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Regulation of chronic colitis in athymic *nu/nu* (nude) mice

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Abstract

The objective of this study was to assess the roles of NK cells, B cells and/or intraepithelial lymphocytes (IEL) in suppressing the development of colitis in nude mice reconstituted with CD4$^+$CD45RB^{high} T cells. BALB/c nude mice were lethally irradiated and reconstituted with bone marrow from different immunodeficient mice to generate athymic chimeras devoid of one or more lymphocyte populations. Transfer of CD4$^+$C45RB^{high} T cells into chimeric recipients devoid of B cells, T cells and IEL produced severe colitis within 6–8 weeks, whereas transfer of these same T cells into B cell- and T cell-deficient or T cell-deficient chimeras produced little to no gut inflammation. In addition, we found that nude mice depleted of NK cells or RAG-1$^{-/-}$ mice reconstituted with IEL failed to develop colitis following transfer of CD45RB^{high} T cells. Severe colitis could, however, be induced in nude mice by transfer of activated/T$_h$1 CD4$^+$CD45RB^{low} T cells. Taken together, our data suggest that IEL, but not B cells or NK cells, play an important role in suppressing the development of chronic colitis in this model. In addition, our data demonstrate that suppression of disease may be due to polarization of naive CD4$^+$ cells toward a non-pathogenic and/or regulatory phenotype.

Introduction

It is well known that cell-mediated immune responses in the intestinal mucosa serve to protect the host from invading pathogens. This is true not only for pathogens of bacterial origin, but also those derived from viruses and parasites (such as helminths), as well as potentially injurious dietary antigens (1,2). Such immune responses involve an ordered series of events that insure not only the clearance of the pathogen, but also induce an immunologic 'memory' that prevents future infections by the pathogen(s). Due to the fact that the small intestine and colon are populated with innocuous bacteria collectively known as the comensal flora, it is not surprising that the gut mucosa has developed the capacity to distinguish between comensal enteric antigens and potential pathogens. Indeed, the lack of an immune response to enteric antigens in the gut strongly suggests that cell-mediated immune responses in mucosal tissues are tightly regulated. There are, however, circumstances where these regulatory processes are either absent or become dysfunctional leading to uncontrolled immune responses to normal enteric antigens, resulting in chronic gut inflammation. This dysregulated immune response to enteric antigens is best characterized experimentally by the CD4$^+$CD45RB^{high} adoptive transfer model of colitis (3).

A large body of recent work has focused on identifying the cell population(s) responsible for maintaining tolerance to enteric antigens as well as the mechanisms responsible for their development and functions (4–6). Recent data suggest that specific subsets of CD4$^+$ T cells may regulate mucosal immune responses by inhibiting effector T cell function through production of regulatory cytokines such as IL-10 and/or transforming growth factor (TGF)-β as well as through receptor–ligand interactions such as CTLA-4 (7–9). Several groups of investigators have shown that this regulatory population resides within the CD4$^+$CD25$^+$CD45RB^{low} subset of T cells, and that IL-10, TGF-β and CTLA-4 are all critical for regulatory function (7,8,10–14). Transfer of this subset either alone or in conjunction with potentially pathogenic naive CD4$^+$CD45RB^{high} cells into SCID or RAG-1$^{-/-}$ mice inhibits the development of experimental colitis (3,4,11).

Based on these studies, we hypothesized that transfer of naive CD4$^+$CD45RB^{high} T cells into T cell-deficient athymic

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nu/*nu* (nude) mice would lead to chronic colitis. However, preliminary work from our laboratory demonstrated that transfer of CD45RB^{high} T cells into specific pathogen free (SPF) nude mice did not lead to the development of colitis (15). Although nude mice lack peripheral T cells, they do retain B cells, NK cells and a unique population of T cells called intraepithelial lymphocytes (IEL) that are thought to develop in the gut in a thymus-independent manner (16–18). These observations suggest that NK cells, B cells and/or IEL may serve as additional regulatory cell populations capable of suppressing cell-mediated immune responses in the gut.

Indeed, there is evidence in the literature to suggest that NK cells and B cells serve to limit the progression and severity of disease in different models of chronic colitis (19–21). NK cells are known for lysis of target cells through a perforin-dependent mechanism. However, NK cells also exert potent immunomodulatory effects via cytokine production, most notably IFN-γ. Recent studies have identified IFN-γ as a key mediator of the inductive phase of experimental inflammatory bowel disease (IBD). Given the importance of IFN-γ in IBD, Fort *et al.* examined the possibility that NK-derived IFN-γ may drive the course of disease in colitis (19). Interestingly, the authors found that depletion of NK cells in immunodeficient RAG$^{-/-}$ hosts receiving CD4$^+$ T cells from IL-10$^{-/-}$ donors had an accelerated onset and severity of disease, suggesting that NK cells play a somewhat protective role in experimental IBD. In addition, transfer of unfractionated CD4$^+$ cells that normally do not produce colitis into NK-depleted RAG$^{-/-}$ recipients also produced a wasting IBD. Using mice deficient in both RAG and perforin (pfp$^{-/-}$/RAG$^{-/-}$) as recipients, it was determined that the protective effect of NK cells was, at least in part, due to perforin-mediated effects on the CD45RB^{high} T cells (19).

Studies by Mizoguchi in the TCR α-deficient (TCR-α$^{-/-}$) model of spontaneous colitis suggest that B cells may also play a suppressive role in experimental IBD. In these studies it was shown that, while colitic animals had significantly higher levels of autoantibodies, some of which were directed against tropomyosin, the absence of B cells led to an accelerated onset and severity of disease (20,21). The authors further showed that either reconstitution with mature B cells or injection of colitic TCR-α$^{-/-}$ × Igμ$^{-/-}$ mice with Ig from TCR-α$^{-/-}$ mice led to attenuation of disease. Together with the observation that the number of apoptotic cells in the colon of TCR-α$^{-/-}$ × Igμ$^{-/-}$ is greatly enhanced and could be reduced after treatment with Ig, the authors propose that production of autoantibodies by B cells suppresses experimental IBD via clearance of circulating self-antigens. This suggests that, within disease free wild-type mice, there exist multiple levels of immune regulation, all of which confer tolerance toward enteric antigens.

Recently, Poussier *et al.* demonstrated that IEL may play a role in regulating the development of colitis secondary to transfer of primary splenic TCRαβ$^+$CD4$^+$CD45RB^{high} T cells into SCID mice (22). The authors demonstrated that prior reconstitution of SCID recipients with TCRαβ$^+$CD4$^-$CD8α$^+$β$^-$ IEL prevented disease and did so in an IL-10-dependent fashion. In contrast, reconstitution with either TCRγδ$^+$ or TCRαβ$^+$CD4$^-$CD8α$^+$β$^+$ intestinal T cells did not prevent colitis.

Based upon these and previous studies from our laboratory, we wished to assess the potential regulatory roles of NK cells, B cells and/or IEL in the CD45RB^{high} T cell/nude transfer model.

Methods

Animals

Wild-type BALB/c and RAG-1$^{-/-}$ (on a BALB/c background) were purchased from Jackson Laboratories (Bar Harbor, ME), whereas BALB/c IL-10$^{-/-}$ mice were obtained from Dr Donna Rennick (DNAX Research Institute, Palo Alto, CA). Breeding pairs of RAG-1$^{-/-}$ and IL-10$^{-/-}$ mice were maintained at our facility. Athymic nude mice (BALB/c) were obtained from Harlan (Madison, WI) and J heavy chain-deficient ($J_H^{-/-}$; BALB/c) were obtained from Taconic (Germantown, PA). All mice were maintained under SPF conditions.

Induction of CD45RB^{high} colitis

Induction of CD45RB^{high}-mediated chronic colitis in RAG-1$^{-/-}$ animals was achieved as previously described (23,24). Briefly, spleens were removed from donor BALB/c female mice and teased into single-cell suspensions in PBS containing 4% FCS. Erythrocytes were removed by hypotonic lysis. For enrichment of CD4$^+$ T cells, the MACS system from Miltenyi Biotec (Auburn, CA) was employed for negative selection by magnetic cell sorting. Briefly, cells were incubated with FITC-conjugated anti-B220, anti-CD8 and anti-MAC-1 mAb (all from PharMingen, San Diego, CA), and subsequently labeled with anti-FITC microbeads (Miltenyi Biotec). Unlabeled cells were separated from labeled cells on a depletion column (column type CS; Miltenyi Biotec) assembled into the magnetic separator (VarioMACS; Miltenyi Biotec). Enriched CD4$^+$ T cells were labeled with biotin-conjugated anti-CD4 mAb GK1.5 followed by streptavidin–670 and phycoerythrin (PE)-conjugated anti-CD45RB mAb (PharMingen, San Diego, CA), and fractionated into CD4$^+$CD45RB^{high} and CD4$^+$CD45RB^{low} fractions by two-color sorting on a FACS Vantage (Becton Dickinson, San Jose, CA). The CD45RB^{high} and the CD45RB^{low} populations were defined as the brightest staining 40% and dullest 15% of CD4$^+$ T cells respectively, and were found to be >98% pure on post-sort analysis. Male athymic nude or RAG-1$^{-/-}$ mice on a BALB/c background at the age of 5–7 weeks (except those used in bone marrow chimera experiments; see below) were injected (i.p.) with 5 × 10^5 CD45RB^{high} T cells suspended in 500 μl of PBS. Clinical evidence of disease (e.g. body wt loss and loose stool/diarrhea) was followed and recorded weekly from the time of the injection.

Preparation of activated/T_h1 CD4$^+$ T cells

For some experiments activated/T_h1 CD4$^+$CD45RB^{low} T cells were obtained from spleens of IL-10$^{-/-}$ or colitic SCID mice that had been injected with CD4$^+$CD45RB^{high} T cells 8 weeks previously as described above. CD4$^+$CD45RB^{low} T cells from spleens of BALB/c IL-10$^{-/-}$ mice were isolated and injected (5 × 10^5 cells i.p.) into male nude recipients as described above. For other experiments, CB.17 SCID mice were reconstituted with BALB/c CD4$^+$ CD45RB^{high} T cells as described previously (23,24). At 8 weeks post-reconstitution those SCID

recipients exhibiting clinical signs of colitis (e.g. >15% weight loss, diarrhea, piloerection) were used as donors for polarized T_h1 cells. The T_h1 cells from these donors were isolated using the same procedure used for CD45RB^{high} isolation and the activated/memory CD45RB^{low} phenotype sorted by flow cytometry. After confirmation of cell viability by post-sort analysis, 5×10^5 T_h1-polarized CD45RB^{low} T cells were injected (i.p.) into male nude recipients. The CD4$^+$CD45RB^{low} T cells obtained from IL-10$^{-/-}$ or colitic SCID mice display an activated/T_h1 phenotype and will produce colitis when transferred into RAG-1$^{-/-}$ or SCID mice (4,8,25). In other experiments, nude mice were reconstituted with wild-type CD45RB^{high} T cells, and after 8 weeks were in turn used as donors of both CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells. These isolated cells were then transferred into SCID mice either alone (CD4$^+$CD25$^-$) or in conjunction with wild-type CD45RB^{high} T cells, and these recipients were monitored for the presence of colitis.

Radiation chimeras

Male nude mice were subjected to whole-body irradiation totaling 1000 rad in a split-dose manner. Mice first received 500 rad of whole-body irradiation and after 4 h received another 500 rad. During the 4-h interal between doses, whole-bone marrow from the appropriate donor mice was isolated and prepared as follows. Donor animals (e.g. male nude, RAG-1$^{-/-}$ or $J_H^{-/-}$ mice on a BALB/c background) were euthanized and the skin removed from both hind legs. Muscle was trimmed away from the leg, and the femurs and tibias removed. The femurs and tibias were flushed with sterile media through a 25-gauge needle inserted into one end of the bone. The bone marrow was collected in a sterile Petri dish and passed through another syringe to disrupt the marrow. Cells were then counted and diluted to a final concentration of 1.5×10^7 cells/ml in sterile Ca^{2+}/Mg^{2+}-free PBS. Following the second 500 rad, recipient mice received 5×10^6 donor bone marrow cells via tail vein injection and were monitored for successful engraftment. Chimeric animals were monitored (e.g. survival and presence or absence of particular cell populations using flow cytometry) for 8 weeks prior to transfer of CD45RB^{high} T cells.

NK cell depletion

Depletion of NK cells in athymic nude mice was performed according to Fort *et al.* (19). Briefly, the anti-asialo-GM_1 mAb (Wako, Richmond, VA) was reconstituted according to the manufacturer's specifications and 50 µl was injected (i.p.) on a weekly basis. Depleting antibodies or saline was given to the mice the day before cell transfer and then weekly for the duration of the experiment. Efficacy of the *in vivo* depleting mAb was determined by flow cytometric analysis of the spleens of treated mice. After red blood cell lysis, spleen cells of treated mice were stained with FITC-conjugated DX5 antibody (PharMingen).

Flow cytometry

The presence or absence of peripheral B cells and NK cells was assessed using flow cytometric analysis. Briefly, a single-cell suspension of splenic leukocytes will be prepared as described above and the leukocytes placed in a 96-well microtiter dish. Approximately 1×10^6 leukocytes were stained with PE-conjugated anti-B220 and FITC-conjugated anti-IgM (both PharMingen) for B cells following blockade of non-specific Fc receptors. Cells were analyzed by two-color flow cytometry using a FACSCalibur system (Becton Dickenson). The percentage of B220$^+$, IgM$^+$ (e.g. functional B cells) was compared to wild-type nude controls. The presence or absence of peripheral NK cells was determined using FITC-conjugated DX5 antibody (PharMingen).

Tissue lymphocyte analyses

Lymphocyte activation and proliferation was determined both peripherally (spleen) as well as locally (intestine and colon) by flow cytometry. Briefly, spleens were removed from either wild-type, nude mice or nude and RAG-1$^{-/-}$ mice reconstituted with various lymphocyte populations, and ground individually between two frosted slides into a single-cell suspension in 4% FACS buffer on ice. The suspension was then passed through a syringe and pelleted. Red blood cells were removed by hypotonic lysis, and the resulting leukocytes, pelleted resuspended in FACS buffer containing anti-Fc receptor antibody at 5×10^7 cells/ml. After incubation with anti-Fc receptor mAb, ~1×10^6 cells were placed into individual wells of a 96-well dish and pelleted. Total T cells were determined by three-color analysis using anti-CD3–FITC, CD4–allophycocyanin (APC) and CD8a–PE (PharMingen), and the degree of activation using CD3–FITC, CD4–APC and CD45RB–PE (PharMingen).

Analysis of intestinal IEL was performed as described previously (26). Briefly, small and large intestines were removed from mice flushed of luminal contents and trimmed of excess connective tissue. Each intestine was opened longitudinally and cut into small (0.5–1.0 cm) pieces in PBS on ice. Pieces were then incubated in pre-warmed (37°C) HBSS/4% FCS/1 mM DTT on a rotating shaker for 45–60 min at 37°C and vortexed. The contents were passed through a sieve and the remaining pieces of intestine incubated again in HBSS/4% FCS/1 mM DTT. The supernatants from the incubations (containing IEL) were pooled and pelleted, resuspended in HBSS/FCS, and passed through a syringe prior to passage over glass wool to remove debris. The cell suspension was then pelleted, resuspended in 40% Percoll and centrifuged on a 40/70% Percoll gradient, and IEL collected from the 40/70% interface. IEL were washed, resuspended in FACS buffer containing anti-Fc receptor mAb and counted. Approximately 5×10^5 cells were placed in individual wells and stained for using anti-CD3–FITC, CD4–APC and CD8a–PE (PharMingen) as well as CD3–FITC, CD4–APC and CD45RB–PE (PharMingen) cocktails prior to analysis on the FACSCalibur (BD Biosciences).

IEL transfer

IEL suspensions were obtained from small intestines as described above. After washing and incubation with anti-FcR mAb, IEL were incubated in FITC-labeled anti-CD8 antibody (PharMingen) and sorted on the FACS Vantage (BD Bioscience). Approximately 5×10^6 CD8$^+$ IEL were injected into RAG-1$^{-/-}$ recipients via the tail vein. One week following IEL transfer, mice were reconstituted with 5×10^5 CD45RB^{high} T cells.

Cytokine mRNA determinations

Colons were removed, cleaned of fecal material and placed in RNA Later stabilization solution (Ambion, Austin, TX) and stored at –20°C until processing. Total RNA was extracted and isolated from tissue in each experimental group using a standard protocol with TRIzol reagent (Gibco/BRL, Grand Island, NY). RNA was then resuspended in RNase-free water and stored at –80°C until analysis. RNA isolates were quantified and examined for integrity using the RNA 6000 Nano LabChip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). After analysis, 250 ng of total RNA from each sample was DNase treated and used in the subsequent reverse transcriptase reaction. The reverse transcriptase reaction was performed using the reverse transcriptase reagent kit according to the TaqMan Gold RT-PCR kit protocol (Applied Biosystems, Foster City, CA). Total RNA (250 ng) was reverse transcribed to cDNA in a 50-μl reaction volume. Quantitative PCR reactions for murine IL-10, TNF-α and IFN-γ were performed using pre-developed probes and primers in the ABI Prism 7700 sequence detector system (PE Biosystems, Foster City, CA). Using the Gene Expression Protocol Revision B (Applied Biosystems, Foster City, CA), DNA amplification was performed in 25 μl of 2 × TaqMan universal PCR Master Mix, 5 μl of cDNA, 17.5 μl RNase-free water and 2.5 μl of 20 × Target Mix. Thermal cycling conditions were as follows: 50°C hold for 2 min, 95°C hold for 10 min, and 45 cycles of 95°C for 15 s and 60°C for 1 min. In separate wells, 18S rRNA was amplified simultaneously with the gene of interest as an internal control for normalization of the gene of interest. Each sample, both target and 18S control, was analyzed using triplicate reverse transcriptase and duplicate no reverse transcriptase reactions. Samples were analyzed using ABI software and the fold expression calculated using the comparative $2^{-\Delta\Delta C_T}$ method described by Livak and Schmittgen (27).

Macroscopic and histological analyses

At 8 weeks following T cell reconstitution, mice were euthanized, and the colons removed, cleaned of fecal material and scored for macroscopic evidence of inflammation using a modification of the method described by Conner *et al.* (28). Briefly, normal colonic morphology was assessed a score of 0; mild bowel wall thickening in the absence of visible hyperemia was given a score of 1; moderate bowel wall thickening and hyperemia was given a score of 2; severe bowel wall thickening with rigidity and marked hyperemia was assigned a score of 3; and severe bowel wall thickening with rigidity, hyperemia and colonic adhesions was given a score of 4. The colons were then divided into proximal and distal sections. A small piece of each section was placed in 10% PBS formalin and stored overnight at 4°C. The fixed tissue was then rinsed with PBS, partially dehydrated in ethanol and embedded in JB-4 (Polysciences, Warrington, PA). These samples were sectioned (5 μm) using glass knives and processed for standard hematoxylin & eosin staining to evaluate the histopathology and the presence of IEL.

Scoring of histopathology was performed in a blinded manner using a modification of the method described by Aranda *et al.* (29). Briefly, eight parameters were used that include: (A) the degree of inflammatory infiltrate in the lamina propria, given a score ranging from 1 to 3; (B) the loss of Goblet cells as a marker for mucin depletion, given a score ranging from 0 to 2; (C) reactive epithelial hyperplasia/atypia with nuclear changes, given a score ranging from 0 to 3; (D) the number of IEL in the epithelial crypts, given a score ranging from 0 to 3; (E) abnormal crypt architecture (distortion/branching/atrophy/crypt loss), given a score ranging from 0 to 3; (F) Number of crypt abscesses, given ascore ranging from 0 to 2; (G) Mucosal erosion to frank ulceration, given a score ranging from 0 to 2; and (H) submucosal spread to transmural involvement, give a score ranging from 0 to 2. The severity of the inflammatory changes in the proximal and distal colon was based on the sum of the scores reported for each parameter. IEL were identified histologically by their characteristic rounded mononuclear morphology, large nucleus:cytosol ratio and location in relation to adjacent epithelial cells (30).

Results

Athymic nude mice are resistant to CD45RB^{high}-mediated colitis

Transfer of 5 × 10^5 CD4$^+$CD45RB^{high} T cells isolated from wild-type BALB/c spleens into RAG-1$^{-/-}$ mice induced a pancolitis within 6–8 weeks, whereas transfer of this same T cell population into nude mice did not induce chronic gut inflammation (24,31,32). Gross inspection of colons from reconstituted RAG-1$^{-/-}$ mice revealed hyperemia, bowel wall thickening and adhesions, as well as increases in weight: length ratios of the colon 8 weeks following reconstitution, whereas colons from nude mice appeared normal (Fig. 1A). Histological inspection of colons from RAG-1$^{-/-}$ mice demonstrated massive transmural leukocyte infiltration, epithelial cell erosions, crypt abscesses, goblet cell drop out and bowel wall thickening, whereas colons from nude mice appeared histologically normal. Quantitative histopathological analysis of each colon showed a dramatic and significant increase in histologic colon scores in RAG-1$^{-/-}$ versus nude mice (Fig. 1B). Using real-time PCR to quantify mRNA for the different cytokines, we analyzed colons from both colitic RAG-1$^{-/-}$ and nude mice reconstituted with CD45RB^{high} T cells. We found that colitis induced in RAG$^{-/-}$ mice was associated with enhanced expression of TNF-α and IFN-γ mRNA compared to unmanipulated RAG-1$^{-/-}$, unmanipulated nude or reconstituted nude mice (Fig. 2A and B). We observed a significant increase in mRNA for IL-10, and a corresponding decrease in message levels for both TNF-α and IFN-γ in the colons of CD45RB^{high} reconstituted nude mice compared to the RAG-1$^{-/-}$ recipients (Fig. 2A and B). In addition, we found that unmanipulated nude and RAG-1$^{-/-}$ exhibited comparable levels of each cytokine examined, suggesting that constitutive overexpression of IL-10 in nude mice is not the reason for lack of disease initiation. Using splenic T cells as an index of systemic T cell activation, we observed a shift from predominantly naive (CD45RB^{high}) in wild-type mice to an activated (CD45RB^{low}) phenotype in T cells obtained from RAG-1$^{-/-}$ mice reconstituted with CD45RB^{high} T cells (Fig. 3). Interestingly, the few CD4$^+$ cells present in unmanipulated nude mice displayed a CD45RB distribution similar to that of wild-type, i.e. only

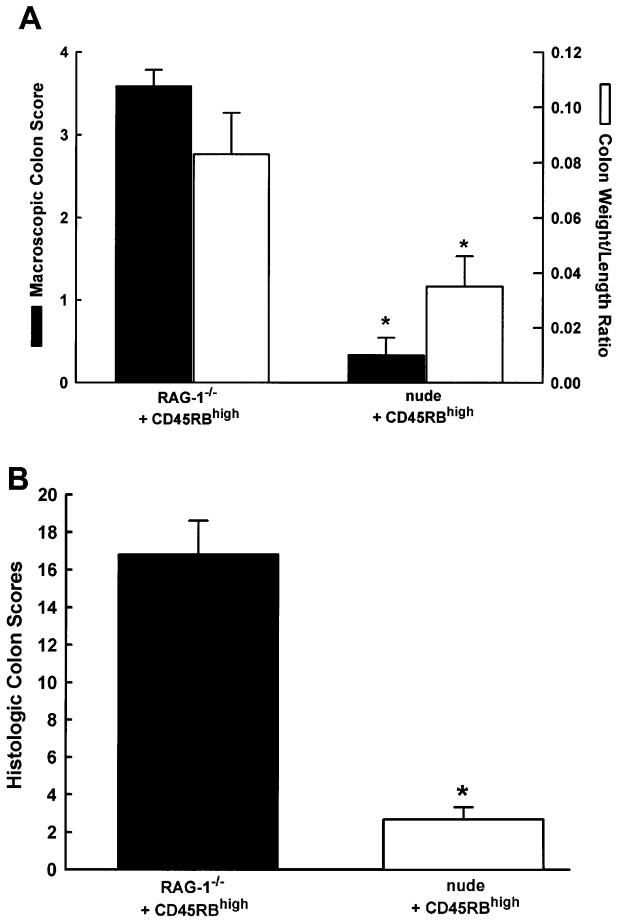

Fig. 1. Macroscopic colon scores and colon weight:length ratios 8 weeks following reconstitution of RAG-1$^{-/-}$ or nude mice with CD45RB^{high} T cells. Macroscopic scoring was performed as described in Methods. JB4 plastic sections (5 μm) of distal colon were stained with hemotoxylin & eosin. Reconstituted nude mice exhibited significantly reduced macroscopic colon scores and colon weight:length ratios compared to RAG-1$^{-/-}$ recipients (A). (B) Blinded histologic scoring of colons for both groups. Histologic scoring criteria are described in Methods. *P < 0.05 compared to RAG-1$^{-/-}$. Data represent the mean + SE for n = 25 RAG-1$^{-/-}$ and n = 25 nude mice. The group (n = 25) of reconstituted RAG-1$^{-/-}$ mice represents a summation of one group of n = 10 which served as a colitic control group for these experiments combined with a second group (n = 15) generated for the NK cell depletion studies (Fig. 4).

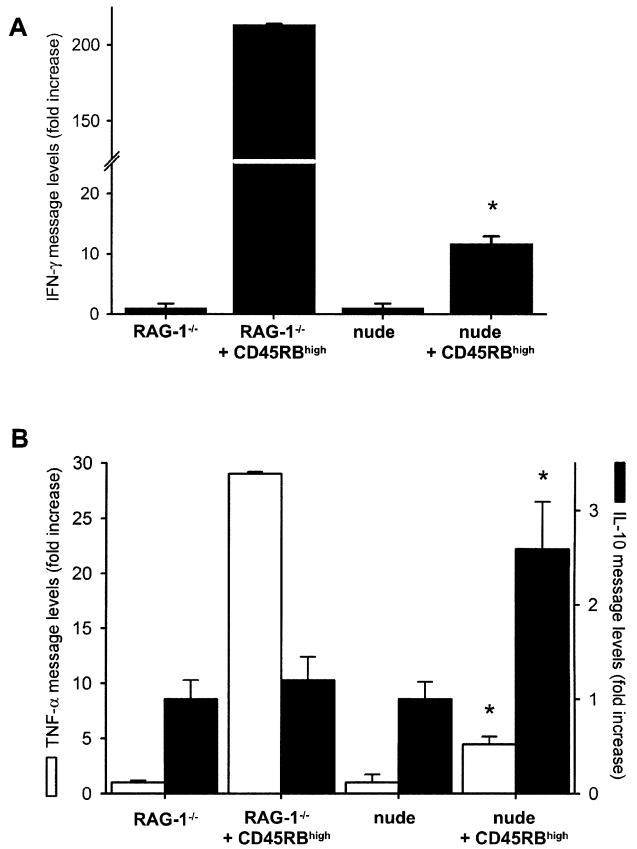

Fig. 2. IFN-γ, TNF-α and IL-10 mRNA of CD45RB^{high} reconstituted RAG-1$^{-/-}$ and nude mice. Total RNA was isolated from colons of each animal, and used for RT-PCR analysis of IFN-γ, TNF-α, IL-10 and GAPDH message levels. (A) A significant reduction in the level of IFN-γ mRNA in the colon of reconstituted nude mice compared to RAG-1$^{-/-}$ recipients. Nude mice, however, have significantly elevated levels of IL-10 mRNA in the colon compared to reconstituted RAG-1$^{-/-}$ recipients, which corresponded to a significant reduction in TNF-α mRNA (B). Data are expressed as a ratio to GAPDH (n = 8 for each group). *P < 0.01 compared to the corresponding group.

~14% of the T cells were CD45RB^{low}, whereas the percentage of CD45RB^{high} cells was similar to that of wild-type (Fig. 3; 30.4 versus 40% for nude versus wild-type respectively). However, CD45RB expression in reconstituted nude mice resembles the distribution observed in reconstituted RAG-1$^{-/-}$ recipients (Fig. 3), suggesting that the naive T cells injected into nude mice were capable of undergoing activation/polarization, yet were unable to induce disease.

Nude mice depleted of NK cells do not develop colitis

As a first step in attempting to identify the specific lymphocyte populations responsible for suppressing the development of chronic colitis in nude mice, we depleted NK cells in nude mice using weekly injections of anti-asialo-GM$_1$ antibody starting 1 day prior to CD45RB^{high} reconstitution and continuing for the duration of the experiment (8 weeks) (19). We found that this protocol resulted in the depletion of >90% of NK cells in nude mice, which was confirmed by flow cytometry using FITC-conjugated DX-5 as a pan-NK cell marker (Fig. 4A, insert). Depletion of NK cells did not, however, result in the development of CD45RB^{high}-medited colitis. Macroscopic as well as histologic scores were comparable to those of saline-injected nude controls, both of which were significantly lower than RAG-1$^{-/-}$ mice reconstituted with CD45RB^{high} T cells (Fig. 4A and B).

Nude mice depleted of B cells do not develop colitis, whereas nude mice depleted of both B cells and IEL develop severe gut inflammation

Several recent reports have suggested that B cells may act to regulate mucosal immune responses and inhibit the initiation and progression of experimental IBD (20,21). Therefore, we sought to address the regulatory role of this lymphocyte

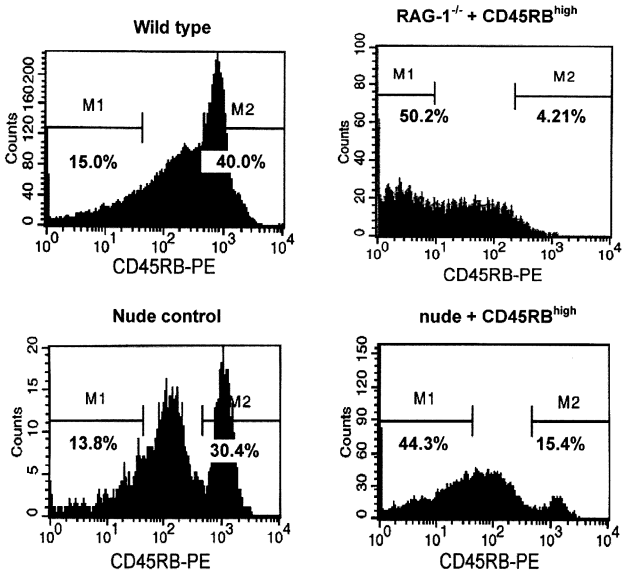

Fig. 3. Activation of peripheral T cells in reconstituted RAG-1$^{-/-}$ and nude mice. Spleen leukocytes were isolated, and stained for CD3, CD4 and CD45RB as described. Analysis by flow cytometry reveals that naive T cells injected into either RAG-1$^{-/-}$ or nude mice are able to undergo polarization into effector (RAG-1$^{-/-}$) or possible regulatory (nude) phenotypes. Representative percentages of CD45RB^{high} and CD45RB^{low} cells are given using wild-type as a standard.

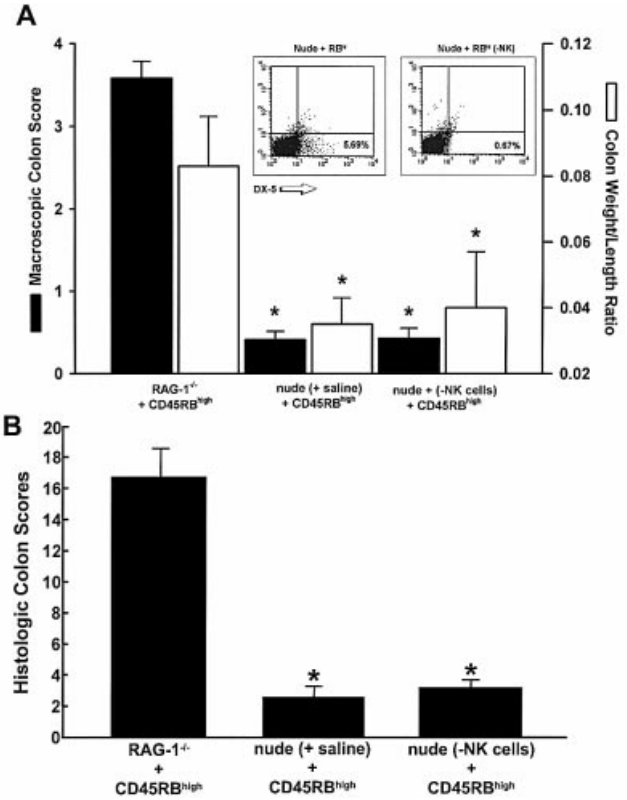

Fig. 4. Macroscopic colon scores and colon weight:length ratios 8 weeks following reconstitution of RAG-1$^{-/-}$, saline-treated nude or NK cell-depleted nude mice with CD45RB^{high} T cells. Nude mice were injected weekly with either saline or anti-asialo-GM$_1$ antibody starting 1 day prior to CD45RB^{high} reconstitution and continuing for the duration of the experiment (8 weeks). Nude recipients depleted of NK cells had significantly reduced macroscopic colon scores and weight:length ratios compared to RAG-1$^{-/-}$ recipients, but were not significantly different from saline-treated reconstituted nude mice (A). NK cell depletion was confirmed by flow cytometry using FITC-labeled anti-DX-5 antibody and is shown to be >90% (A, insert). Macroscopic scoring was performed as described in Methods. (B) Blinded histologic scoring of colons for all three groups. Histologic scoring criteria are described in Methods. *$P < 0.05$ compared to RAG-1$^{-/-}$. Data represent the mean + SE for $n = 25$ RAG-1$^{-/-}$ $n = 9$ nude + saline and $n = 15$ nude (–NK cells). The group ($n = 25$) of reconstituted RAG-1$^{-/-}$ mice represents a summation of one group of $n = 15$ which served as a colitic control group for these experiments combined with a second group ($n = 10$) generated for the nude reconstitution studies (Fig. 1).

population in CD45RB^{high} reconstituted nude mice. To do this, we rescued lethally irradiated nude mice with whole bone marrow derived from *B cell-deficient* J$_H^{-/-}$ mice, RAG-1$^{-/-}$ or nude donors 8 weeks prior to transfer of CD45RB^{high} T cells. J$_H^{-/-}$ → nude chimeras are devoid of B cells, but contain IEL, while nude → nude chimeras contain both B cells and IEL, and RAG-1$^{-/-}$ → nude chimeric mice are devoid of *both* B cells and IEL. Although we were able to deplete mature (B220$^+$/IgM$^+$) B cells by >97% (Fig. 5A, insert), J$_H^{-/-}$ → nude chimeric mice developed little or no colitis when reconstituted with CD45RB^{high}. Indeed, macroscopic colon scores, colon weight:length ratios and histology of J$_H^{-/-}$ → nude colons were very similar to nude → nude chimeras (Fig. 5A and B). When both B cells and IEL were depleted via the generation of RAG-1$^{-/-}$ → nude chimeras, severe colitis developed at 8 weeks following reconstitution with CD45RB^{high} T cells (Fig. 5A and B). Histological inspection of RAG-1$^{-/-}$ → nude chimeras revealed transmural leukocyte infiltration, epithelial cell erosions, goblet cell depletion and bowel wall thickening. Quantification of the histopathology demonstrated large and significant increases in histologic colon scores in the RAG-1$^{-/-}$ → nude chimeric mice compared to J$_H^{-/-}$ → nude or nude → nude chimeras (Fig. 5B).

Resistance to CD45RB^{high} T cell-mediated colitis is associated with the presence of IEL

Histologic analyses of small intestine and colon from each group of mice demonstrated that induction of disease occurred in mice which were devoid of gut-associated IEL (e.g. SCID, RAG$^{-/-}$ and RAG$^{-/-}$ → nude chimeras), whereas resistance to colitis was always associated with the presence of small intestine and colonic IEL (e.g. nude, NK-depleted nude, nude → nude and J$_H^{-/-}$ → nude chimeras; data not shown). Indeed, analysis of the small intestine and colon IEL subsets in RAG-1$^{-/-}$ revealed no CD3$^+$CD4$^+$ or CD3$^+$CD8$^+$ IEL, whereas unmanipulated nude mice contained predominantly (~70%) CD8$^+$ IEL in the small intestine and ~37% in the colon, although at about one-third the total of wild-type mice. Reconstitution of RAG-1$^{-/-}$ mice with CD4$^+$CD45RB^{high} T cells resulted in approximately equal amounts of CD4$^+$ and CD8$^+$ IEL (Fig. 6). In the large bowel there was a shift favoring CD4$^+$ versus CD8$^+$ IEL, more so in the reconstituted RAG-1$^{-/-}$ compared to wild-type (Fig. 6). Reconstitution of nude mice with CD4$^+$CD45RB^{high} T cells showed an increase in the percentage of CD4$^+$CD3$^+$ IEL and a concomitant decrease in

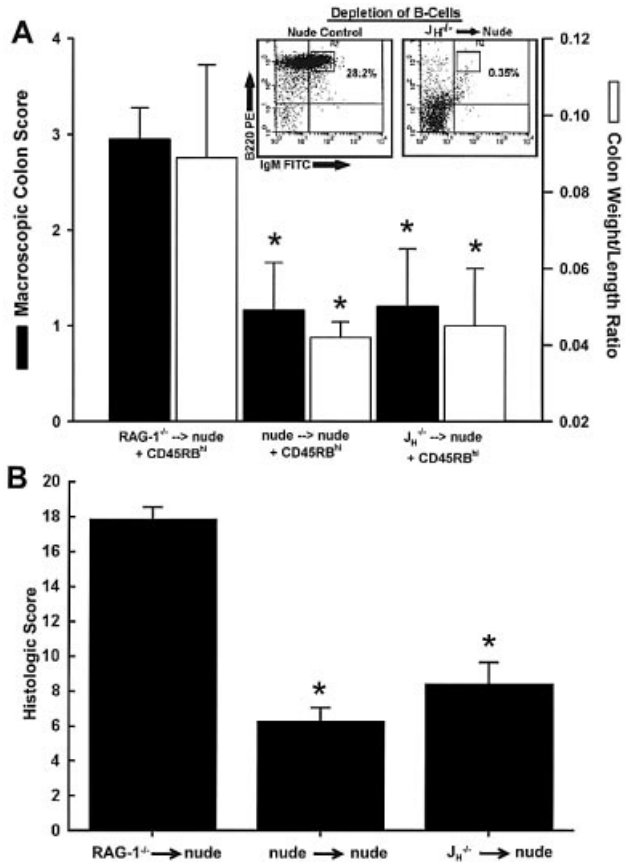

Fig. 5. Macroscopic colon scores and colon weight:length ratios of RAG$^{-/-}$ → nude, nude → nude and $J_H^{-/-}$ → nude radiation chimeras 8 weeks following reconstitution with CD45RB^{high} T cells. Both macroscopic colon scores and weight:length ratios were significantly reduced in nude → nude and $J_H^{-/-}$ → nude chimeras compared to RAG$^{-/-}$ → nude recipients (A). B cell depletion in $J_H^{-/-}$ → nude recipients was confirmed by flow cytometric analysis for mature B cells (B220$^+$/IgM$^+$ cells) and was shown to be >97% (A, insert). Macroscopic scoring was performed as described in Methods. (B) Blinded histologic scoring of colons for both groups. Histologic scoring criteria are described in the Methods section. *$P < 0.05$ compared to RAG-1$^{-/-}$ → nude. Data represent the mean + SE for $n = 10$ RAG-1$^{-/-}$ → nude, $n = 10$ nude → nude and $n = 5$ $J_H^{-/-}$ → nude.

the percent of CD8$^+$ IEL (Fig. 7). In the colon, there was a similar bias toward the CD8$^+$ phenotype in IEL in unmanipulated nude mice (Fig. 7; 11.9 versus 37.7% for CD4 versus CD8 respectively), whereas reconstituted nude mice have almost equal percentages of CD4 and CD8 IEL in the colon (Fig. 7, bottom; 28.9 versus 39% for CD4 versus CD8 respectively). Interestingly, we were also able to demonstrate that IEL from reconstituted nude mice display an activated/memory phenotype as assessed by CD45RB expression (Fig. 8), suggesting that reconstitution of nude mice with naive T cells leads to the activation of resident IEL in the nude mice. Taken together, these data suggest that the presence of IEL is associated with resistance to CD45RB^{high} T cell-induced colitis. To directly test whether IEL are capable of regulating chronic colitis, we isolated and reconstituted RAG-1$^{-/-}$ mice with CD8$^+$ IEL from wild-type donors prior to transfer of naive CD45RB^{high} T cells. As shown in Fig. 9, RAG-1$^{-/-}$ mice receiving 5 × 10^6 CD8$^+$ IEL/mouse 1 week prior to reconstitution with CD45RB^{high} T cells exhibited significantly reduced macroscopic colon scores as well as reduced colon weight:length ratios compared to RAG-1$^{-/-}$ mice receiving CD45RB^{high} cells only (Fig. 9A). In addition, histologic scoring of colons from both groups demonstrated significantly reduced inflammation in RAG-1$^{-/-}$ mice receiving CD8$^+$ IEL prior to CD45RB^{high} transfer compared the recipients of CD45RB^{high} T cells only (Fig. 9B).

Transfer of activated/T_h1 CD4$^+$CD45RB^{low} T cells into nude recipients induces severe colitis

The inability of naive CD4$^+$T cells (CD45RB^{high} T cells) to induce colitis in nude mice suggests that specific populations of regulatory cells (e.g. IEL) may act to directly suppress T_h1 effector cell function or they may promote polarization of the naive CD4$^+$ cells (e.g. CD45RB^{high} T cells) toward a non-pathogenic and/or regulatory phenotype. In order to differentiate between these two possibilities, we transferred CD45RB^{low} cells obtained from spleens of IL-10$^{-/-}$ mice or from colitic SCID mice, that received CD45RB^{high} T cells 8 weeks prior, into nude mice and observed these mice for signs of colitis. It is well established that CD45RB^{low} T cells from IL-10$^{-/-}$ or colitic SCID mice display an activated T_h1 phenotype that produces colitis upon transfer to immunodeficient SCID or RAG$^{-/-}$ recipients (4,8,25). We found that nude mice reconstituted with these polarized T_h1 CD4$^+$CD45RB^{low} T cells developed moderate to severe colitis at 6–8 weeks following transfer, suggesting that IEL in nude mice are *incapable* of directly suppressing activated T_h1 CD4$^+$ cell (CD45RB^{low}) effector cell function (Fig. 10A). Macroscopic and histologic analysis of these mice produced scores very similar to RAG-1$^{-/-}$ mice reconstituted with CD45RB^{high} T cells (Fig. 10B).

Transfer of CD4$^+$CD25$^-$ T cells from CD45RB^{high} reconstituted nude mice produces colitis in SCID recipients; however, CD4$^+$CD25$^+$ T cells from CD45RB^{high} reconstituted nude mice ameliorate CD45RB^{high} colitis

Analysis of spleens from disease-free CD45RB^{high} reconstituted nude mice revealed that there is a substantial increase in the number of CD4$^+$CD25$^-$ T cells as well as the presence of CD4$^+$CD25$^+$ cells. However, the number of CD4$^+$CD25$^+$ T cells in reconstituted nude mice is significantly lower than that of CD4$^+$CD25$^-$ cells (data not shown). Transfer of CD4$^+$CD25$^-$ T cells from disease-free nude mice either alone or with wild-type CD45RB^{high} T cells is capable of inducing colitis in SCID recipients (Fig. 11), suggesting that there exists a polarized pathogenic T cell population in reconstituted nude mice that is prevented from initiating disease. Interestingly, we found that transfer of CD4$^+$CD25$^+$ T cells from reconstituted nude mice was able to inhibit the CD45RB^{high}-mediated colitis in SCID recipients both macroscopically as well as histologically (Fig. 11A and B).

Discussion

A variety of both genetic and immune-manipulated models of IBD have emerged which have facilitated the study of regulatory cell dysfunction in the gut and the resulting chronic colitis that develops. Data obtained from these animal models

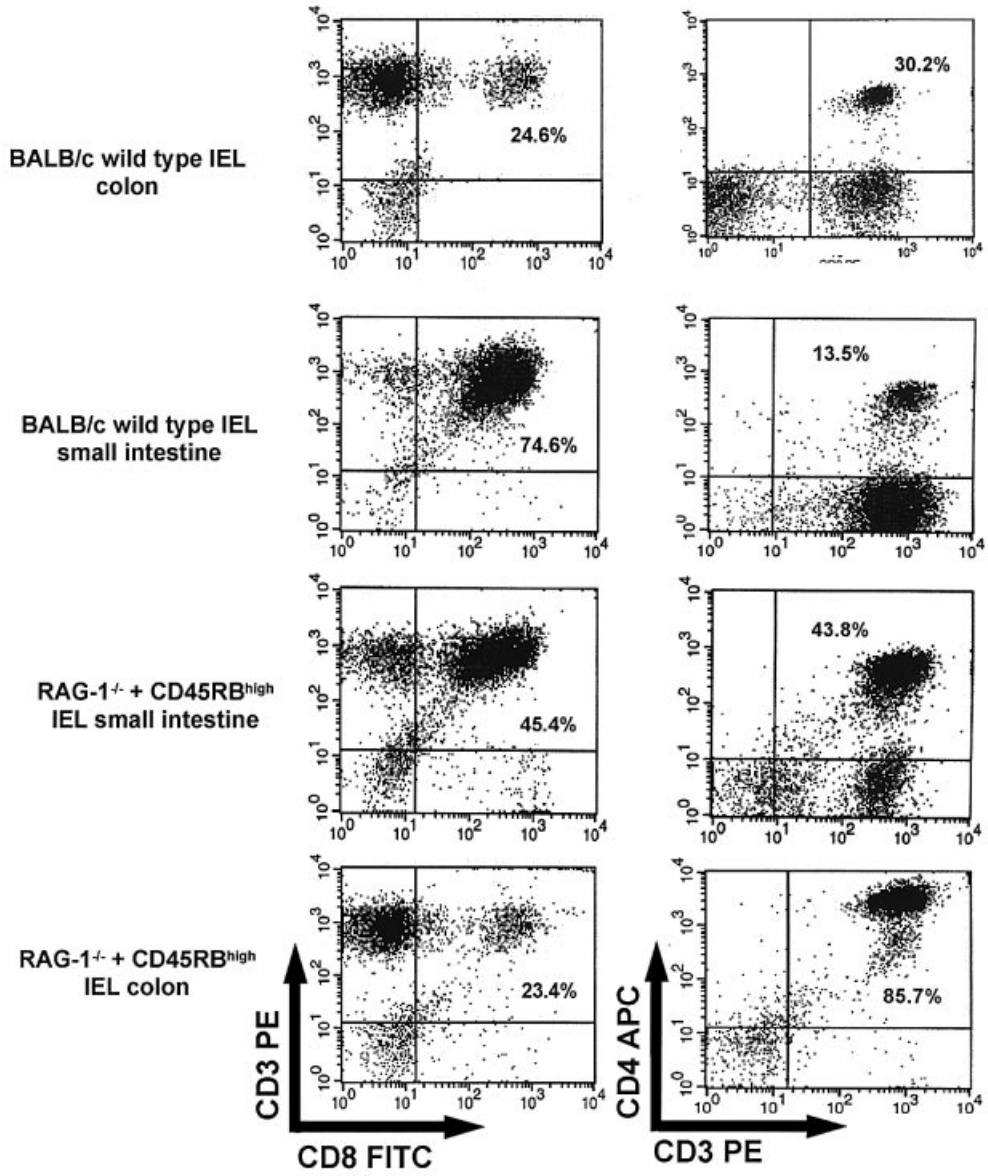

Fig. 6. IEL analysis of small and large intestines obtained from wild-type mice or RAG-1$^{-/-}$ mice reconstituted with CD45RB^{high} T cells. IEL were isolated as described, and stained with anti-CD3–PE, CD4–APC and CD8–FITC prior to analysis on the FACSCalibur. The number of either CD3$^+$CD4$^+$ or CD3$^+$CD8$^+$ is expressed as a percentage of total cells in suspension.

suggest that colitis develops as a result of a dysregulated immune response to normal bacterial antigens (5,7,9,11, 12,33). Several of these studies have focused on the role of specific subsets of peripheral CD4$^+$ T cells that are capable of inhibiting inflammatory T_h1 responses in the gut of immunodeficient SCID or RAG$^{-/-}$ mice mediated by adoptively transferred naive CD4$^+$ T cells (3–5,33). These regulatory T cells (T_{reg}) reside in the CD4$^+$CD25$^+$CD45RB^{low} population and are capable of inhibiting CD45RB^{high}-mediated colitis both prophylactically as well as therapeutically (3,5,14). However, the origin of these T_{reg} cells as well as the exact mechanisms by which they mediate tolerance to enteric antigens remains to be defined. If peripheral T cells are in fact the major determinants of tolerance toward enteric antigens, then one would predict that transfer of naive CD4$^+$CD45RB^{high} T cells into T cell-deficient nude mice would ultimately induce chronic gut inflammation.

Data obtained in the present study clearly demonstrate that nude mice are resistant to CD45RB^{high}-mediated T cells colitis. Because nude mice lack a thymus, they produce few, if any, peripheral CD4$^+$ T cells, but retain B cells, NK cells and IEL. Thus, we reasoned that resistance of nude mice to the disease-producing effects of naive CD45RB^{high} T cells may be due to regulatory functions of one or more of these cells.

IEL represent a unique population of T cells that are found interspersed among epithelial cells of mucosal surfaces such as the small and large intestine. In mouse, it has been estimated that there is one IEL for every three to five epithelial cells or ~3–4 $\times$ 10^7 total cells in the small bowel (30). Studies have shown that the murine small intestine contains 20- to

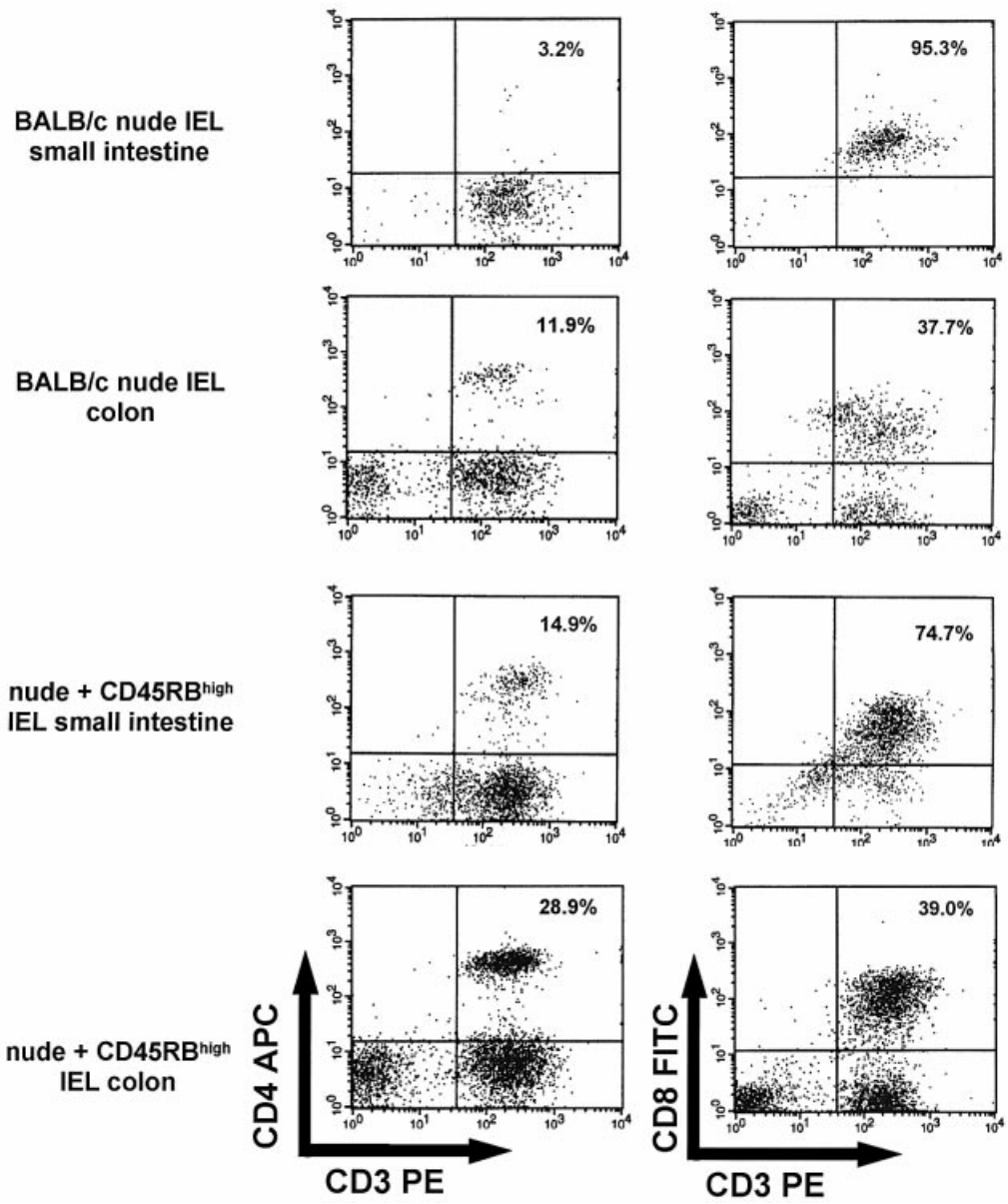

Fig. 7. IEL analysis of small and large intestines obtained from unmanipulated nude mice or nude mice reconstituted with CD45RB^{high} T cells. IEL were isolated as described and stained with anti-CD3–PE, CD4–APC and CD8–FITC prior to analysis on the FACSCalibur. The number of either CD3$^+$CD4$^+$ or CD3$^+$CD8$^+$ is expressed as a percentage of total cells in suspension.

30-fold more IEL than does the colon (30,30,34). Phenotypically, our data (Fig. 6) as well as others demonstrate that murine *small bowel* IEL are 80–90% CD3$^+$, of which 80% are also CD8$^+$ (30). IEL can carry either form of the TCR, with αβTCR cells accounting for 40–70%, while γδTCR cells comprise 30–60% in the small intestine (30,30,34). There are numerous subsets of IEL based on the surface expression of the different isoforms of CD8 (i.e. CD8αα or CD8αβ), as well as expression of CD4, Thy-1 and either TCRαβ or TCRγδ. Based on these markers alone, there are 14 different subsets of IEL that have been identified to date. Recent work has suggested that certain T cell subsets including TCRαβ$^+$CD4$^-$CD8$^-$, TCRαβ$^+$CD4$^-$CD8αα$^+$, TCRαβ$^+$CD4$^+$CD8αα$^+$ and TCRγδ$^+$ CD4$^-$CD8αα$^+$ may develop locally within the intestinal mucosa *independent of any thymic requirements* (35–37). This is an intriguing observation which led to the discovery that the CD8αα$^+$ and CD4$^+$CD8$^+$, thymus-independent, αβ IEL that express TCR with specificity for self antigen are not clonally deleted, in stark contrast to thymus-dependent self-reactive T cells. In addition to absolute numbers of IEL, there appears to be marked differences in the phenotype of IEL residing in the small intestine compared to those in the colon. A study by Beagley *et al.* characterized these phenotypic differences as well as the cytokine expression profiles of IEL residing in the small versus large intestine in three different mouse strains (30). Of IEL expressing CD3, the authors show that there are

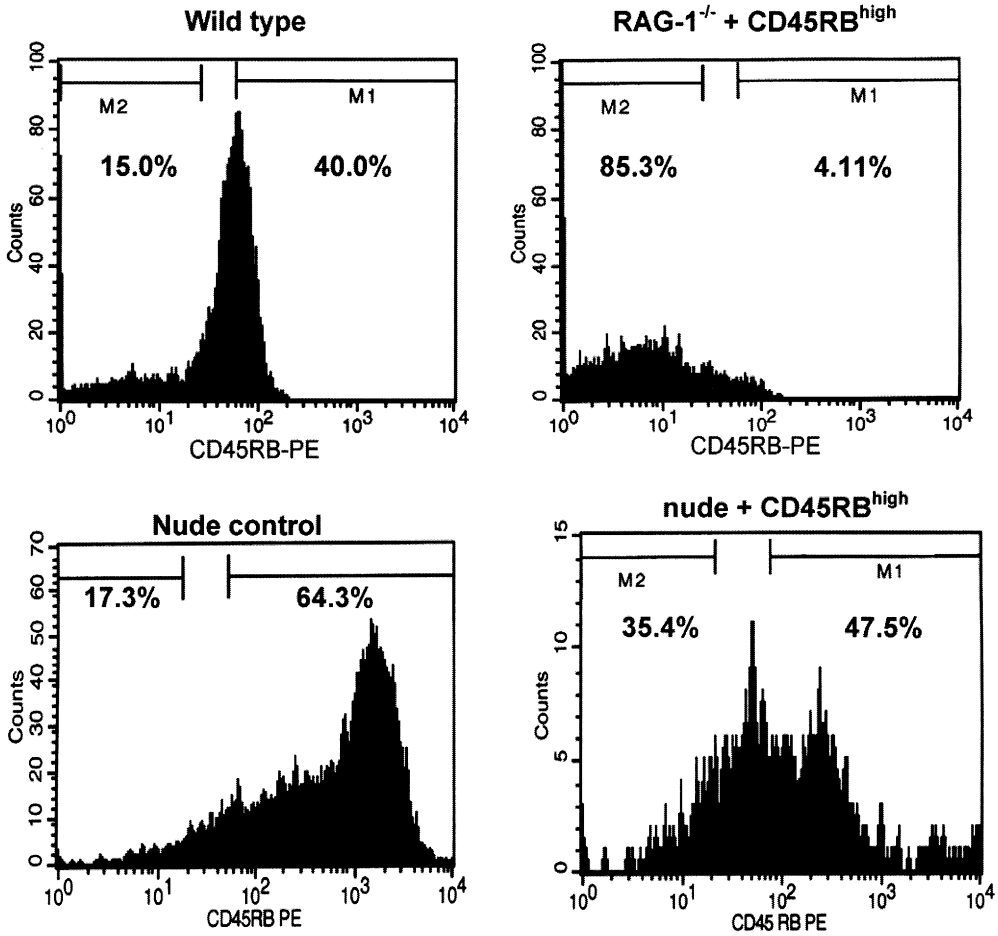

Fig. 8. Reconstituted RAG-1$^{-/-}$ and nude mice posses IEL that display an activated/memory phenotype. IEL were isolated, and stained for CD3, CD4 and CD45RB for three-color analysis. CD45RB expression is shown gated on CD3$^+$CD4$^+$ cells and percentages are shown using wild-type as a standard.

approximately equal numbers of TCR$\alpha\beta$ and TCR$\gamma\delta$ IEL in the small intestine (range 46–55% in the strains examined). However, the large majority of CD3$^+$ IEL isolated from the large intestine are TCR$\alpha\beta$, with much fewer $\gamma\delta$-expressing cells present (~84% TCR$\alpha\beta$ versus ~10% TCR$\gamma\delta$). A similar regional specialization was observed for CD4$^+$ versus CD8$^+$ IEL in small versus large mouse intestine. In the small bowel, >75% of CD3$^+$ IEL are CD8$^+$ compared to ~8% CD4$^+$. Moving into the large bowel, there is a shift in the phenotype of mature IEL in favor of CD4-expressing cells. This phenotypic shift from the small to large intestine is mirrored by the cytokine expression profiles of the different IEL populations in the gut. While IEL of both the small and large intestine produce substantial amounts of TGF-β, IL-1, IFN-γ and TNF-α, there is a noticeable increase in the production of IL-2, IL-4, IL-5 and IL-10 by IEL of the colon (30,34). This regional specialization by IEL, both phenotypically and functionally, suggests that these unique lymphocytes may play an important role in homeostatic as well as pathogenic mucosal immune responses (e.g. IBD).

At first glance, it is difficult to imagine a major regulatory role for IEL in the nude mouse given the fact that the numbers of IEL in the small and large bowel of these mice are ~25% of those found in their wild-type counterparts (38,39). However, several lines of evidence appear to support the concept that IEL represent an important regulatory population within the gut mucosa. First, although reduced in numbers in nude mice, the remaining IEL do in fact express functional TCR (CD3ε) and retain their ability to produce cytokines comparable to wild-type mice (40). Second, colitis has been shown to develop spontaneously, albeit sporadically, in $\beta^{-/-} \times \delta^{-/-}$ double-deficient mice as well as after transfer of CD45RB^{high} T cells (Ostanin, unpublished observations). These mice possess B cells, but no T cells *or IEL* (41,42). In addition, Poussier *et al.* have recently reported that transfer of one specific subset of IEL, i.e. the TCR$\alpha\beta^+$CD4$^-$CD8$\alpha\alpha^+$ subset, remarkably attenuates colitis induced in SCID mice via transfer of CD45RB^{high} T cells (22). Interestingly, these investigators showed that transfer of TCR$\alpha\beta^+$CD4$^-$CD8$\alpha^+\beta^+$ or TCR$\gamma\delta^+$CD4$^-$CD8$\alpha^+\beta^+$ IEL did not suppress colitis, suggesting that small numbers of specific IEL are sufficient to attenuate disease.

The mechanisms by which specific subsets of IEL suppress the development of colonic inflammation have not been delineated; however, at least two possibilities exist. IEL may regulate chronic gut inflammation via direct suppression of T_h1

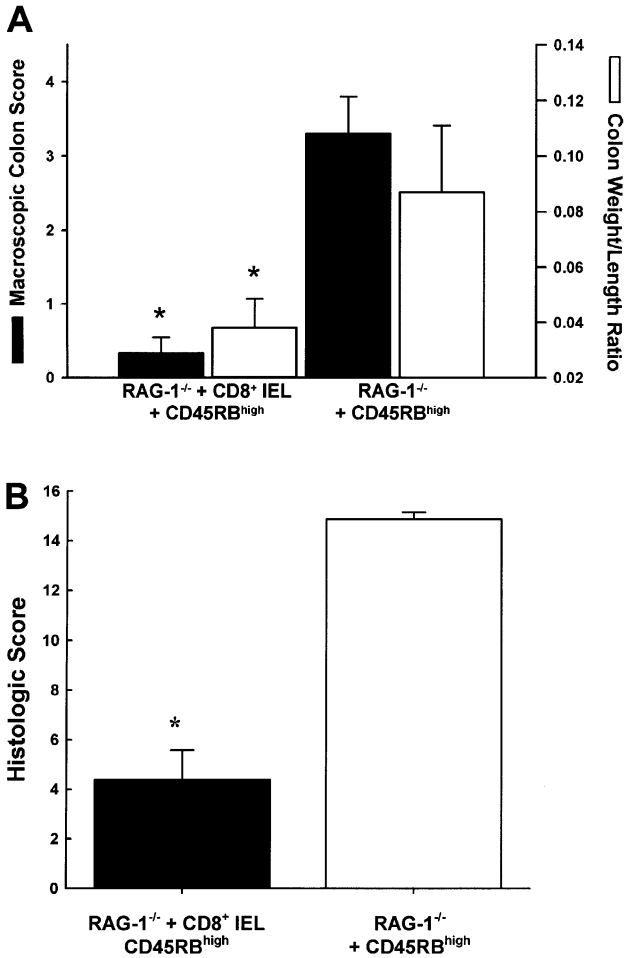

Fig. 9. Transfer of $CD8^+$ IEL into RAG-$1^{-/-}$ mice inhibits development of chronic colitis. RAG-$1^{-/-}$ mice were reconstituted with wild-type $CD8^+$ IEL prior to transfer of $CD45RB^{high}$ T cells and monitored for signs of colitis. RAG-$1^{-/-}$ mice receiving IEL display normal colon morphology compared to control reconstituted RAG-$1^{-/-}$ mice. In addition, IEL-treated RAG-$1^{-/-}$ recipients had significantly lower gross colon scores as well as colon weight:length ratios compared to mice receiving $CD45RB^{high}$ T cells only (A). RAG-$1^{-/-}$ mice receiving IEL also had significantly lower histologic colon scores compared to mice receiving $CD45RB^{high}$ T cells only (B). $P < 0.05$; $n = 6$ for $CD45RB^{high}$ alone and $n = 3$ for RAG-$1^{-/-}$ + $CD8^+$ IEL + $CD45RB^{high}$.

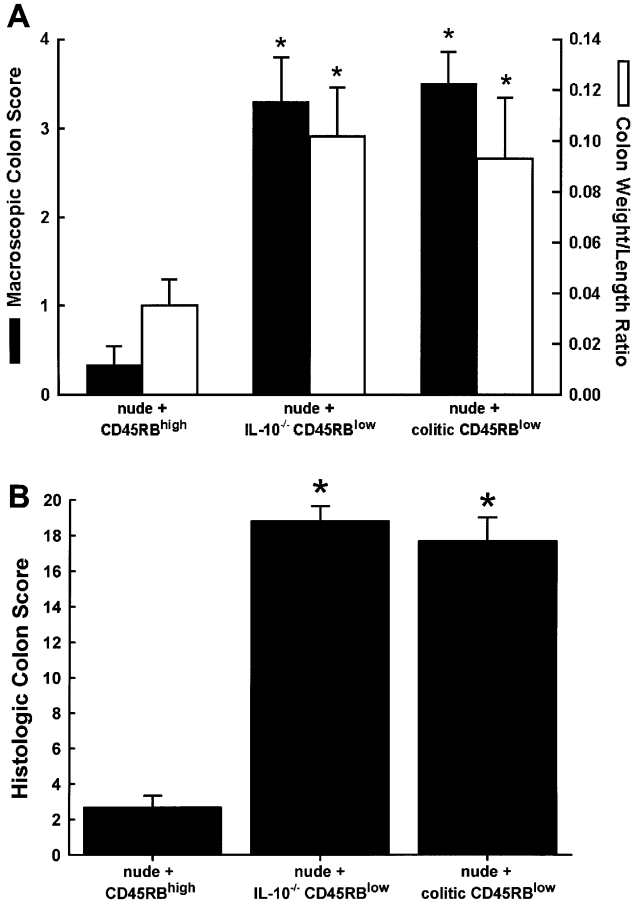

Fig. 10. Macroscopic colon scores and colon weight:length ratios 8 weeks following reconstitution of nude mice with $CD45RB^{high}$ T cells or with $CD45RB^{low}$ T cells obtained from either disease-free IL-$10^{-/-}$ or colitic SCID mice. Methods for generating the colitic SCID mice are described in Methods. Macroscopic scoring was performed as described in Methods. Macroscopic scoring and weight:length ratios reveal that nude mice receiving T_h1-polarized T cells develop moderate to severe inflammation of the colon (A). (B) Blinded histologic scoring of colons for all three groups. Histologic scoring criteria are described in Methods. *$P < 0.05$ compared to nude + $CD45RB^{high}$. Data represent the mean + SE for $n = 10$ nude + $CD45RB^{high}$, $n = 8$ nude + IL-$10^{-/-}$ $CD45RB^{low}$ and $n = 5$ nude + colitic $CD45RB^{low}$.

effector cell function or they may directly or indirectly promote the polarization of the naive $CD4^+$ cells (e.g. $CD45RB^{high}$ T cells) toward a non-pathogenic phenotype. It is equally plausible that IEL serve to maintain a 'tight' epithelial barrier in the gut such that lack of IEL allows for increased access of luminal antigens to the interstitium. In order to differentiate between the first two possibilities we reconstituted nude mice with $CD4^+CD45RB^{low}$ T cells obtained from non-colitic IL-$10^{-/-}$ or colitic SCID mice. Because these specific T cells are already polarized to an activated/T_h1 phenotype (4,8,25), induction of colitis by transfer of these cells into nude would suggest that IEL do not exert their protective effect by suppressing effector cell function, but may promote the polarization of naive $CD4^+CD45RB^{high}$ T cells to a non-pathogenic and/or regulatory phenotype. If, on the other hand, nude recipients do not develop colitis, then this would suggest that IEL may in fact directly suppress effector cell function. We found that transfer of activated/T_h1 T cells into nude mice induced severe colitis that was similar in many respects to the colonic inflammation induced in RAG-$1^{-/-}$ mice (Fig. 10). These data suggested that IEL may suppress colitis in nude mice by promoting the polarization of naive T cells to a non-pathogenic and/or regulatory phenotype. Interestingly, we found that in nude mice, a portion of the injected naive $CD45RB^{high}$ T cells are polarized to a regulatory phenotype capable of suppressing colitis in SCID recipients. Furthermore, we found that the majority of the injected cells are directed toward a potentially pathogenic phenotype that is able to induce colitis in SCID recipients. These data are

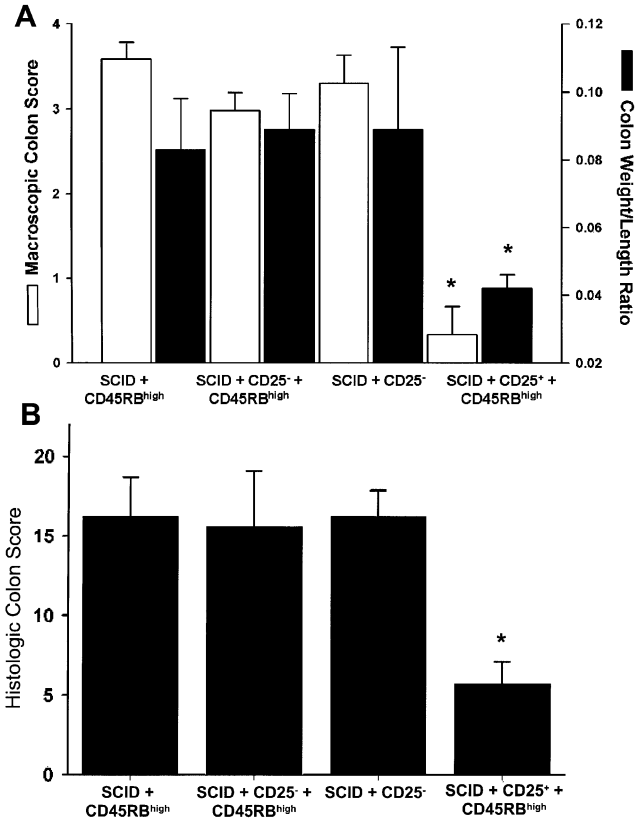

Fig. 11. Macroscopic colon scores and colon weight:length ratios 8 weeks following reconstitution of SCID mice with $CD4^+CD25^-$ (from reconstituted nude mice) alone or with wild-type $CD45RB^{high}$, $CD4^+CD25^+$ (from reconstituted nude mice) with wild-type $CD45RB^{high}$ or with wild-type $CD45RB^{high}$ cells alone. $CD4^+CD25^+$ and $CD25^-$ T cells were obtained from reconstituted nude mice as described in Methods, as was wild-type $CD45RB^{high}$ cells. Macroscopic and histologic scoring reveals that SCID mice receiving $CD4^+CD25^-$ T cells from $CD45RB^{high}$ reconstituted mice develop colitis comparable to recipients of $CD45RB^{high}$ T cells alone (A and B). However, SCID mice receiving $CD4^+CD25^+$ T cells from reconstituted nude mice prior to wild-type $CD45RB^{high}$ cells display significant reduction in macroscopic and histologic inflammatory scores (A and B). $^*P < 0.05$ compared to SCID + $CD45RB^{high}$. Data represent the mean + SE for $n = 8$ SCID + $CD45RB^{high}$, $n = 5$ for SCID + $CD25^-$ + $CD45RB^{high}$, $n = 6$ for SCID + $CD25^-$ and $n = 5$ for SCID + $CD25^+$ + $CD45RB^{high}$.

intriguing in that both regulatory and pathogenic phenotypes are found in disease-free nude recipients, and to our knowledge is the first report of IEL influencing the polarization of peripheral T cells. The mechanisms by which this 'fixed' population of lymphocytes regulate polarization of naive T cells in Peyer's patches and/or mesenteric lymph nodes are not known, but may involve interactions between the IEL and resident antigen-presenting cells (possibly dendritic cells) where the IEL 'educates' the dendritic cell prior to its migration to mesenteric lymph nodes. These 'educated' dendritic cells then direct naive T cells to adopt a non-pathogenic and/or regulatory phenotype. The role of dendritic cells in IEL-mediated suppression of colitis is currently under investigation. In summary, data presented in the present study confirm and extend the work of Poussier *et al.* (22), suggesting a central role for intestinal IEL in the induction of tolerance toward enteric antigens and demonstrate a previously unexplored regulatory role for IEL.

Acknowledgements

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Abbreviation

APC	allophycocyanin
IBD	inflammatory bowel disease
IEL	intraepithelial lymphocyte
PE	phycoerythrin
TGF	transforming growth factor

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