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Activation and Costimulation of Intestinal T Cells: Independent and Collaborative Involvement of CD43, OX40, and Ly-6C

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Abstract

T cells are present in large numbers in the epithelial lining of the small and large intestine of humans and mice. Those cells, referred to as intraepithelial lymphocytes (IELs), are critical for maintaining an effective mucosal immune response against the onslaught of enteric infectious agents and intestinal neoplasia. However, because intestinal immunity must by necessity occur rapidly and efficiently, it is concomitantly important that the local intestinal immune response be curtailed so as not to result in conditions that lead to a destructive inflammatory environment as occurs in inflammatory bowel disease (IBD). Although many aspects of the IEL activation process remain to be understood, emerging evidence indicates that costimulatory molecules on IELs are critical for activation and that they hold the key to regulating intestinal immunity across many levels. In this article, the involvement of three IEL costimulatory molecules (CD43, OX40, and Ly-6C) — working independently or in collaboration—will be discussed in the context of immunity and disease in the human and mouse intestine, and the involvement of those in sustaining the IELs in a uniquely precarious but effective state of activation readiness will be explored.

Keywords

IEL; costimulation; CD43; OX40; Ly-6C; IBD

INTRODUCTION

The goal of this review is to provide information about the activation process of murine and human intestinal intraepithelial lymphocytes in the context of CD43, OX40, and Ly-6C. All three molecules have costimulatory properties. This review will discuss the basic costimulatory activity mediated by those molecules, and will describe what is known about their capacity to modulate IEL function. Clearly, other costimulatory molecules are involved in the IEL immunoregulatory process. By focusing the discussion on CD43, OX40, and Ly-6C, it is hoped that this will prompt additional empirical work aimed at further understanding the role of those molecules in induction and expression of intestinal immunity, as well as with regard to their possible involvement in intestinal immunopathology.

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SMALL INTESTINAL IELS BEAR UNIQUE ACTIVATION PROPERTIES

The Activation Paradox

One of the hallmarks of small intestinal IELs is their paradoxical state of activation. This can be seen by the fact that essentially all IELs express the CD69 early T cell activation marker, as witnessed by the expression of CD69 on IEL isolates stained *ex vivo* (Fig. 1A), and in *in situ*-stained preparations (Fig. 1B) [1,2]. The latter is significant since it indicates that CD69 expression is not an artifact of the IEL extraction process. Because CD69 is upregulated on T cells within hours of antigen-induced activation, it appears that most IELs in the small intestine already have entered into an initial phase of activation. Consistent with CD69 expression, CD8⁺ small intestinal IELs are cytolytic as demonstrated functionally in the redirected cytotoxicity assay (Fig. 1C). This is a well-documented property of both murine and human IELs [3–6], and cytolytic activity is acquired as early as three weeks of age in mice [7]. Notably, cytotoxic activity of small intestinal IELs in mice does not require exposure to commensal antigens as witnessed by studies in which IELs from OT-1 transgenic mice maintained high levels of spontaneous cytotoxic activity against OVApeptide-bearing target cells in the absence of antigen priming [8]. Parallel observations have been reported for IELs from KN6 TCR $\gamma\delta$ transgenic mice [9]. IELs but not spleen cells from those animals were CD69⁺, had cytotoxic activity in redirected assays, and were activated to comparable levels whether from germfree mice or from conventional animals [9]. Those observations coupled with the broadly diverse TCR repertoires of TCR $\alpha\beta$ ⁺ and TCR $\gamma\delta$ ⁺ IELs in normal hosts [10–19], which admittedly may become oligoclonal under conditions of intestinal inflammation, [20–24], speak against a scenario in which IELs are driven into activation in an antigen-specific manner — at least not during the early phase of that process. Despite the presence of cytotoxic activity and CD69 expression, comprehending the state of IEL activation is confounded by a number of properties that do not readily align the IELs with a population of fully-activated effector cells. For example, IELs do not spontaneously secrete cytokines. Yet they can be induced to produce a wide range of both Th1 and Th2 cytokines following immune stimulation [25–27], and once initiated, cytokine synthesis occurs more rapidly and to higher levels for CD8⁺ IELs than for an equivalent number of CD8⁺ lymph node cells as shown in (Fig. 1D) for IFN- γ . Those findings imply that unlike T cells in other peripheral immunological compartments, intestinal IELs are sustained in a precarious state of partial activation, having on the one hand the potential for immediate cytotoxicity while being capable of subsequently procuring additional effector responses that are common to fully-activated T cells. Two basic issues from this remain to be resolved. The first is how are IELs maintained as partially-activated cells without causing untoward damage to the epithelium. The second pertains to the signals that drive IELs further through the activation process, thereby rendering them full-fledged effector cells capable of responding to antigen on short notice.

IEL Activation and Apoptosis

At any given time, an unusually high proportion (25–30%) of small intestinal IELs have entered into early stages of apoptosis, with some cells (1–3% overall) in late stage of apoptosis (Fig. 2). There are at least two potential explanations for the apoptotic behavior of the IELs. First, an anomalous activation scheme consisting of ‘activate first, encounter antigen second’ would render a level of immunological economy onto the intestinal immune system by circumventing the need for cells to process through the usual activation steps required for cytotoxic activity *after* antigen has been engaged. This would afford an immediate protective benefit for the host upon antigen encounter; however, a system such as this would not be without risks. The most obvious being the potential for mucosal tissue destruction due to uncontrolled cytotoxicity. Apoptosis would help to hold that process in check. Moreover, it would imply the presence of a well-regulated sequence of events consisting of: (i) entry of T cells into the intestinal epithelium, (ii) local activation, (iii) utilization (or not) of effector-ready cells, (iv) apoptosis.

Is such a scenario likely and, if so, can one find IELs that exist in this dynamic state. Using staining strategies that define IELs as viable cells, cells in early apoptosis, and cells in late apoptosis, this appears to be the case as seen in (Fig. 2.) Moreover, that pattern holds true for both TCR $\alpha\beta$ and TCR $\gamma\delta$ IELs (Fig. 2), indicating that both of the major IEL populations probably follow a similar generalized activational scheme *in situ*.

However, a second possible role for apoptosis may be not as a means for directly curtailing the IEL effector response rather than as a regulatory process used to maintain a homeostatic balance between immunological activation and suppression. Compelling evidence for this now exists in studies of apoptosis among extraintestinal cell populations. The removal of apoptotic leukocytes by resident macrophages has been shown to exert suppressive effects as seen by an inhibition in the synthesis of the proinflammatory molecules IL-1 β , IL-8, IL-10, GM-CSF, TNF α , leukotriene, C4, and thromboxane B2, with simultaneously elevated levels of the anti-inflammatory molecules TGF β 1, prostaglandin E2, and platelet activating factor [28]. Other studies have demonstrated an inhibition of macrophage inflammatory protein (MIP)-1 α and MIP-2 following the ingestion of apoptotic cells by macrophages [29,30]. Accordingly, it has been proposed that engagement of CD8+ T cells by mature dendritic cells in the absence of CD4+ cells will result in CD8+ T cell apoptosis, whereas engagement of CD8+ cells by dendritic cells in the presence of CD4+ T cells will result in CD8+ T cell activation [31]. Those motifs may have implications for understanding the regulation of IELs owing to the skewed CD4:CD8 T cell ratio in the intestinal epithelium, which can reach levels of 1:15. This would result in a preponderance of CD8+ T cells unaccompanied by CD4+ cells, thereby potentiating the process of apoptosis-mediated suppression of CD8+ IELs.

T CELL ACTIVATION AND COSTIMULATION

Constitutive and Regulated Expression of Murine IEL Costimulatory Molecules

T cell costimulation is important for maximizing activation leading to the generation of a full effector response, and it is critical for rescuing T cells from anergy due to indiscriminate ligation of the TCR to host or foreign antigens. On peripheral T cells, CD28 was the first molecule shown to have costimulatory activity [32]. Since then there has been a steady increase in the number of molecules bearing costimulatory properties. However, with the possible exception of some CD4+ and CD8 $\alpha\beta$ + IELs [33], most IELs express only small amounts of CD28 [34–36]. Thus, other T cell costimulatory molecules are likely involved in determining the efficiency with which IELs proceed into activation. Of the three costimulatory molecules to be discussed here, differences exist as to when they are expressed during the activation process. The CD43 core molecule is expressed constitutively on greater than 95% of IELs (Fig. 3A), including both TCR $\alpha\beta$ and TCR $\gamma\delta$ cells (data not shown). In contrast, Ly-6C and OX40 are upregulated on IELs following CD3 stimulation (Fig. 3B). The temporal difference between when those costimulatory molecules are expressed during activation undoubtedly is reflected in function-associated effects since costimulation via CD43 would occur promptly during the early phase of activation, whereas OX40 and Ly-6C costimulation would require a period of ‘incubation’ for their activational properties to be manifest.

Costimulation by CD43

CD43 expression is distributed across a wide range of hematopoietic cells, including T cells, thymocytes, some B cells, and myeloid and lymphoid precursors in the bone marrow [37–40]. Due to the potential for O-linked glycosylation, the CD43 molecule can exist in a variety of molecular isoforms generated by posttranslational modification of the core protein molecule [41–43]. These consist of a 115 kDa form and a 130 kDa form, the latter being expressed primarily on activated T cells [41,44]. Additionally, the existence of a subset of IELs bearing a ‘Glycan-Low’ version of CD43 may represent a unique type of effector cells in the gut, as

discussed below. Because of its ability to exert both positive and negative effects depending upon the phase of the immune response [44–50], few surface molecules of lymphocytes have been more difficult to understand physiologically and functionally. CD43 costimulation of peripheral T cells as well as small intestinal IELs has been shown to enhance T cell proliferation independent of CD28 activation [49–51]. Additionally, recent studies from our laboratory [27] demonstrate significant functional differences in small intestinal IELs depending upon whether they express the neuraminidase-sensitive CD43 determinant recognized by anti-mouse CD43 mAb S7. Thus, while all IELs express the CD43 core molecule, IELs that expressed the CD43 S7 determinant were significantly more cytotoxic and produced more Th1 and Th2 cytokines than S7-IELs [27]. Consistent with that pattern, gene array studies revealed higher levels of gene expression for the T cell markers CD6, CD44, and CD97 in the S7+ population, whereas expression of genes for the NK activating/inhibitory receptors (Ly49E-GE, Ly49G. 2, CD94/NKG2, Ly49H, Ly49C) were more predominantly affiliated with S7-cells [27]. Most important, S7+ but not S7-IELs from the ileum of IL-10^{-/-} with active intestinal inflammation were polarized in a Th1 direction as seen by spontaneous synthesis of IFN- γ in the absence of IL-4 synthesis [27]. Those findings have implications for IEL immunity and immunopathology since they indicate that expression of the neuraminidase-sensitive CD43 glycoform on IELs correlates with cells that have the greatest potential to elicit a strong antigen-specific effector response, though they also are most likely to contribute to a destructive process during chronic intestinal inflammation. Although comparable studies have yet to be done for human IELs, analysis into the role of CD43 and its accompanying isoforms may provide insights into the disease process during IBD.

OX40 Costimulation as a Regulator of Intestinal Immunity

OX40, a member of the TNF receptor superfamily, is of particular interest for costimulation due to its ability to influence T cell function in a variety of ways. On peripheral T cells, this includes an ability to enhance clonal expansion, proliferation, and cytokine production of CD4⁺ and CD8⁺ T cells [52–54]; augmentation of humoral immunity [55]; and the capacity to disrupt T cell tolerance and mediate autoimmune pathologies [56–59].

OX40/OX40L interactions have been shown to play a role in intestinal inflammation in mice and humans. OX40L-Ig fusion protein enhanced T cell proliferation in normal mice, and in mice with TNBS-induced colitis or in IL-2 knockout mice, treatment with OX40L-Ig resulted in an exacerbation of colitis and an increase in the number of OX40⁺ cells in the Peyer's patches and the lamina propria [60]. Conversely, *in vivo* blockade with OX40-Ig reduced the inflammatory response [60]. Those findings have been confirmed in studies of intestinal inflammation in a model in which CD4⁺ CD45RB high cells were adoptively-transferred into severe-combined immunodeficient mice [61]. Additionally, suppressed levels of IFN- γ , IL-2, and TNF α have been described in intestinal tissues following *in vivo* blockade of OX40L [62]. C57BL/6 mice expressing a transgene for OX40L developed spontaneous IBD (as well as interstitial pneumonia) [63]. Interestingly, similar effects were not observed for BALB/c mice [63], thus pointing to a likely genetic basis for that response. Higher frequencies of OX40 expression have been noted in intestinal biopsies of patients with IBD [64,65] or celiac disease [65] compared to tissues from non-affected individuals.

In an attempt to understand the mechanistic basis for the above observations, studies were done in our laboratory to explore the effects of OX40 costimulation on IEL activation. That work revealed that exposure of IELs to immobilized anti-CD3 mAb induced the upregulation of OX40 expression as early as 24 hours post-stimulation [(Fig. 3), and ref. 1]. Curiously, the OX40L also was expressed on CD8⁺ IELs following anti-CD3 activation [1]. While some OX40L⁺ cells undoubtedly were dendritic cells or other activated antigen-presenting cells, the high overall proportion of CD8⁺ cells displaying acquired OX40L expression suggests that

many of those cells were activated IELs. This is consistent with a report of OX40L expression on T cell clones following TCR stimulation [66]. Functionally, OX40 expression was accompanied by IFN- γ synthesis and elevated levels of cell-mediated cytotoxicity [1]. Interestingly, following the upregulation of OX40, costimulation via that molecule resulted in suppressed IL-10 synthesis, an effect that was confirmed in experiments in which blockade of IEL OX40:OX40L interactions reversed the suppressive effects on IL-10 production [1]. Those findings collectively point to a role for OX40 in driving the IELs in a Th1 direction. Although this would be of considerable value during acute antigenic challenge by virus, bacteria, or parasites, it could be a potentially serious problem that would lead to wide-spread tissue destruction if left unchecked.

Ly-6C — A Costimulatory Molecule that Operates through a CD3-Mediated Signal or in a CD3-Independent Manner with CD43 or Type-I IFN

Ly-6C is a GPI-anchored membrane glycoprotein that is expressed on lymphoid and other hematopoietic cells. Its role on T cells remains uncertain, however [67,68]. Initially thought to be a marker of memory T cells [69–71], it is now evident that factors other than prior antigen exposure can influence the expression of Ly-6C. Type I IFNs have been shown to upregulate the expression of Ly-6C on both CD4⁺ and CD8⁺ T cells *in vitro* and *in vivo* [72,73]. Most convincing, induction of Ly-6C expression occurred in normal mice and in IFN- γ –/– mice but not in IFN- α RI–/– mice following CpG oligodeoxynucleotide stimulation [74]. Compatible with those studies, poly (I:C) has been shown to induce Ly-6C expression on T cells in the absence of antigenic stimulation [75]. Functionally, direct stimulation of CTL clones *via* Ly-6C resulted in proliferation and IL-2 synthesis independent of the presence of antigen-presenting cells, Ly-6C crosslinking, or mitogenic stimulation [76]. Blockade of surface Ly-6C interactions inhibited cytolytic activity and IFN- γ production of CTL [77]. Based on cDNA sequence analysis, human CD59, i.e., protectin, is a structural homologue of murine Ly-6C [78,79], although functional differences have been noted between CD59 and Ly-6C [80]. Evidence supporting a role for CD59 in IBD has been equivocal. In one study, CD59 expression was decreased in intestinal tissues of IBD patients compared to control tissues [81], whereas a second study reported an increase in CD59 expression in IBD patients [82].

Few studies have been done that examine the effects of Ly-6C stimulation of IELs under experimental conditions. In mice, freshly-isolated IELs express little if any Ly-6C [2,83]; however, Ly-6C was upregulated within 24 hrs of anti-CD3 stimulation *in vitro* [(Fig. 3) and ref. 2]. Consistent with the observations described for the extraintestinal CD8⁺ T cells cited above [72,73], expression of Ly-6C also was upregulated on IELs following exposure to IFN- α (Fig. 4). That property appeared to be distinct for Ly-6C since a similar effect was not observed for OX40 under similar IFN- α culture conditions (Fig. 4). Interestingly, when IELs were stimulated for 48 hours *directly* with anti-CD43 mAb in the absence of anti-CD3 stimulation, the Ly-6C antigen was upregulated despite a lack of change in the expression of OX40 (Fig. 3). Those findings suggest that there are at least two pathways to modulate Ly-6C expression on IELs. In the first, Ly-6C expression would be upregulated following stimulation through a classical antigen-mediated activation pathway upon stimulation of the TCR/CD3 complex (Fig. 3B). The second pathway would be an antigen-independent process mediated either by IFN- α or by direct CD43 stimulation. This antigen-independent model is illustrated in (Fig. 5) based on the empirical evidence (Experimental Model) using IFN- α or direct engagement with anti-CD43 mAb. Under natural conditions, this could be manifest in several ways as illustrated in (Fig. 5) (Predicted Natural Model). For example, local virus infection of enterocytes or other intestinal cells would result in the release of IFN- α that would facilitate the expression of Ly-6C on neighboring IELs. Alternatively, antigen-independent upregulation of Ly-6C on IELs under natural conditions could occur upon binding of the CD43 moiety to sialoadhesin, the CD43 ligand [84] expressed on intestinal macrophages. Subsequently, direct

binding of Ly-6C to its natural ligand expressed on B cells or CD11b+ cells [85] in the intestinal epithelium would have two effects. First, it would promote clonal expansion of IELs. Second, it would lead to the synthesis of IL-2 (and possibly to the release of other immunoregulatory or proinflammatory cytokines). Both of those events would occur in the absence of TCR/CD3 stimulation. Importantly, they would constitute an effective mechanism for IEL mobilization prior to the recognition of antigen by IELs. The response of antigen-specific IELs eventually recruited into the affected tissue sites would then lend strength and specificity to the immune response.

SUMMARY

The events involved in determining how IELs proceed through activation continue to be poorly understood. As described here, intestinal T cells in mice are sustained in a novel state of activation, which in many ways is emblematic of the situation observed for human intestinal T cells [6,86]. Understanding that activation process will greatly facilitate approaches aimed at designing more effective intestine-based vaccines, and will contribute to developing immunotherapeutic approaches for controlling wanton intestinal inflammation. By dissecting the involvement of costimulatory molecules in the IEL activation cascade, it will be possible to begin to achieve those goals.

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Fig. (1). Murine small intestinal IELs express CD69 (**A**) *ex vivo* and (**B**) *in situ*. (**C**) Freshly-isolated IELs but not spleen cells are cytotoxic in redirected cytotoxicity assays. (**D**) following anti-CD3 stimulation *in vitro*, CD8⁺ IELs synthesize IFN- γ at higher levels and more rapidly than an equivalent number of CD8⁺ lymph node cells (LNCs).

Fig. (2).

In fresh IELs preparations, 25–30% of all cells are in the early stage of apoptosis as seen by annexin V staining; this includes some of both $TCR\alpha\beta$ and $TCR\gamma\delta$ IELs. A very small percentage of the total IELs, generally <3%, are in the late stage of apoptosis as seen by incorporation of both annexin V and propidium iodide.

Fig. (3). Murine small intestinal IELs constitutively express (A) CD43 but (B) lack expression of the OX40 and Ly-6C costimulatory molecules. Following anti-CD3 stimulation, IELs acquire expression of OX40 and Ly-6C. Direct stimulation of IELs through CD43 in the absence of CD3 stimulation results in the upregulation of Ly-6C but not OX40.

Fig. (4).

In vitro culture of murine small intestinal IELs in the presence of IFN- α induces the expression of Ly-6C but not OX40.

Fig. (5).

Two mechanisms for antigen-independent upregulation of Ly-6C on small intestinal IELs. In the 'Experimental Model', based on the findings described in Figs. 3 and 4, expression of Ly-6C can be upregulated either by direct exposure to IFN- α or by direct ligation of CD43. In the 'Predicted Natural Model', changes in Ly-6C expression would be manifest following exposure to IFN- α released from nearby virus infected tissue cells. Alternatively, upregulation in Ly-6C expression could occur upon direct engagement of CD43 to its ligand, sialoadhesin [84], on macrophages. The presence of Ly-6C expression on IELs would result in TCR-independent IEL proliferation and IL-2 synthesis upon binding to the Ly-6C ligand that is expressed on B cells or CD11b+ cells [85]; both types of cells are present in the small intestine epithelium.