected on the same animals described in Table 1.
functional traits of these cells may also contribute to their regulatory function (see DISCUSSION).

Expression of Gqi2 itself for the functional capabilities of both B (16, 26, 51) and T cells (33, 68), in part because of the critical role of Gqi2 in trafficking mediated by certain chemokine receptors (15, 26). We previously reported that Gqi2−/− CD8+ T cells confer colitis protection in the presence of WT but not Gqi2−/− B cells (67). Extending this finding, CD8+ T cells from Gqi2−/− mice failed to protect in the absence of B cells, WT (B6) CD8+ T cells were protective even in the absence of B cells (Fig. 3). This suggests that WT (but not Gqi2−/−) B cells constitutively induce protective CD8+ T cell in normal mice and that the lack of induction can be rapidly restored when WT B cells are provided in the cotransfer setting.

DISCUSSION

B cells and CD8+ T cells can promote immunoregulation, and in some experimental systems appear to serve complementary roles. The present study evaluates the traits of B cells and CD8+ T cells involved in the immunoregulation of immune colitis. We demonstrate that B cells only need to be competent for peptide-MHC I antigen presentation; neither major costimulatory molecules nor BCR antigen recognition (or cognate immunoglobulin production) is required. CD8+ T cells require both native cognate TCR function and cytolytic capacity, indicating that their regulatory activity is mediated by immune recognition and cytotoxicity targeting a host immune population. These findings, and the comparison of WT and Gqi2−/− mice, suggest that efficient induction of regulatory CD8+ T cells requires direct B cell antigen presentation and may be an ongoing role of B cells in the immunocompetent host.

The regulatory competence of B cells deficient in costimulatory molecules was surprising. CD40 ligation by CD154 (CD40L) of activated T cells is important for programming many aspects of T-B interactions, particularly regarding B cell activation and differentiation (54). Secretory immunoglobulin, whose formation is largely CD40 dependent, is an important factor in controlling enteric microbiota and the immunological impact of their products (39, 42, 72) and has been implicated in B cell regulation of immune colitis (45). In the present study, MD4 B cells, which are deficient in native cognate antibody formation, were also competent for colitis protection. These two findings demonstrate that native antibody production is not an important factor in the role of B cells in this experimental system.

CD80 and CD86 are particularly important for costimulation of CD4+ T cells, a role that is important for naive CD4+ or CD8+ T cells but may be less predominant for activation of
regulatory CD4+ T cells and memory or effector CD4+ or CD8+ T cells (17, 28). Accordingly, it would appear that B cell protection did not involve interaction with naïve T cells but rather preserves the interaction with memory CD4+ or CD8+ T, whose activation may be facilitated by other costimulatory molecules. The preservation of B cell protection in the absence of costimulatory molecules and MHC II, and the lack of a numerical increase in CD4+ Tregs during B cell protection, made it unlikely that their role involved induction of this regulatory population (4).

In studies of colitis regulation by B cells in the TCR-α−/− mouse, genetic experiments also indicated that B cells did not directly interact with CD4+ T cells (MHC II independent) but involved CD1 and costimulatory molecules (CD86 and CD40) (47, 48). The costimulatory findings in part may have reflected experimental design with blocking antibodies, which also would have blocked interactions directly required by colitogenic CD4+ T cells. The requirement for CD1 was genetically confirmed to be B cell autonomous. This difference from the present study may have been related to the TCR-α−/− system itself, which involves novel effector and regulatory TCR-α β+ T cells (61) and CD4+ NKT cells (21) that may be skewed to CD1-dependent immunoregulation observed in other settings of colitis and inflammatory diseases augmented by B cells (13, 20, 23, 45, 47, 59).

In the present system, the exclusive B cell phenotype associated with colitis protection was competence for peptide-MHC1 antigen presentation: dependence on B cell-autonomous expression of TAP (this study) and β2-microglobulin (66). Prior studies of B cell immunoregulation have described a requirement for MHC Ia (4) and certain MHC Ib molecules (38, 50, 62), the latter also implicated in other tissue-selective immunological functions (27). Among these, MR1-dependent invariant T cells are notable because they are highly localized to the intestine, and have strict enteric microbial and B cell requirements. However, this particular T cell population is not likely to be involved in the present findings, since it appears to recognize nonpeptide lipoglycans (27, 38).

B cells and other APCs are competent for direct MHC I cross-presentation of exogenous or endogenous antigens to CD8+ T cells (14, 32, 55, 64). The present study suggests that B cells also present sampled or dendritic cell (DC)-transferred antigen (69), probably gathered from donor microflora, to cotransferred to CD8+ T cells. An important issue will be to determine the pertinent MHC I isomers and the structure and source of antigenic molecules presented by B cells to regulatory CD8+ T cells (e.g., enteric microbiota) or products of cells in response to metabolic or inflammatory stress (64).

The effectiveness of B cells in eliciting CD8+ T cell immunoregulation is unexpected. If B cells are simply serving as APCs, why are native APCs in RAG−/− recipients unable to fulfill this antigen presentation requirement? Dendritic cells in RAG−/− recipients, although not deficient in homing, are reduced in numbers, are less potent in allogeneic T cell stim-
ulation, and express lower MHC and costimulatory molecule levels than WT mice (57). In the present experimental system, the large number of transferred B cells may become predominant for APC function over the impaired resident RAG<sup>−/−</sup> DC compartment.

This preferential APC role by B cells is also observed in murine γ-herpesvirus latency, in which efficient generation of antiviral CD8<sup>+</sup> T cells involves a BCR-independent process requiring presentation of virally encoded antigen (43). Similarly, antibody and BCR-independent function of B cells is also required for efficient CD8<sup>+</sup> T cell clearance of persistent (but not acute) lymphocyte choriomeningitis virus infection, although this appears to also involve their role in anti-viral CD4<sup>+</sup> T cell generation (31). As in the present study, the striking efficiency of B cells in the generation of CD8<sup>+</sup> T cells, above and beyond other resident antigen-presenting populations, was noted, but its mechanistic basis was uncertain.

Expression of Goi2 itself is important in the functional capabilities of B cells (16, 26, 51), owing in part to the critical role of Goi2 in trafficking mediated by certain chemokine receptors (15, 26). Goi2<sup>−/−</sup> B cells are deficient in the capacity to elicit colitis protection by CD8<sup>+</sup> T cells (67). Confirming and extending this finding, the present study demonstrates that immunoregulation by CD8<sup>+</sup> T cells from Goi2<sup>−/−</sup> mice required the addition of WT B cells, whereas WT (B6) CD8<sup>+</sup> T cells were protective even in the absence of B cells. Others have demonstrated similar immunoregulatory competence of CD8<sup>+</sup> T cells in WT mice (44). This suggests that WT (but not Goi2<sup>−/−</sup>) B cells constitutively induce protective CD8<sup>+</sup> T cells in normal mice and that the lack of induction can be rapidly restored when WT B cells are provided in the cotransfer setting. Thus the Goi2<sup>−/−</sup> context provides a unique opportunity to uncover and delineate the mechanisms required for differentiation of immunoregulatory CD8<sup>+</sup> T cells.

The central finding of this study is the robust immunoregulation of colitis conferred by CD8<sup>+</sup> T cells, a function dependent on their expression of a native cognate TCR repertoire and their cytolytic competence. CD8<sup>+</sup> T cell immunoregulation has a notable history (11), mediated through cytokine-mediated suppression (64) or direct cytosis of B cells (50), T cells (35), or APCs (5, 25, 41, 71). Regulatory CD8<sup>+</sup> T cells are inducible by selected APCs in models of experimental autoimmune encephalomyelitis (19, 40) and pulmonary interstitial fibrosis (73). In human IBD, regulatory CD8<sup>+</sup> T cells dependent on MHC Ib interaction with epithelial cells have been identified (1, 52), and defects in this interaction are a feature of patients with IBD (9, 52). As in CD4<sup>+</sup> Treg biology, the specific antigen or antigen-bearing cellular target of CD8<sup>+</sup> T cell immunoregulation is incompletely defined. Clarification of these targets, and the relative role of regulatory CD4<sup>+</sup> and CD8<sup>+</sup> T cells at different stages of an immune response, will be critical to understand immunoregulatory pathogenesis and its therapeutic manipulation.

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GRANTS

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