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Selective upregulation of immune regulatory and effector cytokine synthesis by intestinal intraepithelial lymphocytes following CD43 costimulation

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Abstract

The involvement of the CD43 molecule in the activation of mouse small intestinal intraepithelial lymphocytes (IELs) has been studied using a panel of twenty-two regulatory and effector immune response analytes. In the absence of stimulation in vitro, IELs produced low levels of CCL5 only. Upon CD3 stimulation, the activity of seven of twenty-two analytes were elevated relative to unstimulated cultures, including several proinflammatory cytokines and chemokines. Notably, CD3 stimulation in the presence of CD43 costimulation resulted in elevated levels of five analytes (interleukin-2, interferon- γ , CCL5, granulocyte colony-stimulating factor, and granulocyte-monocyte colony stimulating factor) above that produced by CD3 stimulation alone. That CD43 costimulation was responsible for elevated cytokine/chemokine activity was confirmed at the transcriptional level by real-time PCR for IFN- γ and CCL5, and by ELISA assay for IFN- γ . These findings open the way to a better understanding of the process by which T cells are activated in the intestinal epithelium.

Keywords

intraepithelial lymphocytes; costimulation; immunity; T cell; protein array; real-time PCR

Introduction

Small intestinal intraepithelial lymphocytes (IELs) in mice display many features that distinguish them from other peripheral lymphocytes. For example, the majority of CD8+ IELs express CD69, a marker of recently-activated lymphocytes [1]. Moreover, without in vitro activation, IELs are constitutively cytolytic [2–5], suggesting that they are not classical resting T cells. However, IELs have limited capacity for proliferation and they do not spontaneously produce cytokines [6,7], both of which are properties that belie the likelihood that they are fully-activated T cells. Following stimulation through the T cell receptor (TCR)/CD3 complex, IELs produce cytokines [8,9], and intracellular IFN- γ staining has been demonstrated for IELs from mice with active intestinal inflammation [10]. Thus, the capacity for IEL cytokine production clearly is a regulated event that requires appropriate activating stimuli. Hence, IELs cannot be considered to be fully-activated T cells, but rather they must be viewed as being sustained in a state of partial activation — a hypothesis that is supported by studies of gene

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expression, which reveal that most IELs exist in a state between resting and activated T cells [11,12].

CD43 is a heavily glycosylated cell surface glycoprotein that is widely expressed within the hematopoietic system, including bone marrow cells, thymocytes, peripheral T cells, and some B cells [13–16]. CD43 has been shown to have diverse biological functions. These include the enhanced cytotoxicity of CD8⁺ T cells [17] and the capacity to mediate a costimulatory signal in T cells [18–20]. CD43 differentially influences the expansion and contraction phases of the immune response as seen from studies of lymphocytic choriomeningitis virus-infected mice [21]. Additionally, graft-versus-host disease in the small intestine of mice has been shown to be mediated by a CD43 isoform linked to activated T cells [22].

Costimulation by CD43 occurs upon stimulation of the TCR/CD3 complex with simultaneous ligation of CD43 [18]. This results in enhanced cell proliferation as demonstrated for IELs [19] as well as T peripheral cells [18,20]. In the intestine, costimulation by CD43 would appear to be an important immune-potentiating signal of IELs, particularly due to the generalized lack of the CD28 costimulatory molecule on IELs [23,24]. At present, however, nothing is known about the extent to which CD43 costimulation influences the cytokine synthesis process of small intestinal IELs. Here, we demonstrate that IEL activation in conjunction with CD43 costimulation results in increases in the synthesis of immune regulatory and effector cytokines compared to stimulation by CD3 alone. These findings indicate that murine IELs can be driven into a heightened state of activation, and they identify CD43 as a molecular signal in that process. They also have implications for therapeutic approaches aimed at achieving maximum effects in vaccine delivery systems, and they suggest that control of IEL activation through CD43 may be a pathway for curtailing chronic intestinal inflammation.

Materials and methods

Mice

Adult female C57BL/6 mice, 6–8 wks of age, were purchased from Harlan-Sprague Dawley (Indianapolis, IN). Mice were used in accordance with the University of Texas institutional animal welfare guidelines.

IEL isolation

Small intestine tissues were placed in a 100 mm tissue culture dish containing RPMI-1640 supplemented with FCS (10% v/v), 100 U/ml penicillin-streptomycin, 2 mM L-glutamine, and 5×10^{-5} M 2-ME (all reagents; Sigma-Aldrich, St. Louis, MO). Peyer's patches were discarded and the intestine was flushed of fecal material, opened longitudinally, and cut into 3 mm fragments in supplemented RPMI-1640. Tissues and media were transferred to a 50 ml plastic conical centrifuge tube, mixed, and the medium was removed and replaced 3x with 15–20 ml of Ca⁺⁺, Mg⁺⁺ free PBS followed by 50 ml of Ca⁺⁺, Mg⁺⁺ free PBS containing 2 mM DTT and 5 mM EDTA. Tissues were transferred to a 100 ml beaker, stirred gently at 37°C for 30 min, and passed successively through two 10 cc syringe barrels filled to 5 ml with wetted nylon wool. The cell suspension was separated into two 50 ml tubes, centrifuged, and each cell pellet was mixed in 3 ml of 40% isotonic Percoll (Sigma-Aldrich) and layered onto 4 ml of 70% isotonic Percoll. Gradients were centrifuged at $400 \times g$ for 30 min. Cells were collected from the 40/70% interface areas, combined, washed, resuspended in 3 ml of 40% isotonic Percoll, overlaid onto 4 ml of isotonic 70% Percoll, and centrifuged a second time at $400 \times g$ for 30 min. IELs were collected from the 40/70% Percoll interface, washed, and used for in vitro culture.

Antibodies, in vitro cell culture, and ELISA assay

Purified functional-grade anti-CD3 mAb (145-2C11) was purchased from BD-PharMingen (San Diego, CA). Anti-CD43 mAb (R2/60) [19] was prepared by affinity purification on an anti-rat IgM column (Zymed; South San Francisco, CA) using tissue culture supernatants from hybridoma cells grown in our laboratory. IELs were cultured at a density of 1.5×10^6 cells/ml in 24 or 6 well tissue culture plates with 1 μ g/ml anti-CD3 mAb, 1 μ g/ml anti-CD3 mAb plus 1.0 μ g/ml anti-CD43 mAb, or PBS alone overnight at 4°C. For protein array studies, cells were cultured in serum-free medium LGM medium (Cambrex; Walkersville, MD). Supernatants and cells were harvested after 24 hrs of in vitro culture and used for protein arrays and IFN- γ ELISA (eBioscience; San Diego, CA), and for real-time PCR analysis, respectively.

Multiple analyte protein array assay

Protein array analysis was done using membranes and reagents purchased from RayBiotech (Norcross, GA) with the Mouse Cytokine Array I panel (cat. no. M0308001C). Procedures were conducted according to the manufacturer's protocols. Briefly, membranes were reacted with 1x blocking buffer for 30 min at room temperature. The buffer was removed and 2 ml of sample supernatant was added for 2 hr at room temperature with gentle rotation. The test supernatant was removed and membranes were washed 3x with wash buffer I and 3x with wash buffer II for 5 min each. Membranes were drained and biotin-conjugated anti-analyte antibody cocktail was added overnight at 4°C with rotation. Membranes were washed with wash buffers and HRP-conjugated streptavidin provided was reacted for 60 min at room temperature with rotation. Membranes were drained, washed, and detection buffer provided with the kit was reacted for 5 min at room temperature. The detection buffer was removed, the membranes were placed in plastic sheets, and reacted with photographic film for 1–4 min. Photographic film was developed, scanned, and analyzed using a Gel-Pro Image Analyzer with Gel-Pro Plus software (Media Cybernetics; Silver Spring, MD). Optical density values were normalized between samples using to positive markers included on the membranes.

Real-time PCR

IELs were harvested from cell cultures as described above. Cells were washed in PBS, counted, and RNAs were isolated using an RNeasy kit (Qiagen; Valencia, CA) according to the manufacturer's protocols. RNA concentrations were estimated spectrophotometrically at A₂₆₀. 100 ng of total RNA was used in a 25 μ l/sample master mix consisting of 12.5 μ l of 2x SYBR green and 0.5 μ l I-Script (reverse transcriptase) (BioRad; Hercules, CA) and 1 μ l of PCR primers. Nuclease-free water (BioRad) was added to a final volume of 25 μ l. Analyte primers were purchased from SuperArray Bioscience (Frederick, MD) for IFN- γ (cat. no. PPM03121A; UniGene# Mm.240327; GenBank Accession no. NM_008337.1; band size 95 bp); and CCL5 (cat. No. PPM02960A; UniGene# Mm.284248; GenBank Accession no. NM_013653.1; band size 188 bp). 18s primers were purchased from Maxim Biotech, Inc. (South San Francisco, CA) and used according to the manufacturer's protocols. Mouse QPCR Reference total RNA was purchased from Stratagene (La Jolla, CA), and was used to establish a standard curve for calculating the amplification values of the 18s and analyte products. Real-time PCR amplification was done using a BioRad multi-color i-Cycler real-time PCR thermocycler. Data were collected and analyzed with BioRad i-Cycler software. Comparisons of PCR-amplified products were achieved according to the following formula as described by others [25]:

$$\frac{\text{Analyte RNA (stimulated)}/18\text{s RNA (stimulated)}}{\text{Analyte RNA (unstimulated)}/18\text{s RNA (unstimulated)}} = \text{Fold Increase}$$

Results

Identification of cytokine synthesis by multiple analyte protein arrays

Cell-free supernatants from unstimulated and CD3-stimulated small intestinal IEL cultures were assayed for the 22 cytokine analytes shown in the protein array template grid (Fig. 1). In unstimulated cultures, low-level cytokine production was evident only for CCL5, also known as RANTES (Regulated on Activation, Normal T cell Expressed and Secreted). This is consistent with the fact that CCL5 is normally expressed in low concentrations by unstimulated T cells [26]. However, following CD3-mediated activation, levels of seven cytokine analytes were upregulated (GM-CSF, IL-2, IL-6, IL-12p70, IL-17, IFN- γ , and CCL5) (Fig. 2). After normalization using control values between the unstimulated and CD3-stimulated data, there was an appreciable increase in cytokine production as determined by optical density (Fig. 3A). These findings were of particular interest for several reasons. First, they provide a more comprehensive evaluation of the type of cytokine responses that are elicited by IELs following CD3 signaling. Second, they reveal that several of the cytokine responses activated include proinflammatory or T cell activation mediators. This was especially evident with regard to IL-2, IL-17, IFN- γ , and CCL5, and to a lesser extent, GM-CSF, IL-6, and IL-12p70. The remaining fifteen analyte levels were predominantly unaffected by CD3 stimulation, implying that stimulation through the CD3 complex does not result in a generalized cytokine activation event.

Activation of IEL cytokine synthesis following CD43 costimulation

To evaluate the effects of CD43 costimulation on cytokine synthesis, IELs were exposed to simultaneous CD3 and CD43 stimulation as described in the Materials and methods. Cell-free supernatants from 24 hr cultures were assayed for the 22 cytokine analytes listed in Fig. 1. Because visualization of array blots cannot predict actual differences, densitometry and normalization to control values were used to compare results from anti-CD3 stimulated blots and CD43 costimulated blots. As indicated in Fig. 3B, costimulation resulted in elevated cytokine production for IL-2, IFN- γ , CCL5, GCSF and GM-CSF relative to CD3 stimulation. Note the scaling difference of optical density values in the data from Fig. 3A and Fig. 3B, thus confirming the increase in analyte activities in CD43-costimulated cultures. The levels of cytokine production for the other seventeen analytes remained unchanged in CD43-costimulated cultures (data not shown).

Confirmation of findings from protein array studies by real-time PCR and ELISA

To determine whether the findings observed at the protein level were reflected in transcriptional changes, real-time PCR analysis was done for IFN- γ and CCL5 using RNA extracted from unstimulated, CD3-stimulated, and CD43-costimulated IELs. As shown in Table 1, analyte RNA activity for IFN- γ and CCL5 in CD43-costimulated IELs were elevated over that of CD3-stimulated IELs alone, a finding which was also confirmed at the protein level for IFN- γ in an ELISA assay as shown in Fig. 4. IFN- γ production in unstimulated cultures was <10 pg/ml (data not shown). These findings establish that the observations obtained from protein array studies were consistent at the transcriptional level and at the level of secreted protein, and they indicate that CD43 costimulation of IELs is a mechanism for selectively increasing the production of regulatory and effector immune response molecules.

Discussion

Previous studies have demonstrated that small intestinal IELs can undergo activation following stimulation through the TCR/CD3 complex, leading to modest levels of cell proliferation [6, 7], and that costimulation of IELs through CD43 significantly augments the proliferative signal mediated by CD3 [15]. The present study is the first report of a role for CD43 in the induction

of immune response molecules (cytokines and chemokines) by IELs as determined from a set of molecular analytes known to be instrumental in regulating the immune response. Interestingly, CD3 stimulation alone did not result in the activation of all of the cytokines/chemokines included in the analyte panel. Rather, specific analytes were selectively activated (GM-CSF, IL-2, IL-6, IL-12p70, IL-17, IFN- γ , and CCL5). Other analytes (e.g., IL-3, IL-4, IL-10) may have been slightly elevated compared to non-stimulated cultures; however, levels of those were low. Nonetheless, it is clear that CD3 stimulation resulted in changes in several cytokine/chemokine levels by IELs, all of which are used in the initiation of a strong inflammatory response, indicating a role for CD3 in driving the response in that direction.

A second important feature of this study pertains to the changes in cytokine levels in supernatants of IEL cell cultures following costimulation by CD43 in the presence of CD3 stimulation, which resulted in elevated cytokine/chemokine levels for GCSF, GM-CSF, IL-2, IFN- γ , and CCL5, but no elevation of IL-6, IL-12p70, or IL-17. These findings indicate that CD43 costimulation has differential effects on the synthesis of immune response molecules compared to CD3 stimulation alone. Enhanced analyte expression following CD43 costimulation for IFN- γ and CCL5 also was evident by real-time PCR, thus confirming that effect at the transcriptional level.

Elevated levels of IL-2, IFN- γ , and CCL5 following CD43 costimulation were insightful since all three are involved in the generation of an inflammatory immune response. An increase in IL-2 — a Th1 cytokine — would promote IEL proliferation and result in enhanced cell-mediated immunity, thus accelerating the immune response in conjunction with TCR/CD3 engagement. Similarly, IFN- γ plays a role in local intestinal immunity and inflammation as seen in studies in which IFN- γ is spontaneously produced by as many as 5% of the total IELs from the ileum of IL-10 $^{-/-}$ mice with active intestinal inflammation [10]. Conversely, IFN- γ also may be a contributing factor in the pathogenesis of virus-induced immunodeficiency in mice [27].

CCL5 is likewise a prominent proinflammatory molecule within the intestine. Humans with Crohn's disease and inflammatory bowel disease have been shown to express CCL5 in intestinal lesions and in homogenates from surgical specimens [28,29]. CCL5 also is reported to play a role in the induction of oral tolerance [30,31], suggesting a possible regulatory role during inflammation under normal conditions. Interestingly, IL-17 has been shown to down-regulate CCL5 synthesis mediated by TNF α [32], a finding that may have bearing on the present study given that IL-17 levels were not elevated upon CD43 costimulation. Hence, CD43 costimulation may contribute to the elevated levels of CCL5 directly, but also may lead to elevated CCL5 levels due to a lack of IL-17 synthesis. Infectious agents such as *C. parvum* stimulate CCL5 secretion from intestinal cell lines [33], have been shown to be chemotactic for IELs [34], and lead to increased levels of proliferation of mucosal lymphocytes [35], demonstrating that CCL5 is a critically-important chemokine used to aggressively activate the intestinal immune response.

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References

1. Wang HC, Zhou Q, Dragoo J, Klein JR. Most murine CD8 $^{+}$ intestinal intraepithelial lymphocytes are partially but not fully activated T cells. *J Immunol* 2002;169:4717–22. [PubMed: 12391179]
2. Lefrancois L, Goodman T. In vivo modulation of cytolytic activity and Thy-1 expression in TCR- $\gamma\delta$ + intraepithelial lymphocytes. *Science* 1989;243:1716–1718. [PubMed: 2564701]

3. Viney J, Kilshaw P, MacDonald T. Cytotoxic $\alpha\beta^+$ and $\gamma\delta^+$ T cells in murine intestinal epithelium. *Eur J Immunol* 1990;20:1623–1626. [PubMed: 1974852]
4. Corazza N, Muller S, Brunner T, Kagi D, Mueller C. Differential contribution of Fas- and perforin-mediated mechanisms to the cell-mediated activity of naive and in vivo-primed intestinal intraepithelial lymphocytes. *J Immunol* 2000;164:398–403. [PubMed: 10605035]
5. Montufar-Solis D, Klein JR. Activation and costimulation of intestinal T cells: Independent and collaborative involvement of CD43, OX40, and Ly-6C. *Curr Immunol Rev* 2005;1:13–20. [PubMed: 19081760]
6. Mosley RL, Whetsell M, Klein JR. Proliferative properties of murine intestinal intraepithelial lymphocytes (IEL): IEL expressing TCR $\alpha\beta$ or TCR $\gamma\delta$ are largely unresponsive to proliferative signals mediated via conventional stimulation of the CD3-TCR complex. *Int Immunol* 1991;3:563–569. [PubMed: 1832293]
7. Sydora BC, Mixter PF, Holcombe HR, Eghtesady P, Williams K, Amaral MC, Nel A, Kronenberg M. Intestinal intraepithelial lymphocytes are activated and cytolytic but do not proliferate as well as other T cells in response to mitogenic signals. *J Immunol* 1993;150:2179–2191. [PubMed: 8383717]
8. Iijima H, Takahashi I, Kiyono H. Mucosal immune network in the gut for control of infectious disease. *Rev Med Virol* 2001;11:117–133. [PubMed: 11262530]
9. Wang HC, Klein JR. Multiple levels of activation of murine CD8 $^+$ intraepithelial lymphocytes defined by OX40 (CD134) expression: Effects on cell-mediated cytotoxicity, IFN- γ , and IL-10 regulation. *J Immunol* 2001;167:6717–6723. [PubMed: 11739485]
10. Wang HC, Montufar-Solis D, Teng BB, Klein JR. Maximum immunobioactivity of murine small intestinal intraepithelial lymphocytes resides in a subpopulation of CD43 $^+$ T cells. *J Immunol* 2004;173:6294–302. [PubMed: 15528368]
11. Shires J, Theodoridis E, Hayday AC. Biological insights into TCR $\gamma\delta^+$ and TCR $\alpha\beta^+$ intraepithelial lymphocytes provided by serial analyses of gene expression (SAGE). *Immunity* 2001;15:419–434. [PubMed: 11567632]
12. Fahrner AM, Konigshofer Y, Kerr EM, Gandour G, Mack DH, Davis MM, Chien YH. Attributes of $\gamma\delta$ intraepithelial lymphocytes as suggested by their transcriptional profile. *Proc Natl Acad Sci USA* 2001;98:10261–10266. [PubMed: 11526237]
13. Baecher CM, Infante AJ, Semcheski KL, Frelinger JG. Identification and characterization of a mouse cell surface antigen with alternative molecular forms. *Immunogenetics* 1988;28:295–302. [PubMed: 3169878]
14. Gulley ML, Ogata LC, Thorson JA, Dailey MO, Kemp JD. Identification of a murine pan-T cell antigen which is also expressed during terminal phases of B-cell differentiation. *J Immunol* 1988;140:3751–3757. [PubMed: 2897394]
15. Moore T, Huang S, Terstappen LW, Bennett M, Kumar V. Expression of CD43 on murine and human pluripotent stem cells. *J Immunol* 1994;153:4978–4987. [PubMed: 7525720]
16. Rosenstein Y, Santana A, Pedraza-Alva G. CD43, a molecule with multiple functions. *Immunol Res* 1999;20:89–99. [PubMed: 10580634]
17. Mosley RL, Hamad M, Whetsell M, Klein JR. A novel marker of murine bone marrow hematopoietic cells that is expressed on peripheral T cells and is associated with a functionally important molecule on activated cytotoxic T lymphocytes. *Hybridoma* 1994;13:353–358. [PubMed: 7860091]
18. Sperling AI, Green JM, Mosley RL, Smith P, DiPaolo RJ, Klein JR, Bluestone JA, Thompson CB. CD43 is a murine T cell costimulatory receptor that functions independently of CD28. *J Exp Med* 1995;182:139–146. [PubMed: 7790813]
19. Bagriacik EU, Tang M, Wang HC, Klein JR. CD43 potentiates CD43-induced proliferation of murine intestinal intraepithelial lymphocytes. *Immunol Cell Biol* 2001;79:303–307. [PubMed: 11380685]
20. Hamad M, Mosley RL, Wang J, Klein JR. Stimulation via the CD43 coreceptor augments T cell proliferation during the early phase of antigen-induced activation. *Dev Comp Immunol* 1996;20:77–82. [PubMed: 8738938]
21. Onami TM, Harrington LE, Williams MA, Galvan M, Larsen CP, Pearson TC, Manjunath N, Baum LG, Pearce BD, Ahmed R. Dynamic regulation of T cell immunity by CD43. *J Immunol* 2002;168:6022–6031. [PubMed: 12055210]

22. Bagriacik EU, Armstrong MD, Okabe M, Klein JR. Differential expression of CD43 isoforms on murine T cells and their relationship to acute intestinal graft versus host disease: studies using enhanced-green fluorescent protein transgenic mice. *Int Immunol* 1999;11:1652–1662.
23. Gelfanov V, Lai YG, Gelfanov V, Dong JY, Su JP, Liao NS. Differential requirement of CD28 costimulation for activation of CD8+ intestinal intraepithelial lymphocyte subsets and lymph node cells. *J Immunol* 1995;155:76–82. [PubMed: 7602124]
24. Reimann J, Rudolphi A. Co-expression of CD8a in CD4+ T cell receptor $\alpha\beta$ + T cells migrating into the murine small intestine epithelial layer. *Eur J Immunol* 1995;25:1580–1588. [PubMed: 7614985]
25. Loke P, Zang X, Hsuan L, Waitz R, Locksley RM, Allen JE, Allison JP. Inducible costimulator is required for type 2 antibody isotype switching but not T helper responses in chronic nematode infections. *Proc Natl Acad Sci (USA)* 2005;102:9872–9877. [PubMed: 15994233]
26. Schall TJ, Jongstra J, Dyer BJ, Jorgensen J, Clayberger C, Davis MM, Krensky AM. A human T cell-specific molecule is a member of a new gene family. *J Immunol* 1988;141:1018–1025. [PubMed: 2456327]
27. Uehara S, Hitoshi Y, Numata F, Makino M, Howard M, Mizuochi T, Takatsu K. An IFN- γ -dependent pathway plays a critical role in the pathogenesis of murine immunodeficiency syndrome induced by LP-BM5 murine leukemia virus. *Int Immunol* 1994;12:1937–1947. [PubMed: 7696211]
28. Oki M, Ohtani H, Kinouchi Y, Sato E, Nakamura S, Matsumoto T, Nagura H, Yoshie O, Shimosegawa T. Accumulation of CCR5+ T cells around RANTES+ granulomas of Crohn's disease: a pivotal site of Th1-shifted immune response. *Lab Invest* 2005;85:137–145. [PubMed: 15492753]
29. McCormack G, Moriarty D, O'Donoghue DP, McCormack PA, Sheahan K, Baird AW. Tissue cytokine and chemokine expression in inflammatory bowel disease. *Inflamm Res* 2001;50:491–495. [PubMed: 11713901]
30. DePaolo RW, Lathan R, Karpus WJ. CCR5 upregulates high dose oral tolerance by modulating CC chemokine ligand 2 levels in the GALT. *J Immunol* 2004;173:314–320. [PubMed: 15210789]
31. Lee JB, Matsumoto T, Shin YO, Yang HM, Min YK, Timothy O, Bae SJ, Quan FS. The role of RANTES in a murine model of food allergy. *Immunol Invest* 2004;33:27–38. [PubMed: 15015830]
32. Andoh A, Fujino S, Bamba S, Araki Y, Okuno T, Bamba T, Fujiyama Y. IL-17 selectively down-regulates TNF- α -induced RANTES gene expression in human colonic subepithelial myofibroblasts. *J Immunol* 2002;169:1638–1637.
33. Maillot C, Gargala G, Delaunnay A, Ducrotte P, Brasseur P, Ballet JJ, Favenec L. *Cryptosporidium parvum* infection stimulates the secretion of TGF- β , IL-8, and RANTES by Caco-2 cell line. *Parasitol Res* 2000;86:947–949. [PubMed: 11133108]
34. Ebert EC. Human intestinal intraepithelial lymphocytes have potent chemotactic activity. *Gastroenterology* 1995;109:1154–1159.
35. Lillard JW Jr, Boyaka PN, Taub DD, McGhee JR. RANTES potentiates antigen-specific mucosal immune responses. *J Immunol* 2001;166:162–169. [PubMed: 11123289]

Fig. 1 Template for Protein Array Grids

	a	b	c	d	e	f	g	h
1	Pos	Pos	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
2	Pos	Pos	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
3	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12	IL-12p70	IL-13
4	IL-4	IL-5	IL-6	IL-9	IL-10	IL-12	IL-12p70	IL-13
5	IL-17	IFN- γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF- α
6	IL-17	IFN- γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF- α
7	TPO	VEGF	Blank	Blank	Blank	Blank	Blank	Pos
8	TPO	VEGF	Blank	Blank	Blank	Blank	Blank	Pos

Fig. 1.
Template design for analyte array panels shown in Fig. 2.

Fig. 2.

Multiple analyte protein array analysis of (A) unstimulated, (B) CD3-stimulated, and (C) CD3 plus CD43 costimulated IEL cultures. Cell-free supernatants were collected from 24 hr IEL cultures and were reacted with analyte membranes as described in Materials and methods. Increases were determined in analyte synthesis following CD3 stimulation and CD3 plus CD43 costimulation after normalization as shown in Fig. 3. Data are representative of two experiments.

Fig. 3.

CD43 costimulation induces high level of cytokines and chemokines elicited by CD3 stimulation alone. (A) Graphic representation of data from Fig. 1 after normalization based on positive control values included with array membranes, showing induction of cytokine production for GM-CSF, IL-2, IL-6, IL-12p70, IL-17, IFN- γ , and CCL5 by CD3-stimulated IELs compared to results of unstimulated cultures. (B) Following costimulation, the activity of five analytes (G-CSF, GM-CSF, IL-2, IFN- γ , and CCL5) were markedly upregulated; however, three cytokine levels produced by CD3 stimulation alone (IL-6, IL-12p70, and IL-17) were unchanged (data not shown). Note the difference in scaling of graphs between panels A and B. These findings indicate that CD43 costimulation selectively alters the synthesis level of specific regulatory and effector immune response molecules produced by intestinal IELs. The data are representative of two experiments.

Fig. 4. CD43 costimulation results in higher levels of IFN- γ in cell-free supernatants of IELs after 18, 24, and 42 hours of culture as determined by ELISA. IFN- γ levels for unstimulated cultures were <10 pg/ml (data not shown). Data are representative of four experiments.

Table 1 Effect of CD43 costimulation of IFN- γ and CCL5 gene expression by real-time PCR*

Analyte	Fold increase	
	CD3 stimulated	CD3 +CD43stimulated
	Unstimulated	Unstimulated
IFN- γ	91.0	93.5
CCL5	4.9	97.2

* Ratio of analyte RNA/18s RNA values