Immunoregulatory Functions for Murine Intraepithelial Lymphocytes: γ/δ T Cell Receptor-positive (TCR$^+$) T Cells Abrogate Oral Tolerance, while α/β TCR$^+$ T Cells Provide B Cell Help

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Summary

Past work has shown that a subset of effector T cells with unique characteristics could abrogate hapten- or antigen-induced tolerance, and the reconstitution of this immune response has been termed contrasuppression. We have studied contrasuppression in a model of oral tolerance (OT) in which adoptively transferred antigen-specific T contrasuppressor (Tcs) cells reverse OT and result in antibody responses to the eliciting antigen. In the present study, we show that murine intraepithelial lymphocytes (IELs) from mice orally immunized with sheep red blood cells (SRBC) contain T cells that exhibit Tcs cell activity. This effect was mediated by CD3$^+$ γ/δ T cell receptor-positive (TCR$^+$), but not α/β TCR$^+$ T cells, and γ/δ TCR$^+$ Tcs cells were associated with both the CD4$^-$,CD8$^+$ and CD4$^-$,CD8$^-$ (double-negative) IEL fractions. The CD4$^-$,CD8$^+$ γ/δ TCR$^+$ IELs were further separated into *Vicia villosa*-adherent and -nonadherent fractions. Adoptive transfer of *V. villosa*-adherent γ/δ TCR$^+$ T cells to mice with OT to SRBC resulted in splenic IgA, IgM, and IgG subclass anti-SRBC responses, while *V. villosa*-nonadherent γ/δ TCR$^+$ T cells were without activity. The γ/δ TCR$^+$ IELs did not support in vitro antibody responses in B cell cultures, while α/β TCR$^+$ IELs were effective T helper cells. Further, cytokine production by the γ/δ TCR$^+$ IELs was examined, and the γ/δ TCR$^+$ *V. villosa*-adherent fraction, which possessed contrasuppressor function, contained low levels of IL-5 mRNA and small numbers of IL-5-producing cells when compared with α/β TCR$^+$ IELs and *V. villosa*-nonadherent γ/δ TCR$^+$ IELs. Our results now show that mouse IELs contain two distinct types of T cells that function in the immune response, e.g., α/β TCR$^+$ T cells that produce IL-5 and function as helper cells, and γ/δ TCR$^+$ T cells that restore antibody responses in mice that had been orally tolerized with antigen.

An important feature of the mucosal immune system in the gastrointestinal (GI)[1] tract is the distinct lymphoid aggregates, the Peyer's patches (PP), which are major IgA inductive sites. These differ from the effector regions, which include the lamina propria, where large numbers of CD4$^+$ T cells and IgA-producing plasma cells are present (reviewed in reference 1). Less well understood effector sites include the lymphocyte population, which resides in the epithelial layer, and the intraepithelial lymphocytes (IELs), which first encounter the myriad of antigens that are present in the GI tract. It is important to better understand the function of lymphocytes in these effector regions, since >10^{10} Ig-producing cells occur per meter of human intestine and one of every six cells in the epithelium is an IEL (1, 2). These IELs possess a number of unique features that are distinct from lymphocytes residing in other lymphoid tissues, including their phenotype of surface markers, use of heterodimeric chains of TCR, and biologic functions.

Generally, 80–90% of mononuclear cells isolated from the

[1] *Abbreviations used in this paper:* DN, double negative; DP, double positive; ELISPOT, Enzyme-linked immunospot; GI, gastrointestinal; IEL, intraepithelial lymphocyte; OT, oral tolerance; PFC, plaque-forming cell; PP, Peyer's patch; SFC, spot-forming cell; Tcs, T contrasuppressor.

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mouse intestinal epithelium express CD3 and an associated TCR, and ~75% of these are $CD4^-,CD8^+$ (3, 4). Other subsets include $CD4^+,CD8^+$ (double positive; DP), $CD4^+,CD8^-$ and $CD4^-,CD8^-$ (double negative; DN) in approximately equal frequencies (5, 6). In mice, a high frequency of $CD3^+$ IELs use the TCR composed of a γ chain disulfide linked to the δ chain (γ/δ TCR) (3, 4). Thus, the present study and the work of others have shown that 45–65% of $CD4^-,CD8^+$ IELs use the γ/δ TCR, while 35–55% of this subset express α/β TCR (7, 8). The IEL compartment contains a subpopulation of T cells that develop in the intestinal epithelium itself without thymic influence (9–11). Specifically, the γ/δ TCR-bearing T cells in IELs have been shown to be of extrathymic origin (9, 10). Further, more recent work has shown that these $CD8^+$ γ/δ TCR^+ IELs express the homodimeric form (α/α) of the CD8 molecule, are derived from bone marrow, and are distinct from a subpopulation of α/β TCR^+ T cells in IELs. In this regard, α/β TCR^+ IELs express heterodimeric α/β CD8 chains, are of thymic origin, and are recent emigrants from the PP (11). In addition, the DN subset, representing ~8% of $CD3^+$ IELs, exclusively use the γ/δ TCR, while DP and $CD4^+,CD8^-$ IELs only express the α/β TCR (8). Recent studies have shown that the TCR^+ T cells in IELs are encoded by the $V_{\gamma 7}$ and $V_{\delta 1}$ (or $V_{\delta 4}$) genes, which are distinct from the γ/δ TCR^+ cells in other tissues (3, 4, 7, 12). Most studies to date have associated IELs with cytotoxic functions, in part due to their predominant expression of CD8 (13). In this regard, IELs with cytotoxic activity were induced both in vivo and in vitro by immunization with allogeneic antigens (14–16). Further, IELs contain subsets with natural killer function and spontaneous cytotoxic activity (17, 18). Based upon immune, physiologic, and anatomical considerations, the IELs are continuously exposed to environmental antigens from the gut lumen but also are present above the lamina propria regions, where large numbers of plasma cells produce Igs, largely of the IgA isotype (1). Thus, IELs are in sentinel locations to potentially serve as regulatory cells for mucosal immune responses.

Our studies have focused on immunoregulatory functions for both α/β TCR^+ and γ/δ TCR^+ T cells in IELs. We have used a model that determines the ability of different T cell subsets to abrogate a state of systemic unresponsiveness, termed oral tolerance (OT), which is induced by the repeated oral administration of T-dependent antigens. Thus, adoptive transfer of antigen-specific T contrasuppressor (Tcs) cells to recipients, rendered tolerant to the same antigen, results in restoration of systemic antibody responses (6, 19–23). In the present study, we have directly isolated the γ/δ TCR^+ T cells from both $CD4^-,CD8^+$ and DN IEL subsets of mice orally immunized with SRBC and compared them with α/β TCR^+ IELs for their regulatory functions in abrogation of OT and for help in in vitro antibody responses. Our results are discussed from the standpoint that IEL T cells function in the immune response. Further, γ/δ TCR^+ IEL T cells exhibit an important potential for the maintenance of appropriate mucosal antibody responses in the presence of OT and mediate these antigen-specific regulatory functions in mucosal effector regions where antigens are normally encountered from the environment.

Materials and Methods

Mice. C3H/HeN mice were obtained from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD) and were maintained in horizontal flow cabinets and provided sterile food and water ad libitum. All mice used in this study were between 8 and 12 wk of age.

Induction and Abrogation of Oral Tolerance. SRBC or horse (H)RBC (Colorado Serum Co., Denver, CO) were washed in HBSS and resuspended to 50% in eight parts HBSS and two parts sodium bicarbonate (6, 19–23). Mice were orally tolerized by the administration of this suspension (0.25 ml) daily via gastric intubation for 28 consecutive days (6, 19–23). To study immunoregulatory functions of IELs, mice were orally primed with SRBC or HRBC by daily gastric intubation for three consecutive days (6, 21). In some studies, T cells were isolated from IELs of mice orally immunized with SRBC for 28 d. As controls, T cells were obtained from spleens of mice orally immunized with SRBC for 3 or 28 d. 1 wk later, mice were killed and different subsets of IELs were obtained as described below. Individual IEL T cell fractions (5×10^4 to 1×10^6 cells) were adoptively transferred intravenously to mice that had been orally tolerized with SRBC (6, 19–23). These mice were then immunized by the intraperitoneal injection of 0.1 ml of a 20% suspension of SRBC. After 4 d, the mice were killed, their spleens removed, and the number and isotype of the anti-SRBC plaque forming cells (PFC) determined as described below.

Isolation of IELs and Spleen Cells. IELs were isolated by a protocol described previously (6, 8, 24). Briefly, the small intestines were obtained from four to five mice and the mesenteric lymph nodes, connective tissues, and PP were carefully removed. The intestines were then opened longitudinally, cut into short segments (~1 cm), and washed extensively in RPMI 1640 containing sodium bicarbonate, nonessential amino acids, sodium pyruvate, L-glutamine, penicillin, streptomycin, and gentamicin (incomplete medium) containing 2% FCS (6, 8, 24). To obtain the IELs, the tissue was first incubated in incomplete medium for 30 min at 37°C with stirring, and then transferred to a 50-ml centrifuge tube, shaken vigorously for 15 s, and the medium containing cells was removed. This process was repeated two additional times. The harvested cells were pooled, washed, resuspended in incomplete medium containing 5% FCS, and then passed through a small column of cotton and loosely packed glass wool to remove dead cells and tissue debris. Discontinuous density gradients were prepared using Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden), in 15-ml centrifuge tubes by layering from the bottom 75% (2 ml) and then 40% (2 ml) Percoll. Approximately $3–4 \times 10^7$ cells in 3 ml medium were layered onto the gradient and centrifuged at 25°C for 20 min at 600 *g*. The interface between the 75% and 40% layers contained lymphocytes with a cell viability of >95%. Approximately $5–7 \times 10^6$ lymphocytes/mouse were obtained from these gradients. Spleens were aseptically removed and single cell suspensions were prepared by gently teasing these tissues through sterile stainless steel screens into incomplete medium. Clumps were removed by settling at 1 *g*, and the cell-rich supernatant was removed, washed in incomplete medium, and resuspended in appropriate medium (6, 8, 19–24).

Purification of IEL T Cell Subsets by Magnetic-activated Cell Sorting (MACS) and FACS® IELs were resuspended in warm (37°C) in-

complete medium with 5% FCS and washed four times in this medium. To remove adherent cells, the washed cells were resuspended to 5 × 10^6 cells/ml, added to tissue culture plates, and incubated for 3 h at 37°C. The nonadherent cells were >99% viable, and purified $CD3^+$ or $CD8^+$ T cells were obtained with the MACS system (Stefan Miltenyi Biotec GmbH, Mortzfeld Bergisch-Gladbach, Germany) by using anti-CD3 mAb (145-2C11; 25) or anti-CD8 antibody (53.6-72; 26) (6, 8, 24). This procedure resulted in a preparation containing >99% viable $CD3^+$ T cells. For the isolation of γ/δ TCR^+ or α/β TCR^+ T cells, the $CD3^+$ cell fraction was incubated with FITC-conjugated anti-γ/δ TCR (UC7-13D5; 27) and biotinylated anti-α/β TCR (H57-597; 28) mAbs followed by avidin-PE and then subjected to FACS® sorting (FACStar$^{Plus®}$; Becton Dickinson & Co., Sunnyvale, CA). In some experiments, $CD3^+$ IELs were incubated with biotinylated anti-CD8 (53.6-72; 26) and with FITC-anti-γ/δ TCR (UC7-13D5; 27) or FITC-anti-α/β TCR (H57-597; 28) mAbs followed by avidin-PE to obtain the $CD3^+$,$CD8^+$ γ/δ TCR^+ or $CD3^+$,$CD8^+$ α/β TCR^+ cell subsets. To obtain $CD3^+$ DN T cells, IELs were reacted with FITC-anti-CD3 (145-2C11; 25) and arsanilic acid (ARS)-conjugated anti-CD4 (GK 1.5; 29) and ARS-anti-CD8 (53.6-72; 26) mAbs followed by PE-conjugated anti-ARS before FACS® sorting. These cells were then incubated with biotinylated anti-γ/δ TCR (UC7-13D5; 27) or anti-α/β TCR (H57-597; 28) mAbs followed by an avidin-PE-Texas red tandem (avidin-tandem) to determine the nature of the TCR on $CD3^+$ DN IELs. Further, an aliquot of $CD3^+$,$CD8^+$ T cells were stained with FITC-anti-γ/δ TCR (UC7-13D5; 27) and biotinylated *Vicia villosa* lectin (E. Y. Laboratories, San Mateo, CA) followed by avidin-PE and then subjected to cell sorting by using a FACStar$^{Plus®}$. These FACS® separations allowed a purity of >99% for each of the IEL T cell subsets.

In Vitro Immune Responses. Spleen cells were prepared from mice orally tolerized with SRBC. Spleen cells were resuspended in complete MEM (Gibco Laboratories, Grand Island, NY) containing 2-ME (5 × 10^{-5} M) (20–22). The cells were cultured (5 × 10^6/0.5 ml/well) in 24-well macroculture plates (Linbro Chemical Co., Hamden, CT) either in the presence or absence of murine rIL-5 (5 U/ml; 30, 31) and were immunized with SRBC (2 × 10^6/well). In addition, some wells containing spleen cells from mice rendered orally tolerant to SRBC received γ/δ TCR^+ IEL T cells (5 × 10^4/well) isolated from mice orally primed with SRBC (20–22) (see above). In some experiments, splenic B cell cultures were prepared from normal mice for the assessment of Th cell function. Spleens were removed from mice that had received 0.1 ml of undiluted goat anti-thymocyte serum (Calbiochem-Behring Co., La Jolla, CA) by intravenous injection 2 d previously (31). Single spleen cells were then treated with a monoclonal anti-T cell cocktail consisting of anti-CD4 (GK1.5; 29), anti-CD8 (53.6-72; 26), and anti-Thy-1.2 (30-H12; 26) for 30 min at 4°C followed by incubation with monoclonal anti-rat κ chain (MAR 18.5; 32), then incubated for 30 min at 37°C with a 1/10 diluted baby rabbit complement (Pel-Freeze Biologicals, Rogers, AR) (31). Cells obtained by this procedure were >96% sIg^+ (31). Different subsets of T cells (1–2 × 10^6/well) were then added to individual B cell cultures (5 × 10^6 cells) containing SRBC (1–2 × 10^6 cells/well). These cultures were incubated for 5 d at 37°C in an atmosphere of 7% O_2, 10% CO_2, and 83% N_2 (20–22). Nonadherent cells were removed from in vitro cultures and the number, and isotype of the anti-SRBC PFC were determined as described below.

PFC Assay. Single cell suspensions from the spleens of immunized mice or in vitro cultures were washed and resuspended in HBSS before assay. Spleen cell suspensions were assayed in triplicate for IgM, IgG1, IgG2b, and IgA anti-SRBC or -HRBC PFC responses using the modified Jerne-Nordin slide method as previously described (6, 19–23). Our past studies have shown that no SRBC-specific IgG2a or IgG3 subclass responses occur in this adoptive transfer system (6, 19–23).

Enumeration of Cytokine-producing Cells by Enzyme-linked Immunospot (ELISPOT) Assay. For the quantitation of IFN-γ- or IL-5-producing cells in IELs, the cytokine-specific ELISPOT assay was used (8, 24). In this assay, a 96-well plate with a nitrocellulose base (Millititer HA; Millipore Corp., Bedford, MA) was coated with mAb anti-IFN-γ (R4-6A2; 33) and/or anti-IL-5 (TRFK-5; 34), as previously described (8, 24). Different subsets of IELs (10^3 to 10^5 cells/well) in complete medium (incomplete medium containing 10% FCS) were added to individual wells. After incubation for 20 h, the plates were washed and then incubated with biotinylated mAb anti-IFN-γ (XMG 1.2; 35) or biotin-anti-IL-5 (TRFK-4; 34) followed by avidin-peroxidase and the substrate 3-amino-9-ethyl-carbazole. The spots were counted with the aide of a dissecting microscope. To estimate the frequency of T cells that produce either IFN-γ or IL-5 or both (termed double cytokine producers), nitrocellulose plates were coated with both TRFK-5 (34) and R4-6A2 (33). Different subsets of IELs were added and spot-forming cells (SFC) developed with either biotinylated TRFK-4 (34) or XMG 1.2 (35), or with both antibodies together (24). The percentage of double producer cells was calculated from the following formula (24): 100× [(total calculated − actual total)/(actual total)]; where total calculated = the SFC enumerated by anti-IFN-γ only plus the SFC enumerated by anti-IL-5 only, and actual total = the SFC enumerated by both anti-IFN-γ and anti-IL-5. Cycloheximide treatment of T cells reduced by 85–90% the numbers of cytokine-specific SFC, clearly showing the de novo synthesis of these cytokines.

mRNA-cDNA Dot Blot Analysis. The cDNA probe for murine IFN-γ (Clontech Laboratories, Inc., Palo Alto, CA) was used according to the conditions recommended by the suppliers. The cDNA for the IL-5 PstI fragment was also used in this study (36) and the IL-5 cDNA was isolated from *Escherichia coli* transformed with the plasmid pcD-SRa-5 as described (37). Standard mRNA-cDNA dot blot hybridization was used (8, 38). Briefly, subsets of purified IELs were lysed by NP-40 (5%) buffer. After removal of cell nuclei and debris by centrifugation, the supernatants were mixed with SSC buffer in the presence of 7.5% formaldehyde and incubated for 15 min at 65°C. An appropriate dilution range of mRNA was dotted and immobilized on nitrocellulose paper using a Manifold® device (Schleicher & Schuell, Inc., Dassel, Germany). Pre- and actual hybridization of filters were performed as described by others (39). The ^{32}P-labeled denatured cDNA was added and incubated at 42°C for 24–48 h. Filters were washed with 2× SSC followed by 0.1× SSC buffer containing SDS and were then exposed to x-ray films using an intensifier screen. Developed films were scanned using a scanner (Apple Computer, Inc., Cupertino, CA) to quantitate the level of hybridization. To insure the quality of mRNA isolated from IELs, the same filters were hybridized with an actin-specific cDNA probe.

Statistics. The results of PFC and SFC responses were expressed as the mean ± SD. The significance of the difference in means was determined by the student's t test.

Results

γ/δ TCR^+ IELs Abrogate Oral Tolerance. To directly test whether γ/δ TCR-bearing T cells in IELs possess an immunoregulatory function (e.g., contrasuppression), purified

$CD3^+$ IELs from mice orally primed with SRBC for 3 d were separated into γ/δ TCR^+ and α/β TCR^+ fractions by FACS® using the anti-γ/δ TCR (UC7-13D5) and anti-α/β TCR (H57-597) mAbs. The FACS® profile revealed two distinct nonoverlapping populations of $CD3^+$ γ/δ TCR^+ and α/β TCR^+ T cell subsets in IELs (Fig. 1 *A*). Thus, according to the FACS® profile, 100% pure γ/δ TCR^+ or α/β TCR^+ T cells were separated and used in this experiment. When purified $CD3^+$ γ/δ TCR^+ or α/β TCR^+ T cells were adoptively transferred to mice orally tolerized with SRBC, conversion of OT to IgM, IgG1, IgG2b, and IgA responses was only seen in mice that received γ/δ TCR^+ cells and the homologous antigen (Fig. 1 *B*). On the other hand, the α/β TCR^+ T cell fraction did not abrogate OT. These results are the first direct proof that T cells that use the γ/δ TCR function in antigen-specific immunoregulation, e.g., these T cells mediate contrasuppression. Further, γ/δ TCR^+ T cells isolated from IELs of mice orally immunized with SRBC for 28 d also abrogate OT (see below). To ensure the antigen specificity of Tcs cells in IELs, $CD3^+$ T cells isolated from mice orally primed with either SRBC or HRBC were adoptively transferred to mice that were orally tolerant to SRBC. Abrogation of OT to SRBC-specific immune responses was only achieved by the transfer of $CD3^+$ T cells from SRBC-primed mice, but not from mice that were primed with HRBC (Fig. 1 *B*). Further, $CD3^+$ γ/δ TCR^+ IELs from normal mice did not abrogate OT. These findings confirmed our previous observations that Tcs cells, which abrogate OT, possess antigen-specificity (6, 18–20).

Both $CD4^-$,$CD8^+$ and DN γ/δ TCR^+ T Cell Subsets Possess Contrasuppressor Function. Based on the studies of others and our own work (6–8), γ/δ TCR^+ T cells are associated with the $CD4^-$,$CD8^+$ and DN IEL fractions. Thus, $CD4^-$,$CD8^+$ γ/δ TCR^+ or DN γ/δ TCR^+ IELs were isolated from SRBC orally primed and normal mice, and their ability to abrogate OT was examined (Fig. 2, *A* and *B*). When $CD3^+$,$CD8^+$ IELs were stained for γ/δ TCR expression, 45–65% of the ~4 × 10^6 $CD3^+$,$CD8^+$ IEL T cells (per mouse) were γ/δ TCR^+, while 35–55% were α/β TCR^+ (Fig. 2 *A*, *left*). When the converse experiment was performed by using mAb anti-γ/δ TCR, 35–55% of $CD3^+$,$CD8^+$ IELs were α/β TCR^+ (Fig. 2 *A*, *right*), while the remaining 45–65% of $CD3^+$,$CD8^+$ IELs did not express this receptor. FACS® analysis of $CD3^+$, DN IELs from SRBC orally primed and normal mice revealed that 100% of the cells were γ/δ TCR^+ (Fig. 2 *B*). This fraction represents ~8% of ~5.5 × 10^6 IEL $CD3^+$ T cells per mouse. γ/δ TCR^+ cells were not found in the $CD4^+$,$CD8^-$ and DP fractions; however, these cells did express α/β TCR (data not shown). These results showed that γ/δ TCR^+ T cells are only found in the $CD4^-$,$CD8^+$ and DN fractions of the IELs from SRBC orally primed and normal mice (7, 8).

When these subsets of γ/δ TCR^+ IELs were examined for contrasuppressor activity, the $CD3^+$,$CD8^+$ γ/δ TCR^+ T cell fraction converted OT to IgM, IgG1, IgG2b, and IgA anti-SRBC PFC responses upon adoptive transfer (Fig. 2 *C*). In contrast, the $CD8^+$ α/β TCR^+ T cell fraction did not reverse OT to SRBC-specific immune responses. In addition, the adoptive transfer of γ/δ TCR^+, DN T cells in IELs from SRBC orally primed mice also resulted in conversion

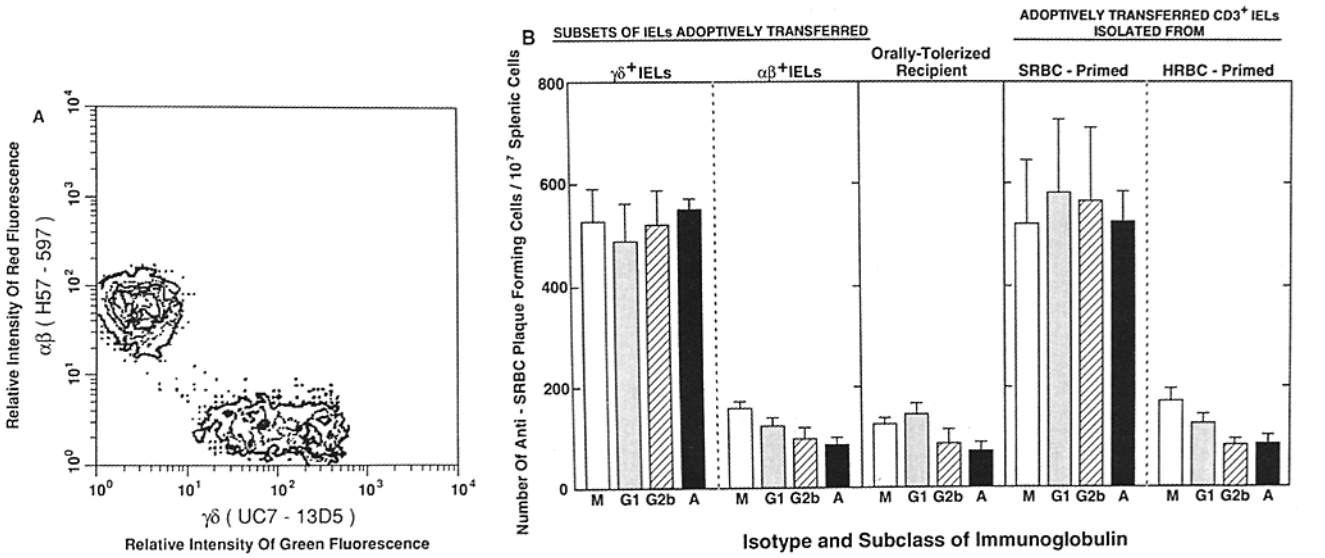


Figure 1. $CD3^+$ γ/δ TCR^+ IELs from SRBC orally primed mice abrogate OT to antigen-specific IgM, IgG1, IgG2b, and IgA responses. Purified $CD3^+$ IELs were incubated with FITC-anti-γ/δ TCR (UC7-13D5) and biotinylated anti-α/β TCR (H57-597) followed by avidin-PE and then subjected to analysis by FACStar^{Plus}® (*A*). FACS-separated γ/δ TCR^+ and α/β TCR^+ T cells (10^6 cells) were adoptively transferred to SRBC orally tolerized mice and these mice were then systemically challenged with SRBC. Splenic anti-SRBC of IgM (□), IgG1 (▨), IgG2b (▨), and IgA (■) isotypes were assessed 4 d later (*B*). In separate experiments, $CD3^+$ IELs were isolated from SRBC or HRBC orally primed mice and then adoptively transferred to SRBC orally tolerized mice. These mice were systemically immunized with SRBC, and IgM, IgG1, IgG2b, and IgA responses were assessed in an identical manner (*B*).

of OT to anti-SRBC PFC responses (Fig. 2 *C*). These findings provide direct proof that γ/δ TCR$^+$ T cells in the CD4$^-$,CD8$^+$ and DN IEL subsets possess contrasuppressor function. Since the major fraction of γ/δ TCR$^+$ T cells resided in the CD4$^-$,CD8$^+$ T cell subset of IELs, functional studies of this subset were performed as described below.

γ/δ TCR$^+$ IELs Mediate Contrasuppression while α/β TCR$^+$ Cells Exhibit Helper Function. Since γ/δ TCR$^+$ IELs from SRBC orally primed mice support antigen-specific immune responses in OT, it was important to determine if these γ/δ TCR IELs possess Th cell function. In this regard, γ/δ TCR$^+$ and α/β TCR$^+$ IELs, which represent 55% and 45% of 5.5 × 10^6 CD3$^+$ IEL T cells (per mouse), were isolated from orally primed mice and then cultured with purified splenic B cells from normal mice in the presence of accessory cells and antigen. As positive controls, CD4$^+$ Th cells were isolated from spleens of mice orally primed with SRBC and added to identical B cell cultures. The splenic CD4$^+$ α/β TCR$^+$ T cells enhanced IgG1 and IgA SRBC-specific immune responses (Fig. 3). Of interest was the finding that α/β TCR$^+$ IELs also provided significant Th cell function for IgG subclass and IgA responses. Enriched, α/β TCR$^+$ T cells from IELs always supported higher in vitro PFC responses than comparable splenic Th cells (Fig. 3). The α/β TCR$^+$ IELs and α/β TCR$^+$ CD4$^+$ splenic T cells from nonimmunized mice did not support any SRBC-specific B cell responses. We also assessed the α/β TCR$^+$ T cell subset for cytokine production, and this fraction contained a high message for IL-5 in addition to IFN-γ (see Fig. 5 *B*) and separate Northern analysis showed that full-length IL-5 mRNA was present. Further, the α/β TCR$^+$ subset contained both IFN-γ- and IL-5-producing cells. Independent analysis showed that >90% of cells producing these two cytokines produced either IFN-γ or IL-5, but not both (manuscript in preparation). On the other hand, γ/δ TCR$^+$ IELs that possess contrasuppressor function did not support any SRBC-specific responses in B cell cultures (Fig. 3). These results show that α/β TCR$^+$ IELs exhibit significant helper functions when compared with γ/δ TCR$^+$ IELs (Table 1).

V. villosa–adherent γ/δ TCR$^+$ IELs Contain Both Tcs Cells and Low Numbers of IL-5-producing Cells. A distinct feature of Tcs cells is their high affinity for the lectin *V. villosa* (reviewed in reference 40). When CD8$^+$ γ/δ TCR$^+$ IELs were incubated with biotin-conjugated *V. villosa* followed by avidin-PE, ~10–15% of 2.2 × 10^6 CD8$^+$ γ/δ TCR$^+$ IELs (per mouse) bound to this lectin (Fig. 4 *A*). When contrasuppressor function was examined in these two subsets of γ/δ TCR$^+$ IELs from mice orally primed with SRBC for 3 d, the *V. villosa*–adherent fraction abrogated OT to IgM, IgG1, IgG2b, and IgA anti-SRBC PFC responses (Fig. 4 *B*). On the other hand, the *V. villosa*–nonadherent subset did not possess contrasuppressor function. In the next experiment, γ/δ TCR$^+$, *V. villosa*–adherent and –nonadherent T cells were isolated from IELs and spleens of mice given SRBC for 28 d by the oral route; separate experiments with mice from this group showed that the animals were unresponsive to the antigen. Each T cell subset from IELs or spleen of these mice was then adoptively transferred to syngenic mice rendered orally tolerant. Only the γ/δ TCR$^+$, *V. villosa*–adherent T cells from IELs abrogated OT (Fig. 4 *C*). On the other hand, γ/δ TCR$^+$, *V. villosa*–nonadherent IEL T cells as well as *V. villosa*–adherent and –nonadherent splenic T cells from the same mice did not convert OT to immune responses (Fig. 4 *C*). These findings showed that γ/δ TCR$^+$, *V. villosa*–adherent T cells in IELs are the only T cell fraction that was capable of abrogating OT. Further, this would suggest that these γ/δ TCR$^+$ IEL T cells maintain appropriate IgA responses at mucosal effector sites in the presence of systemic unresponsiveness.

The frequency of IFN-γ- and IL-5-producing cells were also assessed in *V. villosa*–adherent and –nonadherent γ/δ TCR$^+$ fractions; large numbers of IFN-γ- or IL-5-producing cells were seen in the latter subset (Fig. 5 *A*). Further, mRNA isolated from γ/δ TCR$^+$, *V. villosa*–nonadherent fraction possessed messages for these two cytokines, most notably for IL-5 (Fig. 5 *B*). On the other hand, the γ/δ TCR$^+$, *V. villosa*–adherent fraction contained a lower number of IL-5-producing cells (Fig. 5 *A*). The mRNA-cDNA dot-blot hybridization also revealed a low level of IL-5-specific message (Fig. 5 *B*). In addition, our study showed that exogenous IL-5 was not sufficient to reverse antigen-specific unresponsiveness in cultures containing spleen cells from OT mice (Table 1).

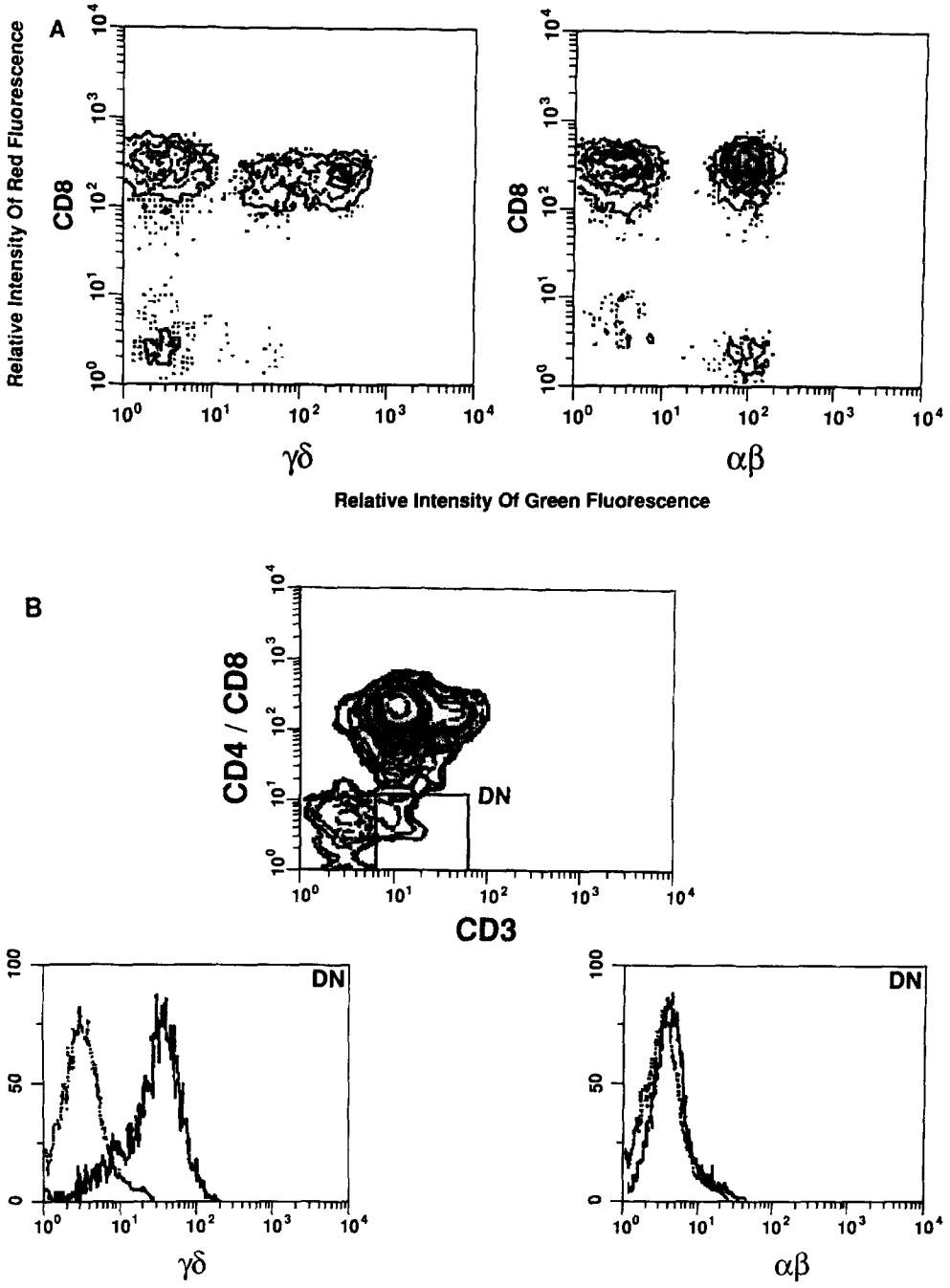
Table 1. *Unique Characteristics of α/β TCR$^+$ IELs with Helper Function and V. villosa–Adherent γ/δ TCR$^+$ IELs that Abrogate OT*

Characteristics	T cells and rmIL-5 tested (IgA PFC/culture and IL-5 SFC/10^4 cells)		
	γ/δ TCR	α/β TCR	rmIL-5
Abrogate OT*	582 ± 64	43 ± 5	36 ± 12
Helper function for B cells‡	26 ± 7	912 ± 63	31 ± 3
IL-5 production§	45 ± 7	198 ± 17	N/A

* Splenic cells (5 × 10^6/well) from SRBC orally tolerized mice were cocultured with either γ/δ TCR$^+$ or α/β TCR$^+$ IELs (5 × 10^4/well) from mice orally primed with SRBC in the presence of SRBC. The other wells were cocultured with rmIL-5 (5 U/ml) and SRBC. After 5 d of incubation, cells were harvested and SRBC-specific IgA responses were determined by PFC. Spleen cell cultures containing SRBC alone provided 35 ± 9 IgA anti-SRBC PFC.

‡ Splenic B cells (5.0 × 10^6/well) from normal mice were cocultured with either γ/δ TCR$^+$ or α/β TCR$^+$ IELs (10^6/well) from mice orally primed with SRBC in the presence of SRBC. The other wells were cocultured with rmIL-5 (5 U/ml) and SRBC. After 5 d, IgA responses were determined by PFC as described above. Spleen B cell cultures alone containing SRBC provided 24 ± 10 IgA anti-SRBC PFC.

§ γ/δ TCR$^+$ and α/β TCR$^+$ cells were isolated from IELs of SRBC orally primed mice by FACS® and then subjected to IL-5-specific ELISPOT assay.



Although numbers of cytokine-producing cells are low in *V. villosa*-adherent γ/δ TCR$^+$ IELs, a high percentage of those cells that produced cytokines were distinguished by their ability to secrete both IFN-γ and IL-5. Thus, these results suggest that γ/δ TCR$^+$, *V. villosa*-adherent IELs function in antigen-specific immunoregulation, but are not classical Th cells (Table 1). Taken together, these results show that γ/δ TCR$^+$ Tcs cells possess an affinity for *V. villosa*, and a smaller percentage of these cells produce cytokines when compared with the *V. villosa*-nonadherent fraction. It should also be noted that α/β TCR$^+$ IELs, which contain helper function, possess high levels of message for both IL-5 and IFN-γ. Thus, we now suggest that IELs contain a subset of T cells that provide helper function.

Discussion

One unique feature of the mucosal immune system is the maintenance of appropriate IgA responses to orally encountered antigens at mucosal surfaces in the presence of systemic unresponsiveness to these antigens, commonly termed OT. Thus, oral administration of protein or bacterial antigens simultaneously induces antigen-specific OT in systemic sites and S-IgA responses in mucosal secretions (41). To protect

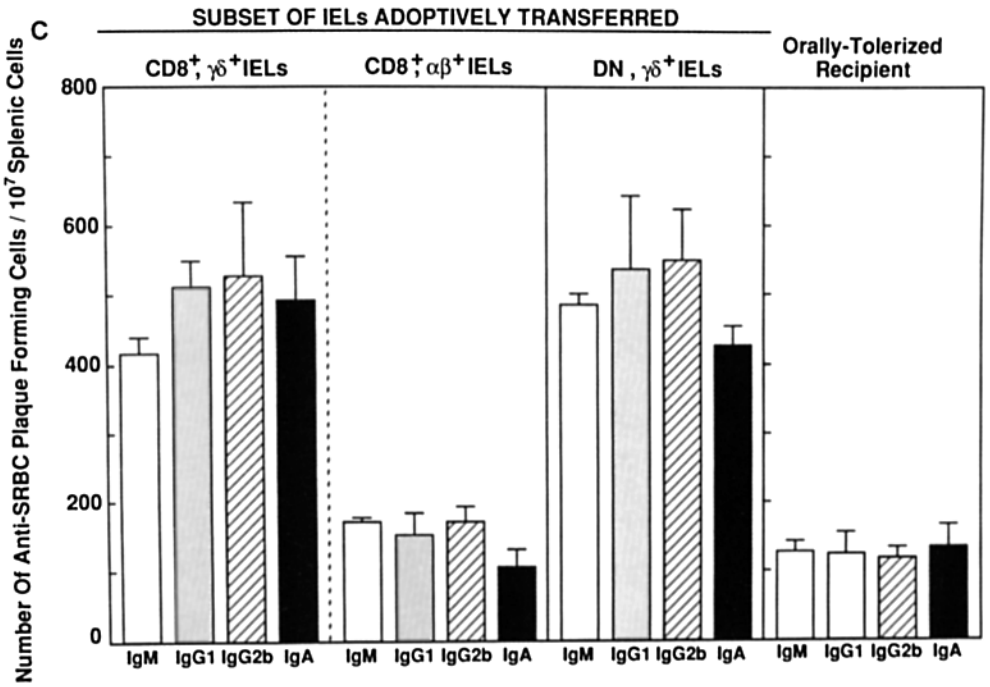


Figure 2. γδ TCR bearing CD4⁻,CD8⁺ and DN IELs possess contrasuppressor function. Purified CD3⁺ IELs, from SRBC orally primed mice were incubated with biotinylated-anti-CD8 (53.6-72) and FITC-anti-γ/δ TCR (UC7-13D5) (*A*, *left*) or anti-α/β TCR (H57-597) (*A*, *right*) followed by avidin-PE before FACS® analysis and sorting. To obtain DN T cells, IELs were incubated with FITC-anti-CD3 (145-2C11) and ARS-conjugated anti-CD4/anti-CD8 followed by PE-anti-ARS and then subjected to FACS® (*B*, *top*). Further, aliquots of DN T cells were reacted with biotinylated anti-γ/δ TCR (*B*, *bottom left*) or anti-α/β TCR (*B*, *bottom right*) followed by avidin tandem. FACS®-separated CD4⁻, CD8⁺ γ/δ TCR⁺ or α/β TCR⁺ as well as DN γ/δ TCR⁺ IELs (5 × 10⁴ cells) were adoptively transferred to SRBC orally tolerized mice intravenously, and then immunized intraperitoneally with SRBC. 4 d later, splenic IgM (□), IgG1 (⊡), IgG2b (▨), and IgA (■) anti-SRBC PFC responses were enumerated (*C*).

IgA responses at mucosal sites such as the lamina propria regions of the GI tract, where an environment of suppression normally exists, it would be important to determine if IELs contain T cells that function in the immune response, and in effect maintain this compartment for continued immune responses to orally encountered antigens. Thus, the T cells that reside in the basolateral surfaces of epithelial cells, commonly termed IELs, may provide important functions in mucosal immune responses. In this study, we tested the idea that IELs may possess unique antigen-specific functions

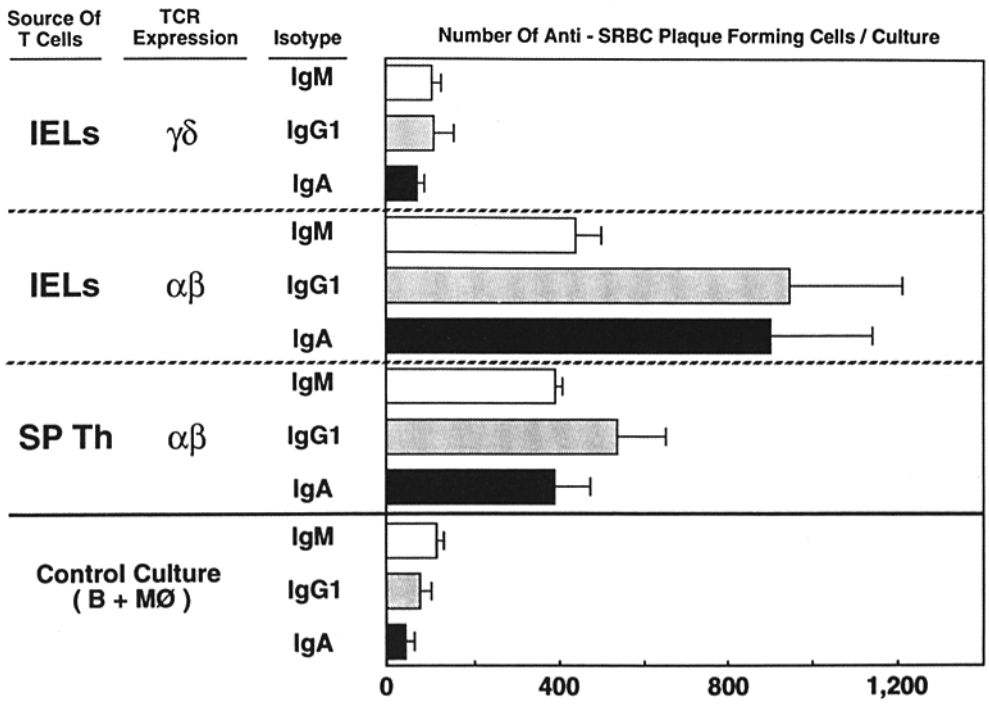
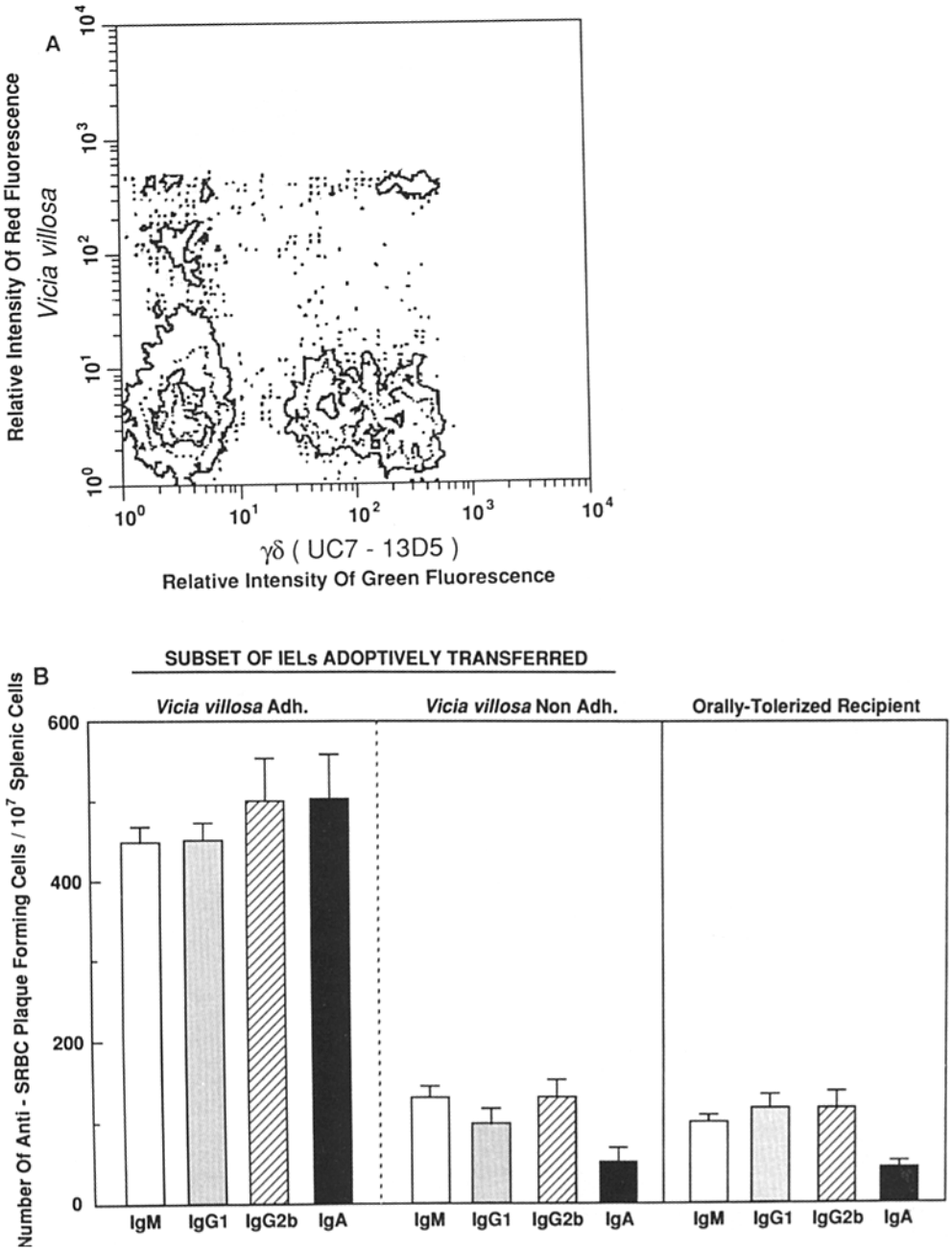


Figure 3. γ/δ TCR⁺ contrasuppressor IELs do not possess Th cell function while α/β TCR⁺ IEL fractions provide B cell help. FACS®-separated γ/δ TCR⁺ or α/β TCR⁺ IELs (10⁶ cells) from SRBC orally primed mice were incubated with purified B cells (5.0 × 10⁶) and SRBC (2 × 10⁶) for 5 d. Cells were harvested and IgM (□), IgG1 (⊡), and IgA (■), and anti-SRBC PFC responses were examined. As positive controls, splenic CD4⁺ Th cells from spleens of the same mice were used.



to either support or protect antibody responses at IgA effector sites from the influence of systemic unresponsiveness, since IELs are continuously exposed to the myriad of antigens from the gut lumen.

The present study has shown that the adoptive transfer of $CD3^+$ γ/δ TCR^+ T cells from IELs of mice orally immunized with SRBC to recipient mice that are unresponsive to this antigen results in the conversion of OT to SRBC-specific IgM, IgG1, IgG2b, and IgA responses (Figs. 1 and 4 *C*). Further, γ/δ TCR^+ T cells in both the $CD4^-$,$CD8^+$ and DN fractions of the IELs possess this contrasuppressor function. These findings are the first direct evidence that $CD3^+$,$CD4^-$,$CD8^+$, and $CD3^+$ DN T cells, which use the γ/δ heterodimer chains of the TCR, possess an antigen-specific regulatory function. Of further significance was the finding that γ/δ TCR^+ T cells from IELs can rescue antigen-specific antibody responses from the influence of systemic unresponsiveness, an effect that is especially pronounced for the IgA isotype. Further, it is generally considered that $CD4^+$ Th cells in the underlying lamina propria regions induce the terminal differentiation of B cells into IgA-producing plasma cells. In this regard, our recent studies have shown that high numbers of Th2-type $CD4^+$ T cells, which produce key cytokines such as IL-5 and IL-6 for terminal differentiation

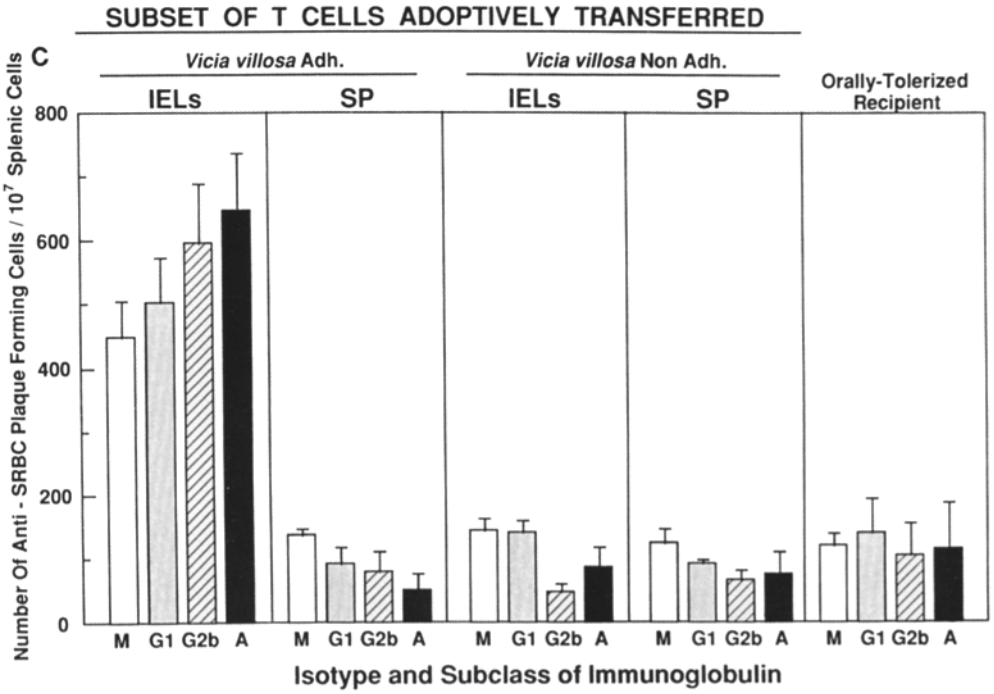


Figure 4. *V. villosa* (*V.u*)-adherent γ/δ TCR$^+$ IELs possess contrasuppressor function. Purified CD8$^+$ T cells from IELs of mice orally primed with SRBC were stained with FITC-anti-γ/δ TCR (UC7-13D5) and biotinylated anti-*V.u* followed by avidin-PE and then subjected to FACS® analysis (*A*). FACS®-separated, *V.u*-adherent and -nonadherent CD8$^+$ γ/δ TCR$^+$ IELs (5×10^4) from mice orally primed with SRBC for 3 d were adoptively transferred to mice orally tolerized with, and then immunized with, SRBC. 4 d later, splenic IgM (□), IgG1 (▨), IgG2b (▨), and IgA (■) anti-SRBC PFC responses were enumerated (*B*). Flow cytometry isolated, *V.u*-adherent and -nonadherent γ/δ TCR$^+$ T cells (5×10^4) from IEL and spleen of mice orally immunized with SRBC for 28 d were adoptively transferred to mice rendered tolerant to SRBC. These mice were then immunized with SRBC. 4 d later, splenic IgM (□), IgG1 (▨), IgG2b (▨), and IgA (■) anti-SRBC PFC responses were determined (*C*).

of surface IgA$^+$ (sIgA$^+$) B cells to become high Ig-producing cells, are seen in lamina propria regions when compared with other tissues (23, 31, 42). However, a subset of γ/δ TCR$^+$ IELs do not serve as Th cells for antibody responses, but serve as an enhancing cell type that can overcome immunologic tolerance (Table 1). Taken together, the anatomical proximity of CD8$^+$ and DN Tcs cells and CD4$^+$ Th cells at IgA effector sites would make physiological sense, since this would provide maintenance of efficient IgA responses for the protection of mucosal surfaces. Further, it is also possible that γ/δ TCR$^+$ Tcs cells in the IELs could also enhance (or protect) cytotoxic T cells from the influence of suppression, since it has been shown that a subset of IELs in human jejunum and colon do not express the cytotoxic cell-specific surface antigen H366 and exhibit suppressor function (43). However, this point remains to be established and will be addressed in future studies.

To further characterize γ/δ TCR-bearing Tcs cells in IELs, γ/δ TCR$^+$ T cells from SRBC orally primed mice were separated into *V. villosa*-adherent and -nonadherent fractions, since it has been shown that a unique feature of Tcs cells is a strong affinity for this plant lectin (40). When these two fractions of γ/δ TCR$^+$ IELs were adoptively transferred to mice with OT, only the *V. villosa*-adherent cells abrogated OT to SRBC-specific immune responses (Fig. 4 *B*). This result provided further evidence that γ/δ TCR$^+$ IELs, which share with Tcs cells the property of binding to *V. villosa*, can mediate contrasuppression. Furthermore, the γ/δ TCR$^+$ Tcs cells were also observed in the *V. villosa*-adherent fraction of mice rendered orally tolerant to SRBC (Fig. 4 *C*). This would suggest that these cells are indeed functioning in the epithelium of mice that are receiving toleragenic doses of antigen. Further, this subset of CD3$^+$ T cells in IELs may play an important role in the maintenance of IgA responses in the presence of suppression in the gut.

This characteristic of *V. villosa* binding was also important since it allowed us to separate functional Tcs cells from IELs that differed in cytokine production. Thus, when cytokine secretion and mRNA levels (e.g., IFN-γ and IL-5) by γ/δ TCR$^+$, *V. villosa*-adherent and -nonadherent cells were examined, the *V. villosa*-adherent fraction with contrasuppressor function exhibited low IL-5 levels (Fig. 5). On the other hand, the *V. villosa*-nonadherent fraction contained higher numbers of IL-5- (and IFN-γ)-producing cells. This finding does not support the idea that Tcs cells are a form of Th cells or "super" Th cells, since γ/δ TCR$^+$, *V. villosa*-adherent Tcs cells in IELs do not produce IL-5. On the contrary, Th2 type cells in IgA effector tissues (e.g., the lamina propria region of intestine) secrete significant amounts of IL-5 (24). Further, by use of Th cell assays, the γ/δ TCR$^+$ IELs from SRBC-primed mice did not support antigen-specific PFC responses, while α/β TCR$^+$ IELs and splenic CD4$^+$ Th cells from the same mice enhanced both IgG and IgA anti-SRBC responses (Fig. 3). In fact, the α/β TCR$^+$ IELs supported IgG and IgA responses that exceeded those seen with splenic CD4$^+$ Th cells (Table 1 and Fig. 3). These findings have provided important new evidence that α/β TCR$^+$ IELs are capable of providing helper function, while γ/δ TCR$^+$ IELs contain subsets capable of abrogating oral tolerance (e.g., contrasuppression).

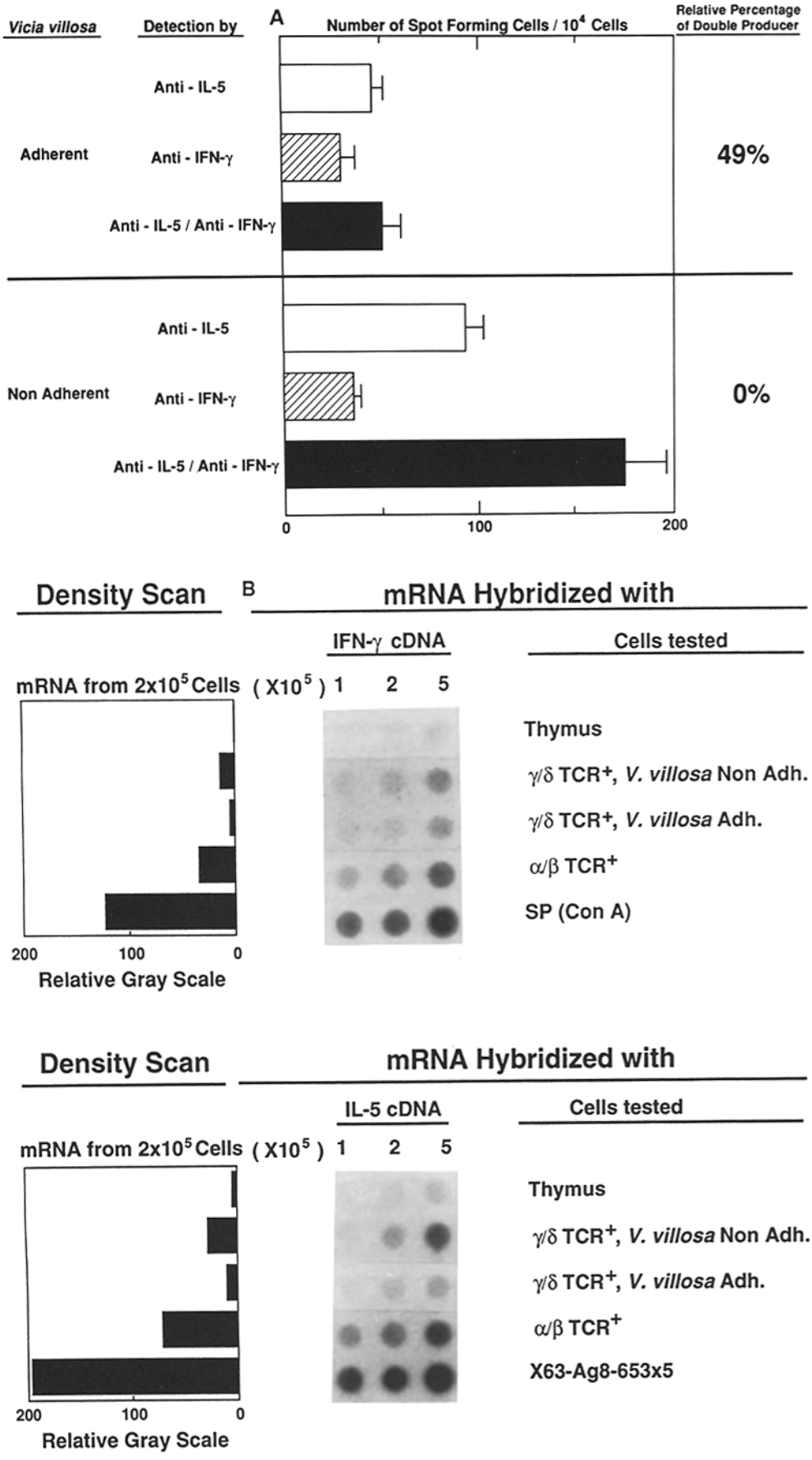


Figure 5. *V. villosa* (*V.v*)-adherent γ/δ TCR$^+$ IELs contain low levels of IL-5 message while α/β TCR$^+$ IELs possess high IL-5 expression. Purified CD8$^+$ T cells from IELs of SRBC orally primed mice were stained with FITC-anti-γ/δ TCR (UC7-13D5) and biotinylated anti-*V.v.* followed by avidin-PE and then subjected to FACS® analysis (see Fig. 4 *A*). FACS®-separated, *V.v.*-adherent and -nonadherent CD8$^+$ γ/δ TCR$^+$ IELs were subjected to IFN-γ- and IL-5-specific ELISPOT assay (*A*). Extracted mRNA from individual subsets of IELs including α/β TCR$^+$ T cells and *V.v.*-adherent and -nonadherent CD8$^+$ γ/δ TCR$^+$ T cells were hybridized with IFN-γ- and IL-5-specific cDNA probes (*B*). As positive controls, mRNA from Con A–stimulated splenic T cells or IL-5-transfected X63-Ag8-653X5 were used. mRNA extracted from freshly isolated thymocytes and nonstimulated splenic T cells were used as negative controls. Levels of hybridization were examined by scanning and expressed as relative gray scale (score of 0–200). The quality of mRNA was verified by use of an actin-specific cDNA probe.

As indicated above, it was significant that γ/δ TCR$^+$, *V. villosa*–adherent Tcs cells in IELs contained low numbers of cytokine producing cells. Further, of the *V. villosa*–adherent cells that were secreting IFN-γ and IL-5, at least 50% were shown to produce both cytokines simultaneously (double cytokine producers) (Fig. 5 *A*). This feature is clearly distinct from classical Th cell clones, which can be separated into IFN-γ producing Th1-type and IL-5 secreting Th2-type cells (44). Taken together, these results have also provided compelling evidence that γ/δ TCR$^+$ IELs with contrasuppressor function are not a form of Th cells. In this regard, our previous studies have also shown that CD3$^+$ DN Tcs cells isolated from spleens of mice orally primed with SRBC are distinct from classical Th cells (20). It should be emphasized that only small numbers (as few as 5×10^4 T cells) of Tcs cells are required for abrogation of OT upon adoptive transfer. On the other hand, adoptive transfer of Th cells into nude mice have shown that 5×10^6 to 5×10^7 T cells are required for T-dependent responses to SRBC (manuscript in preparation). Thus, we can now conclude that Tcs cells do not function as Th cells both in vitro and in vivo (Table 1).

The finding of low numbers of cytokine producing cells in γ/δ TCR$^+$, *V. villosa*–adherent T cells in IELs has raised another important issue, since it has been shown that CD4$^+$ T cells from murine PP, an important IgA inductive tissue, can be separated into two subsets based upon their ability to bind *V. villosa* (45). In this study, IL-5 production was only seen in the *V. villosa*–adherent fraction, but not in the *V. villosa*–nonadherent fraction (45). In contrast, our present study has shown that *V. villosa*–adherent Tcs cells in IELs produced little IL-5. Further, it was suggested that these *V. villosa*–adherent γ/δ TCR$^+$ cells were of major importance in contrasuppression. Our studies have also provided evidence that these cytokines cannot break tolerance since addition of an optimal concentration of rIL-5 to cultures containing splenocytes from SRBC orally tolerized mice and homologous antigen did not support IgA anti-SRBC responses (Table 1). Although we cannot yet explain the discrepancy between these two studies, the most likely explanation involves the major differences in TCR usage and in CD8 expression between these two subsets (CD8$^+$ γ/δ TCR$^+$ IEL T cells vs. CD4$^+$ α/β TCR$^+$ PP T cells), both of which exhibit an affinity for *V. villosa* lectin. Further, it is also possible that the tissue environment (e.g., IgA effector [IELs] vs. inductive [PP]) sites may result in T cell subsets (with different functions) that possess different affinity for this lectin.

It will be important to next determine the precise mechanism for the abrogation of OT by γ/δ TCR$^+$ IEL T cells. These γ/δ TCR$^+$ Tcs cells are not active cytokine (e.g., IFN-γ and IL-5)-producing cells. Thus, γ/δ TCR$^+$ Tcs cells may require direct cell-to-cell contact with CD4$^+$ Th cells in order to provide their contrasuppressor function. In this regard, it is possible that γ/δ TCR$^+$ T cells may recognize the TCR idiotype expressed by the V regions of α/β TCR on CD4$^+$ Th cells. γ/δ TCR$^+$ IELs may also interact with CD4$^+$ Th cells via the CD1 molecule since it has been shown that γ/δ T cells isolated from immunodeficient patients recognized a CD1$^+$ target T cell line (46, 47). Alternatively, γ/δ TCR$^+$ IELs may communicate with α/β TCR$^+$ CD4$^+$ T cells via a heat shock protein–like molecule expressed on activated latter cells. Further, it is possible that CD8$^+$ γ/δ TCR$^+$ T cells may interact with antigen-presenting cells, including B cells in an MHC class II–restricted manner, since a recent study has provided evidence that CD8$^+$ γ/δ TCR$^+$ T cells in IELs exhibit MHC class II–restricted antigen recognition (48). Thus, the selection of a certain family of Vγ genes (Vγ4) was always associated with a distinct haplotype of I-E expression (48). Moreover, it has been shown that antigen-specific γ/δ TCR$^+$ T cells can respond to tetanus toxoid antigen in a class II–restricted manner (49). Although the present study has provided a new and novel feature of γ/δ TCR$^+$ Tcs cells in IELs, the exact mechanisms for the abrogation of OT by this subset of regulatory T cells remains to be elucidated.

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