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Leukocyte-Associated Ig-Like Receptor-1 Functions as an Inhibitory Receptor on Cytotoxic T Cells[1]

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Leukocyte associated Ig-like receptor-1 (LAIR-1) is a surface molecule expressed on human mononuclear leukocytes that functions as an inhibitory receptor on human NK cells. In addition to NK cells, LAIR-1 is expressed on T cells, B cells, macrophages, and dendritic cells. Most cells express two biochemically distinct forms of LAIR-1, which we now show are likely alternative splice variants of the same gene. Cross-linking of LAIR-1 on human T cell clones results in inhibition of cytotoxicity only in T cell clones that lack CD28 and are able to spontaneously lyse certain targets in vitro. Moreover, the cytolytic activity of freshly isolated T cells, which is thought to be mainly due to "effector" T cells, can be inhibited by anti-LAIR-1 mAb. Thus, LAIR-1 functions as an inhibitory receptor not only on NK cells, but also on human T cells. This indicates that LAIR-1 provides a mechanism of regulation of effector T cells and may play a role in the inhibition of unwanted bystander responses mediated by Ag-specific T cells. ***The Journal of Immunology*****, 1999, 162: 5800–5804.**

Mechanisms that terminate the immune response are important for the maintenance of the balance of the immune system. To ensure tolerance and to prevent autoimmunity, tight regulation of effector functions is necessary. Several mechanisms are known to provide this regulation. These include active cell death, death by lack of growth factors, anergy, and active negative feedback mechanisms mediated by cytolytic T lymphocyte-associated antigen-4 and cytokines (reviewed in Ref. 1). Negative regulation of immune cells can also be provided for by inhibitory receptors that, by virtue of the immunoreceptor tyrosine based inhibitory motifs (ITIMs)[3] in their cytoplasmic tails, can lead to dephosphorylation of positive signal transduction events upon ligand binding (2).

The role of inhibitory receptors has been extensively studied on NK cells. Ligation of human killer cell inhibitory receptors (KIRs) on NK cells by MHC class I molecules on the target cells, leads to inhibition of target cell lysis (reviewed in Ref. 3). KIRs are also expressed on a small subset of human T cells, predominantly $CD28^-$ memory cells, and are functional in inhibiting cytotoxicity and cytokine production of these cells (4–7). Recently, additional families of inhibitory receptors have been identified both in humans and mice (reviewed in Ref. 8). Many of these inhibitory receptors are related to the KIRs (3). In mice, the gp49B molecule (9) functions as an inhibitory receptor on mouse mast cells and NK cells (10, 11). Similarly, cross-linking of gp91 or paired Ig-like receptors leads to inhibition of B cell receptor-mediated activation of B cells in vitro (12–15). The ligands of gp49 and paired Ig-like receptors are not known yet.

In humans, a novel group of inhibitory molecules has been identified that is mainly expressed on monocytes and B cells, although some family members are found on NK and T cells. These molecules have been called Ig-like transcripts, leukocyte Ig-like receptors, monocyte/macrophage Ig-like receptors, or HM18, by the laboratories that reported the first cDNA sequences (16–21). Some of the family members bind MHC class I molecules or viral MHC class I homologues (18, 19, 22). Cross-linking of the receptors by mAb inhibits the Ag presentation by APC (17), as well as activation of B cells, T cells, NK cells, and macrophages (22, 23).

We previously reported on the identification of leukocyte-associated Ig-like receptor-1 (LAIR-1) (24). LAIR-1 is a member of the Ig superfamily that is expressed on the majority of human PBMCs, including NK, T, B, monocytes, and dendritic cells, as well as the majority of thymocytes. It is a type I transmembrane glycoprotein with a single Ig-like domain in the extracellular region and a cytoplasmic tail containing two ITIMs. Cross-linking of LAIR-1 on human NK cells by mAb delivers a potent inhibitory signal that is capable of decreasing target cell lysis by both resting and activated NK cells in vitro (24).

Compared with other inhibitory receptors reported so far, LAIR-1 is unique with its broad expression pattern, which implies that it will play a role in a variety of immune interactions. Here we describe another member of the LAIR family and demonstrate that LAIR-1 can function as an inhibitory receptor on effector T cells.

Materials and Methods

Cells

Peripheral blood from healthy donors was purchased from Stanford Blood Center (Stanford, CA). PBMC were isolated by Ficoll-Hypaque centrifugation. T cell clones (TCCs) were established as described (4) and maintained in culture using the method of Yssel et al. (25).

The HLA class I-deficient EBV-transformed lymphoblastoid cell line 721.221 has been described (26). DT287 is a stable transfectant of 721.221, expressing human FcγRII (CD32), generated in our laboratory. 293 cells expressing SV40 large T Ag (293T) were generously provided by Dr. T. Kitamura (DNAX, Palo Alto, CA). Jurkat and P815 were obtained from the

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[3] Abbreviations used in this paper: ITIM, immunoreceptor tyrosine-based inhibitory motif; KIR, killer cell inhibitory receptor; TCC, T cell clone; LAIR, leukocyte-associated Ig-like receptor.

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American Type Culture Collection (Manassas, VA). YT-2C2 is a NK-like tumor cell line that was kindly provided by Dr. K. Smith (27).

Monoclonal Abs

The mAb against LAIR-1 (DX26, IgG1) (24) has been described. The anti-CD3 mAb (CLB-T3/4.1, IgG1) was a kind gift of Dr. René van Lier (CLB, Amsterdam, The Netherlands). All other mAbs were generously provided by Becton Dickinson Immunocytometry Systems (San Jose, CA).

Cytotoxicity assay

Cell lines were labeled with ^{51}Cr and used as targets in a 4- or 7-h radioisotope release assay, as described (28). Data are expressed as the mean of triplicate cultures. Spontaneous radioisotope release did not exceed 10% of maximum release as determined by lysis of target cells with 10% Triton-X 100. The percentage of specific lysis was calculated as: [(cpm specific ^{51}Cr release − cpm spontaneous ^{51}Cr release)/(cpm maximum ^{51}Cr release − cpm spontaneous ^{51}Cr release)] × 100.

Expression cloning

A cDNA library from Jurkat T cells was generously provided by Dr. Terrill McClanahan (DNAX). cDNA cloning by transient expression in 293T cells was performed as described (29) with modifications (30).

cDNA was sequenced using the dideoxy termination technique with Sequenase 2.0 kit (United States Biochemical, Cleveland, OH) and by using an automated nucleic acid sequencer (Applied Biosystems, Foster City, CA).

Generation of stable transfectants of LAIR-1

Stable transfectants of LAIR-1a and -1b were generated as described (31). In short, LAIR-1a and LAIR-1b-encoding cDNAs were subcloned into the pMX puro retroviral vector (provided by T. Kitamura, DNAX), and transfected into 293T cells together with a packaging vector (31, 32). The supernatant was used to infect YT.2C2 cells. Cells were drug selected and transfectants with high expression of LAIR were sorted, using the FACStar flow cytometer (Becton Dickinson).

Biochemistry

To biochemically characterize the Ags recognized by anti-LAIR-1 mAb, cells were labeled with ^{125}I and Ags immunoprecipitated by the procedure described previously (33). Immunoprecipitates were separated by SDS-PAGE and visualized by exposure to a Phosphor Imager screen.

Results

Jurkat T cells express LAIR-1b

We previously reported that human NK cells express two biochemically distinct forms of LAIR-1. The majority of the LAIR-1 protein in NK cells has an apparent molecular weight of ~40 kDa, but we also detected a form of LAIR-1 of ~34 kDa (24). Molecular cloning of LAIR-1 from an NK cell library, revealed a cDNA that encoded a protein of ~40 kDa (now designated LAIR-1a), in agreement with the majority of the immunoprecipitated LAIR-1 protein in NK cells (24). Immunoprecipitation of LAIR proteins from the human T cell tumor Jurkat revealed exclusive expression of the ~34 kDa species, while human PBMC (containing a majority of T cells) expressed both, but seem to have more of this short form (Fig. 1).

To study the nature of the LAIR-1 protein in Jurkat T cells, 293T cells were transfected with a cDNA library prepared from Jurkat T cells and a cDNA encoding LAIR-1 was expression cloned (30). As shown in Fig. 2*A*, a single cDNA was obtained that encoded a protein recognized by anti LAIR-1 mAb. We designated this cDNA *LAIR-1b*. The *LAIR-1b* cDNA was 1537 bp containing an 813-bp open reading frame encoding a type I membrane protein with a 21-aa leader sequence, 125-aa extracellular domain, 23-aa transmembrane domain, and a 101-aa cytoplasmic region. When the nucleotide and amino-acid sequence were aligned to that of the previously cloned LAIR-1a, it showed that the LAIR-1b protein is identical to LAIR-1a, with the exception of a 17-aa deletion in the stalk region, between the Ig domain and the transmembrane region (Fig. 2*B*).

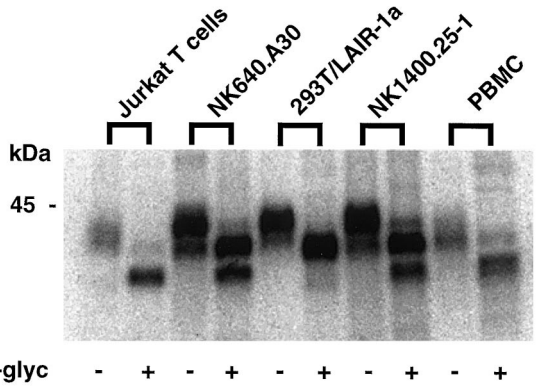

FIGURE 1. Two biochemically distinct LAIR-1 proteins. 293T cells, transfected with the LAIR-1a-encoding cDNA (293T/LAIR-1a), two different human NK cell clones (NK640.A30 and NK1400.25-1), Jurkat T cells, and human PBMC were labeled with ^{125}I, lysed, and LAIR-1 Ag was immunoprecipitated with anti-LAIR-1 mAb. The immunoprecipitates were treated with *N*-glycosidase where indicated and analyzed by SDS-PAGE under reducing conditions.

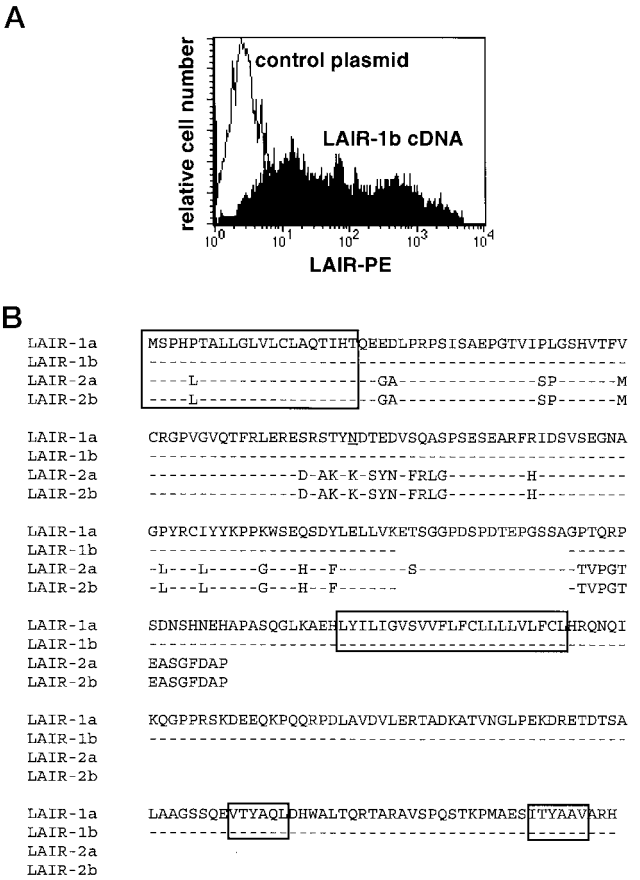

FIGURE 2. cDNA cloning of LAIR-1b. *A*, LAIR-1b expression on 293T cells transfected with the pMET7 vector containing an irrelevant insert (open histogram) or *LAIR-1b* cDNA (shaded histogram). *B*, Alignment of the predicted amino acid sequences of LAIR-1a, LAIR-1b, LAIR-2a, and LAIR-2b. The leader sequence, putative transmembrane domain, and ITIM sequences in the cytoplasmic domain are boxed. The potential *N*-linked glycosylation site in the extracellular domain is underlined. Conserved residues are indicated by dashes; gaps are indicated by spaces. The LAIR-1b sequence data are available from EMBL/GenBank/DDBJ under accession number AF109683.

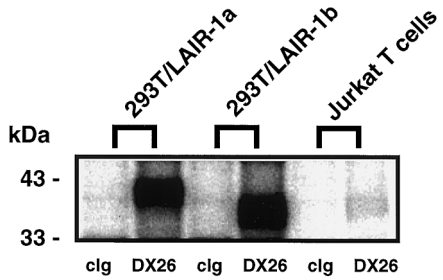

FIGURE 3. LAIR-1b is expressed on Jurkat T cells. 293T cells, transfected with the LAIR-1a-encoding cDNA or the LAIR-1b-encoding cDNA and Jurkat T cells were labeled with ^{125}I, lysed, and LAIR-1 was immunoprecipitated with anti-LAIR-1 mAb (DX26) or control IgG1 (cIg).

Immunoprecipitation of LAIR-1a and LAIR-1b proteins from cells transfected with the respective cDNAs and from Jurkat T cells showed that LAIR-1b indeed runs at the same position as the ~34 kDa LAIR-1 protein in Jurkat cells (Fig. 3).

LAIR-1b is able to function as an inhibitory receptor in NK cells

To compare the capacity of LAIR-1a and LAIR-1b to function as inhibitory receptors, the LAIR-1-negative NK cell line, YT.2C2, was stably transfected with LAIR-1a or LAIR-1b. Anti-LAIR-1 mAb efficiently inhibited the cytotoxic activity of both the LAIR-1a and LAIR-1b-transfectants but not the wild-type YT.2C2 against the human FcγRII (CD32)-transfected EBV-transformed B cell line, 721.221/CD32 (Fig. 4, *lower panels*). Cytolysis of the FcR-negative parent line, 721.221, however, was not affected by anti-LAIR-1 mAb, indicating that FcR cross-linking of LAIR-1a or -1b was required to deliver the negative signal (Fig. 4, *upper panels*). These data confirmed that the inhibitory effect of anti-LAIR-1 mAb on cells naturally expressing LAIR-1 (24) was due to LAIR-1 cross-linking. Furthermore, it showed that both LAIR-1a and LAIR-1b were able to function as inhibitory receptors in an NK cell line upon mAb cross-linking.

LAIR-1 functions as an inhibitory receptor on human CD28$^-$ T cell clones

Since LAIR-1 is expressed on the majority of human T cells, we set out to study whether it is also capable of inhibiting T cell

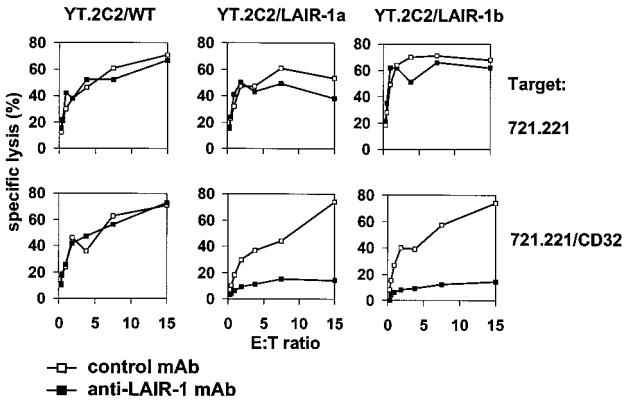

FIGURE 4. Both LAIR-1a and LAIR-1b function as an inhibitory receptor in an NK cell line. YT.2C2 cells (*left panels*) or YT.2C2 cells stably transfected with LAIR-1a (*middle panels*) or LAIR-1b (*right panels*) were assayed in a 4-h ^{51}Cr-release assay for lysis of 721.221 or 721.221 cells expressing CD32, in the presence of anti-LAIR-1 mAb (●) or control IgG1 (○). Anti-LAIR-1 mAb and control IgG1 were used at 5 μg/ml. Data are representative of three independent experiments.

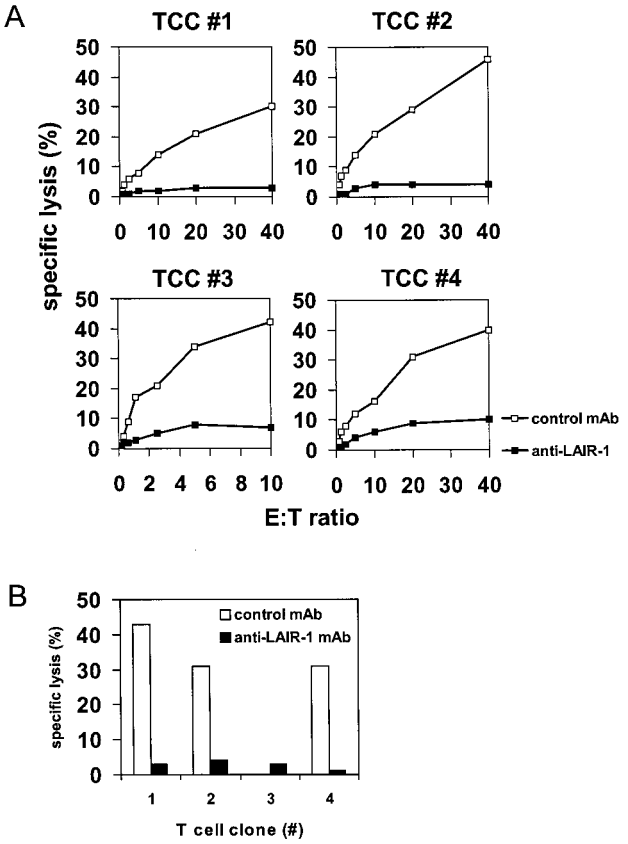

FIGURE 5. Anti-LAIR-1 mAb inhibits the spontaneous cytotoxicity of CD28$^-$ TCC. Human TCC, derived from CD8$^+$CD28$^-$ T cells from peripheral blood from a healthy donor were assayed in a 4-h ^{51}Cr-release assay for lysis of 721.221 transfectants expressing human CD32 (*A*) or FcR$^+$ P815 (*B*) in the presence of anti-LAIR-1 mAb (filled symbols) or control IgG1 (open symbols). Anti-LAIR-1 mAb and control IgG1 were used at 5 μg/ml. Data are representative of three independent experiments.

function, in addition to its inhibitory function on NK cells. Initially, we did not see any effect of anti-LAIR-1 mAb on T cell function in vitro. Proliferation of peripheral blood T cells in response to recall Ag, alloantigen, or anti-CD3 mAb could not be inhibited by anti-LAIR-1 mAb (data not shown). Cytotoxic activity against FcR-bearing targets mediated by several TCCs stimulated by anti-CD3 or superantigen could also not be inhibited by anti-LAIR-1 mAb (data not shown).

However, we did see inhibitory effects of the anti-LAIR-1 mAb when we used TCCs generated from the CD28-negative subset of CD3$^+$CD8$^+$ T cells from peripheral blood. Some of these TCCs are able to exhibit spontaneous cytotoxic activity against targets without addition of anti-TCR/CD3 mAb. Cytotoxic activity of these TCCs against the EBV B cell line 721.221, transfected with the human FcγRII, was effectively inhibited by anti-LAIR-1 mAb (Fig. 5*A*). Three clones were able to also spontaneously lyse P815 target cells, and this cytotoxicity was also inhibited by the addition of anti-LAIR-1 mAb (Fig. 5*B*). This demonstrates that LAIR-1 can function to inhibit the cytotoxic activity of human CD28$^-$ TCCs in vitro.

LAIR-1b functions as an inhibitory receptor on freshly isolated T cells

Peripheral blood T cells are able to lyse target cells in vitro when stimulated via the TCR/CD3 complex. This cytotoxic activity is thought to be due mainly to the "effector" population of CD8$^+$ T

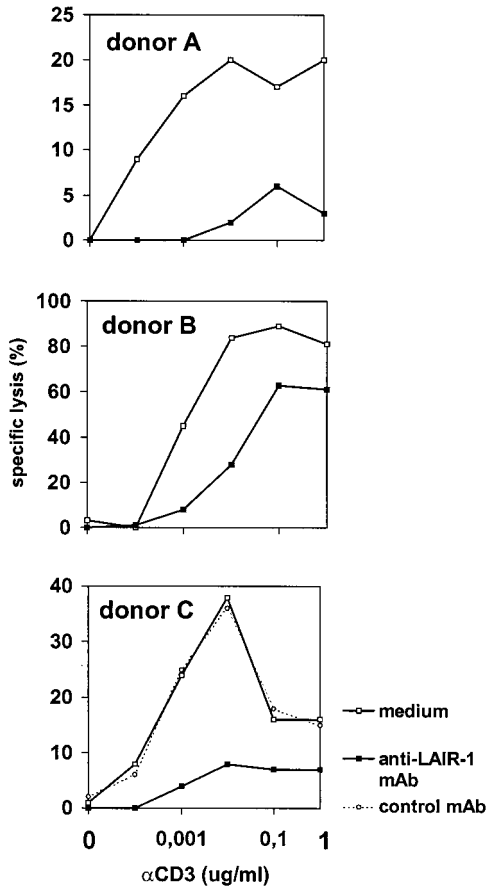

FIGURE 6. Anti-LAIR-1 mAb inhibits the CD3-induced cytotoxicity of peripheral blood T cells. Freshly isolated PBMCs were used as effector cells and assayed for killing of P815 cells in the presence of increasing amounts of anti-CD3 mAb as indicated on the *x*-axis. Cells were incubated with or without 5 μg/ml anti-LAIR-1 mAb (filled symbols) or with control IgG1 (dotted line). The supernatant was harvested after 7 h and ^{51}Cr release was measured. Data are representative of 12 different donors tested in 8 independent experiments.

cells (34). PBMCs from 15 healthy donors were assayed directly upon isolation in a 7-h ^{51}Cr release assay for lysis of P815 cells in the presence of anti-CD3 mAb. A total of 12 of 15 donors showed significant specific lysis. This lysis was inhibited up to 90% by the addition of anti-LAIR-1 mAb in all but one of these donors (Fig. 6), demonstrating that LAIR-1 is able to inhibit the cytotoxic activity of circulating effector T cells. The cytolysis was not inhibited by a control IgG1 mAb added in the same concentrations, excluding the possibility that the inhibition was due to competition of anti-CD3 and anti-LAIR for FcRs (Fig. 6*C*).

Discussion

We characterized a second transmembrane protein recognized by the anti-LAIR-1 mAb, which is identical to LAIR-1 with the exception of a 17-aa deletion in the stalk region of the extracellular domain, designated LAIR-1b. The presence of O-linked glycosylation sites in this 17-aa stretch may account for the considerable size difference on SDS-PAGE between LAIR-1a and LAIR-1b. Given their identical nucleotide sequence, with the exception of the deletion, LAIR-1a and -1b are likely to be generated by alternative splicing. Alternative splicing of the related KIR genes can also generate isoforms lacking the membrane proximal stalk region (35).

NK cells express mainly LAIR-1a and to a lesser extent LAIR-1b (Fig. 1) (24). Jurkat T cells express LAIR-1b, and peripheral T cells seem to have a preference for LAIR-1b (Fig. 1). However, this is not absolute, since RT-PCR analysis of mRNA of T cells from several different healthy donors revealed the presence of both LAIR-1a and LAIR-1b transcripts (data not shown). Being alternative splice variants, the expression of LAIR-1a and LAIR-1b, could, in principle, be regulated in a cell-type specific manner but could also represent simply a stochastic process. Our data suggest that LAIR-1a and LAIR-1b do not differ functionally. When transfected into the NK cell line YT.2C2, both forms are capable of inhibiting the cytotoxic function of these cells. One cannot exclude, however, that absence of the 17-aa stretch in the stalk region might lead to a different conformation of the extracellular domain, which could influence ligand-binding.

We previously reported on the molecular cloning of LAIR-2 (LAIR-2b in Fig. 2*B*) (24), a putative secreted family member of LAIR-1, with 84% amino acid homology in the Ig domain. Searching a database of expressed sequence tags (EST) revealed a LAIR-2-like clone (Genbank accession number: AA133246; LAIR-2a in Fig. 2*B*). The EST clone was sequenced and was found to be identical to LAIR-2b, with the exception of a 17-aa insertion (data not shown). The 17-aa stretch present in LAIR-2a and missing in LAIR-2b is in exactly the same position as the 17-aa stretch in LAIR-1 (Fig. 2*B*). This finding suggests that LAIR-1 and LAIR-2 are derived from a common ancestral gene. The biological role of the secreted LAIR family members is as yet unclear.

We also demonstrate here that LAIR-1 is not only an inhibitory receptor on NK cells, but that anti-LAIR-1 mAb is also able to inhibit cytotoxic responses in T cells, indicating that LAIR-1 provides a mechanism of regulation for T cells. However, there is a restriction as to which T cell functions can be inhibited via LAIR-1. Inhibition was only observed when T cells derived from the effector T cell population were used in cytotoxicity assays. First, inhibition of TCCs was only observed in the case of spontaneous cytotoxicity in vitro, which can only be mediated by a minority of the $CD8^+CD28^-$ TCC. Similarly, Poggi et al. (36) reported on a restricted capacity of Abs recognizing the p40 molecule to inhibit T cell function. Anti-p40 mAb inhibited anti-Vβ8-induced, but not anti-CD3-induced cytotoxicity of only some V$\beta8^+$ TCC (36). Recent studies have shown that anti-p40 mAbs stain LAIR-1 transfectants and therefore define the same Ag as anti-LAIR-1 mAb (L.M., unpublished observations).

We now also demonstrate inhibition of the cytotoxic activity of freshly isolated T cells from peripheral blood. This cytotoxicity is reported to be due mainly to the effector CTLs, which express the phenotype $CD8^+CD45RA^+CD27^-$ and are different from memory and naive cells (34). These T cells are all $CD28^-$ T cells and are known to have low proliferative potential and high perforin-mediated cytotoxic activity in vitro (37, 38). The number of $CD28^-$ cells increases with age (39) and these cells have shorter telomere lengths, reminiscent of an active replicative history (40). Our finding that LAIR-1 cross-linking can inhibit the cytotoxicity of these T cells suggests that LAIR-1 provides a mechanism for down-regulation of ongoing immune responses against tissues bearing ligands for this receptor.

Acknowledgments

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