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The editors of The Journal of Experimental Medicine have been notified by Dr. Linde Meyaard of the University Medical Center Utrecht, The Netherlands, that she and the other authors of the above Brief Definitive Report wish to retract the paper.

The authors state:

“Recent studies by our laboratory have revealed that EpCAM is not a ligand for human LAIR-1 and LAIR-2. The prior experiments showing that LAIR-1–hIg and LAIR-2–hIg fusion proteins bind to EpCAM transfectants were an artifact resulting from contamination of the fusion proteins with an anti–human EpCAM monoclonal antibody, which was affinity purified using the same protein A columns.”

The authors extend their apologies to the scientific community.
The Epithelial Cellular Adhesion Molecule (Ep-CAM) Is a Ligand for the Leukocyte-associated Immunoglobulin-like Receptor (LAIR)

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Abstract

Human leukocyte-associated immunoglobulin-like receptor (LAIR)-1 is expressed on many cells of the immune system and is predicted to mediate inhibitory functions based on the presence of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in its cytoplasmic domain. Although the role of LAIR-1 in the regulation of immune responses in vivo is unknown, LAIR-1 cross-linking by monoclonal antibodies inhibits various immune cell functions in vitro. Here, we identify the colon carcinoma-associated epithelial cellular adhesion molecule (Ep-CAM) as a ligand for LAIR-1 and LAIR-2, a related soluble LAIR-1 family member. Ep-CAM interacts with the LAIR molecules through its epidermal growth factor domain; Ep-CAM-specific antibodies can abrogate the binding. Intraepithelial T lymphocytes express LAIR-1 and Ep-CAM may contribute to mucosal tolerance and that

Introduction

Inhibitory receptors, bearing immunoreceptor tyrosine-based inhibitory motifs (ITIMs), play a pivotal role in balancing the immune response (for a review, see reference 1). Rather than relaying activation signals, these receptors suppress cellular functions. One or more ITIMs in the cytoplasmic tail are responsible for the inhibitory signal upon ligation of the receptors by their ligand. ITIMs are short amino acid motifs with the consensus sequence I/V/L/SxYxxL/V, which are able to recruit cytoplasmic protein tyrosine phosphatases upon phosphorylation of the central tyrosine residue. These phosphatases abrogate signaling through activating receptors, thereby preventing cellular immune functions such as cytotoxicity or proliferation. In recent years, many novel inhibitory receptors have been identified and recognized to be necessary for the immune system to prevent excessive activation or autoimmunity.

The leukocyte-associated Ig-like receptor (LAIR)-1 (2) is a member of the Ig superfamily that is expressed on the majority of peripheral blood mononuclear cells, including NK cells, T cells, B cells, monocytes, and dendritic cells, as well as the majority of thymocytes. Cross-linking of LAIR-1 by mAb in vitro delivers a potent inhibitory signal that is capable of inhibiting cellular functions of NK cells, effector T cells, B cells, and dendritic cell precursors (2–5). In addition to LAIR-1, we identified LAIR-2, a putative secreted protein that is 84% homologous to LAIR-1 (3).

LAIR-1 has two ITIM motifs and is structurally related to human killer cell Ig-like receptors (KIRs) and the immunoglobulin-like transcripts (ILTs/LIRs). The KIRs and some of the ILTs recognize MHC class I and are thought to play a role in the prevention of autoimmunity (for a review, see reference 6). Many of the ITIM-bearing inhibitory receptors are members of multigene families that con-
tain genes encoding activating receptors. The activating isoforms are characterized by having short cytoplasmic domains and basic amino acids within their transmembrane regions. These receptors signal through their association with immunoreceptor tyrosine-based activating motif (ITAM)-bearing transmembrane adaptor molecules, such as FcεRIγ or DAP12 (7, 8).

Within the family of ITIM-bearing receptors, LAIR-1 is unique because it is extremely broadly expressed and does not recognize MHC class I (2). Furthermore, sequencing of the genomic region where the LAIR genes are located suggests that there are only two LAIR genes, neither of which is predicted to encode an activating receptor (9). To delineate the biological function of LAIR-1, identification of the natural ligand is imperative. We here report the identification of epithelial cellular adhesion molecule (Ep-CAM) as a binding partner for LAIR.

Materials and Methods

Cell Lines. 293T cells were provided by T. Kitamura (DNAX Research Institute). HT29 cells were obtained from American Type Culture Collection.

Abs. The mouse anti-LAIR-1 mAb DX26 was described previously (2). The 8A8 (IgG1) producing hybridoma was generated by fusing the Sp2/0 myeloma cell line with splenocytes from a BALB/c mouse immunized with purified LAIR-1L protein.

323/A3 is a mouse anti–human Ep-CAM mAb (10) and UBS54 a BALB/c mouse immunized with purified LAIR-1. The 8A8 (IgG1) producing hybridoma was generated and identified by fusing the Sp2/0 myeloma cell line with splenocytes from a BALB/c mouse immunized with purified LAIR-1L protein.

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Detection of LAIR-1 Ligand. The leader sequence and the first 162 amino acids (1–162) fused to the Fc portion of human IgG1 (LAIR-1-hIg) was produced by transient expression in 293T cells. Subsequent purification was achieved by using protein A sepharose columns. Cell lines were tested for the presence of a putative LAIR-1 ligand, 10^6 cells were incubated for 1 h at room temperature (RT) with 0.5 μg/mL LAIR-1-hIg, 1% BSA, 2% FCS, and 10 μg/mL anti–Ep-CAM mAb. Upon washing, 10 μg/mL biotin-conjugated goat anti–human-IgG1 (Caltag Laboratories) was added for 15 min at RT, followed by washing and 15 min incubation with phycoerythrin-conjugated streptavidin. Cells were analyzed on a FACScalibur with the addition of propidium iodide to exclude dead cells. As control IgG, either 2% pooled human serum (HPS) or a mouse CTLA4-hIg protein was used, both giving similar results.

Cloning of LAIR-1 Ligand. The colorectal carcinoma cell line HT29 was found to highly express LAIR-1 ligand as assayed by LAIR-1-hIg binding. A cDNA library from this cell line was constructed into the pCDNA3.0 vector using oligo-dT–primed cDNA. cDNA cloning by transient transfection into 293T cells was performed as described (12) with modifications (13). Four independent cDNA clones were obtained and sequenced.

Generation of Ep-CAM Deletion Mutants. Deletion mutants of the human Ep-CAM cDNA were constructed by using PCR. PCR fragments of the extracellular domain of Ep-CAM were cloned in frame into a pCDNA3.1 vector with an NH2-terminal Ep-CAM leader sequence, a COOH-terminal transmembrane region, and an intracellular domain of Ep-CAM, followed by a Myc epitope tag. All constructs were confirmed by nucleotide sequencing. After transfection into 293T cells, expression of the protein was checked by Western blotting using an anti-Myc mAb. Membrane expression and transfection efficiency was monitored by staining of methanol-fixed transfected cells with anti-Myc mAb.

Isolation and Staining of Intratumoral Lymphocytes. Intratumoral lymphocytes (IELs) were isolated from the colon of donors that underwent partial colon resection because of malignancies. Unaffected parts of the colon were used to isolate IELs as described previously (14). Cells were stained immediately after isolation with anti-CD3 and anti–LAIR-1 Abs and analyzed by flow cytometry. All tissues were handled according to the guidelines of the ethical committee of the University Medical Center Utrecht on the use of human subjects in medical research.

Immunohistology. Human ileum sections were snap frozen in liquid nitrogen and stored at −70°C. Frozen sections (6 μm) were cut, mounted on glass slides, dried at RT, and fixed in 3.7% formaldehyde in PBS at RT for 10 min. The sections were washed with PBS containing 1.5% glycine and incubated with biotin–conjugated UBS54 (anti–Ep-CAM) and a mixture of anti–Ep-CAM mAb UBS54 and 8A8 for 1 h at RT. After washing, sections were incubated with alkaline phosphatase–conjugated streptavidin and with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti–human-IgG1 Abs and FITC-conjugated streptavidin. Tissue sections were counterstained with propidium iodide (molecular probe) and mounted in fastar (Vector Laboratories).

Cells and DNA. Ep-CAM is a LAIR-1 ligand. To identify the natural ligand we constructed a fusion protein of the extracellular domain of LAIR-1 and the Fc portion of human IgG1 (LAIR-1-hIg). This protein was used as a staining reagent to screen cell lines. Human colon carcinoma cell lines bound this fusion protein but not a control Ig fusion protein. The results of these experiments are shown in Figure 1. The natural ligand for LAIR-1 was identified as Ep-CAM.

Figure 1. LAIR-1L is identical to Ep-CAM. (A) LAIR-1L-hIg binding to the colon carcinoma cell line HT29, demonstrating the presence of a putative LAIR-1L. (B) LAIR-1L-hIg binding to 293T cells transfected with the LAIR-1L cDNA from the HT29 cell line. (C) LAIR-1L-hIg binding to 293T cells transfected with the LAIR-1L cDNA from the HT29 cell line. (D) LAIR-1L-hIg binding to 293T cells transfected with an irrelevant cDNA. (B and D).
Ep-CAM is a 38-kD transmembrane glycoprotein that is expressed on the surface of human simple epithelia in close proximity to Ep-CAM-expressing intraepithelial lymphocytes (16, 17). Therefore, we investigated whether LAIR-1-expressing cells in the intestine might be able to interact with Ep-CAM on the basolateral surface of the majority of human simple epithelial cells. Isolated intraepithelial lymphocytes were stained for LAIR-1 and anti–Ep-CAM Abs demonstrating expressing intestinal intraepithelial lymphocytes in close proximity to Ep-CAM–expressing epithelial cells (Fig. 5 B). This suggests that these cells might be subject to downregulation through Ep-CAM–LAIR-1 interactions, which might provide a mechanism to regulate mucosal immunity in the intestine.

**Ep-CAM Binds Both LAIR-1 and LAIR-2.** We previously described a gene highly related to LAIR-1, designated LAIR-2 (2). LAIR-2 has 84% amino acid homology in the Ig domain with LAIR-1, and cDNA for two different splice variants were identified (3). LAIR-2 is not recognized by the LAIR-1–specific mAbs (data not shown). We conclude that Ep-CAM binds to the LAIR molecules through its first EGF-like repeat.

Ep-CAM Contains a Domain with Epidermal Growth Factor-like Domain. To determine which domain of Ep-CAM was responsible for binding to the LAIR molecules, deletion constructs of Ep-CAM were designed. Ep-CAM contains two epidermal growth factor (EGF)-like repeats in its extracellular domain and has a short cytoplasmic domain (15). Various PCR fragments containing the different domains of the extracellular part of Ep-CAM were produced (Fig. 4 A). These were ligated into a vector containing the leader sequence of Ep-CAM at the NH2 terminus and the transmembrane region and cytoplasmic tail at the COOH terminus. All constructs were Myc-tagged at the COOH terminus. Upon transfection in 293T, protein expression was compared by anti–Myc Western blotting and transfection efficiency was assessed by staining of methanol-fixed cells with anti–Myc Abs. Microscopic analysis of the stained cells indicated membrane expression of the proteins encoded by the various constructs and protein expression and transfection efficiency was comparable for all constructs (data not shown). The only construct, apart from full-length Ep-CAM, that retained binding of LAIR-1-hlg, was a construct containing the first EGF-like repeat from Ep-CAM (Fig. 4 B). Similar results were obtained for LAIR-2-hlg (data not shown). We conclude that Ep-CAM binds to the LAIR molecules through its first EGF-like repeat.

This observation is in accordance with the finding that the anti–Ep-CAM mAb 323/A3 was able to block Ep-CAM–LAIR interaction. The epitope for this mAb maps to the first EGF-like domain of Ep-CAM (15).

**Intraepithelial Lymphocytes Express LAIR-1.** Ep-CAM is involved in Ep-CAM binding to the LAIR molecules, which might provide a mechanism to regulate mucosal immunity in the intestine.
Ep-CAM Is a Ligand for LAIR

Figure 4. LAIR binds to the first EGF domain of Ep-CAM. (A) Myc-tagged deletion constructs of human Ep-CAM (LK57-LK63) and wild-type human Ep-CAM (LK17) were transfected into 293T cells and binding of LAIR-1-hlg and LAIR-2-hlg was assessed by flow cytometry. Results are indicated with ++ for strong binding and – for absence of binding. The domains of Ep-CAM are indicated with boxes (15). Amino acids (aa) are indicated with numbers, starting with the first methionine. Leader, leader peptide (aa 1–22); EGF-1, EGF-like domain 1 (aa 27–59); EGF-2, EGF-like domain 2 (aa 66–135); TM, transmembrane region (aa 267–288); myc, myc tag. (B) Representative histograms of LAIR-1-hlg binding to cells transfected with the constructs indicated. Cells were stained with LAIR-1-hlg (filled histograms) or control hIg (open histograms). The middle panel represents the binding to cells transfected with constructs LK57 through LK62. Similar results were obtained with LAIR-2-hlg (data not shown).

Figure 5. LAIR-1 is expressed on human intestinal intraepithelial lymphocytes. (A) IELs isolated from colon stained with anti-CD3 and gated on CD3-positive cells. Filled histogram represents staining with anti-LAIR-1 mAb and open histogram with isotype control mAb. (B–E) Sections from human ileum were stained with anti-LAIR-1 mAb (red), anti-Ep-CAM mAb (green), and DAPI (blue), and analyzed on a fluorescence microscope. C and E are higher magnifications of the areas indicated in B and D, respectively. Intraepithelial lymphocytes are indicated with white arrows.
The physiological role proposed for Ep-CAM so far is in intercellular adhesion (18). It has been shown to function as a Ca\(^{2+}\)-independent homophilic intercellular adhesion molecule (19). Ep-CAM resembles known adhesion molecules, although the adhesion mediated by Ep-CAM is relatively weak and its role in maintaining tissue integrity is uncertain (15). Ep-CAM is also abundantly and homogeneously expressed on human carcinomas of different origins. Recently, Ep-CAM has been noted as a promising target for immunotherapy (20). The advantage for tumor cells to express Ep-CAM and the mechanisms of action of anti-Ep-CAM therapy have not yet been elucidated. It is tempting to speculate that Ep-CAM expression might equip tumor cells with a tool to suppress antitumor responses through its interaction with LAIR-1. Preliminary studies have failed to demonstrate diminished NK cell lysis against Ep-CAM-bearing target cells. Further studies are required to address this issue.

We demonstrate that the widely expressed immune inhibitory receptor LAIR-1 has an abundantly expressed ligand, Ep-CAM. Therefore, tight regulation of this interaction is probably needed, which might be provided at different levels. First, LAIR-1 expression can be regulated on cells in different stages of differentiation or upon activation, as we previously demonstrated for B cells on which LAIR-1 expression is tightly regulated (4). In addition, we have demonstrated that LAIR-2 can bind the same ligand as LAIR-1, suggesting regulation of LAIR-1 function by LAIR-1 competition for the same ligand.

Ep-CAM molecules have been detected in several species, including mice (21). In addition, some mouse Ep-CAM (data not shown). An alignment of natural homologues of LAIR molecules identified in human and murine homologues of LAIR-1 have been identified in rodents. The human LAIR gene is located on chromosome 19q13.4, in close proximity to the KIR and ILT genes. These gene families are not conserved in evolution, such that counterparts of these human receptors have not been identified in mice (23). However, KIRs have functional homologues in the mouse, the structurally unrelated Ly49 family of molecules. It will be interesting to pursue the possibility that mouse Ep-CAM has a cellular receptor of unrelated sequence, yet with similar function to human LAIR.

In summary, our data reveal a novel interaction between two previously known proteins. We propose that Ep-CAM, through interaction with the inhibitory receptor LAIR-1, plays a role in the control of mucosal immune responses, possibly preventing excessive inflammatory responses in regions, like the intestine, with high antigen exposure. To further establish the biological importance of the interaction of LAIR with Ep-CAM, experimental evidence that Ep-CAM binding can stimulate the inhibitory function of LAIR-1 is needed.

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