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Transcriptional Regulation of ILT Family Receptors

Hideo Nakajima, Azusa Asai, Aki Okada, Lin Ping, Fumiyasu Hamajima, Tetsutarô Sata, ‡ and Kenichi Isobe

Ig-like transcripts (ILT/leukocyte Ig-like receptor/macrophage Ig-like receptor or CD85) are encoded on human chromosome 19q13.4, designated the human leukocyte receptor complex, and are predominantly expressed on myeloid lineage cells. We investigated the transcriptional regulation of ILT1, ILT2, and ILT4 genes to elucidate control mechanisms operating on the specific expression of ILT receptors. Inhibitory ILT2 and ILT4 both have a similar genomic structure, in which the ~160-bp 5′-flanking regions function as core promoters with critically important PU.1 binding sites. However, an Sp1 family-binding GC-box is more influential in trans-activation of ILT2 than ILT4. Additionally, ILT4 transcription is tightly regulated by chromatin modifications accompanied by histone acetylation, which strictly controls expression within myeloid lineage cells. Activating ILT1 carries a core promoter corresponding to the intronic region of ILT2 and ILT4, where PU.1 and Runx1 binding sites are essential, but a downstream heat shock element also augments promoter activity. Thus, each ILT is regulated by a distinct transcriptional mechanism, although PU.1 acts as a common trans-acting factor. We also found that human CMV infection strongly trans-activates inhibitory ILT2 and ILT4 genes through the expression of immediate-early proteins. The Journal of Immunology, 2003, 171: 6611–6620.

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Abbreviations used in this paper: ILT, Ig-like transcript; Ach3, acetylated histones H3; Ach4, acetylated histones H4; AZA, 5-aza-2′-deoxycytidine; ChIP, chromatin immunoprecipitation; DN, dominant negative; HCMV, human CMV; HDAC, histone deacetylase; HSF, heat shock transcription factor; IE, immediate early; KIR, killer cell Ig-like receptor; MZF-1, myeloid zinc finger gene-1; TSA, trichostatin A.

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HCVM can modulate the immune responses through up-regulation of inhibitory ILT genes.

Materials and Methods

Cells

Human embryonic kidney cell line 293 and mouse fibroblast cell line NIH-3T3 were maintained in DMEM with 10% FCS. Human myeloid cell lines (THP-1, U937, and K562), B cell lines (C1R and 721.221), and a T cell line (Jurkat) were grown in RPMI 1640 with 10% FCS.

Plasmid constructs

The ILT promoter constructions were generated by PCR using human genomic DNA as a template (Promega, Madison, WI). According to published genomic sequences, −1-kb 5′-flanking regions of ILT1 (−1013 to +74), ILT2 (−994 to +27), and ILT4 (−1007 to +14) were amplified and ligated into the pGL3-basic vector (Promega) to generate ILT promoter-fire luciferase reporter constructs. Various lengths of DNA fragments were further amplified and inserted. Reporter plasmids were mutated at the transcription factor binding positions using PCR-based site-specific mutagenesis as follows (mutated residues are shown in lowercase letters): ILT2-Mu1, TCCaGaGTCGTTGGGGT; ILT4-Mu1, CCCGGGtGAGGGGA; ILT2/4-Mu2, AAAGaGaAa; ILT2/4-Mu3, AGAGGaG; ILT2/4-Mu4, CA AAAGCa; ILT1-Mu5, GTTGCAagGCTT; ILT1-Mu6, GTGTGAAaT; and ILT1-Mu7, AGAGgATGC. PU.1 was amplified from a human leukocyte cDNA library, and PU.1 and PU.1-Ets were made by deletion of trans-activation domain Δ33–99 and all but the Ets domain Δ2–162, respectively, via PCR with appropriate primer pairs. All PU.1 constructs were ligated into pCDNA3.1 (Invitrogen, Carlsbad, CA). Transcription factor expression and backbone vectors were as follows: Sp1-pGCM (gift from Dr. T. Shenk, Princeton University, New Haven, CT), Runx1-pEF-BOS (provided by Dr. Y. Ito, Kyoto University, Kyoto, Japan), and myeloid zinc finger gene 1 (MZF-1),Ab-pCDNA3.1myc-his (received from Dr. J. Morris, Medical College of Wisconsin, Madison, WI). HCMV IE expression vector pEQ274 (IE1), pEQ276 (IE1+2), pEQ326 (IE2), and control vector pEQ336 containing only the HCMV IE promoter without IE proteins were provided by Dr. A. Geballe (Fred Hutchinson Cancer Research Center, Seattle, WA).

Complementary DNA synthesis and RT-PCR amplification

Total RNA was isolated from 5 × 10⁶ cells with RNeasy mini kits (Qiagen, Hilden, Germany) according to the manufacturer’s instructions, and 10 μg of total RNA was subjected to cDNA synthesis using First-Strand cDNA synthesis kits (Amersham Pharmacia Biotech, Little Chalfont, U.K.). Mixtures containing 1 μl of serial 3-fold dilutions of synthesized cDNA were amplified by PCR for 20–30 cycles (94°C for 30 s, 60°C for 30 s, 72°C for 30 s) after denaturation for 3 min at 94°C. ILT1–4, PU.1, β-actin, GAPDH, and HCMV IE1 were amplified with specific sets of primers. Quantitative real-time PCR was performed using the Smart Cycler System (Takara, Shiga, Japan). The relative amounts of cDNA in each sample were determined using a standard curve of known concentration. After normalization to GAPDH, the difference in quantity of a specific gene was calculated as the fold change from the control samples.

Luciferase assays

In general, 1 × 10⁶ cells were plated in 500 μl of OPTI-MEM (Invitrogen) with 10% FCS in 24-well plates. Firefly luciferase reporter constructs (5 μg) and pRL-thymidine kinase control vector (pRL-TK; Promega; 0.25 μg) were mixed with 5 μl of Lipofectamine 2000 (Invitrogen) to form a complex. In cotransfection studies, mixtures also contained 1 μg of transcription factor expression plasmids, HCMV IE expression plasmids, or control vectors. Mixtures were added to cells, and after a 4-h incubation, 1 ml of fresh medium was added. Next day, the cells were harvested and assayed by the Dual-Luciferase Reporter Assay System (Promega), using a luminometer (EG&G, Berthold, Germany). The relative light units were calculated after normalization against Renilla luciferase activities of the pRL-TK internal control vector.

Primer extension

A 33-mer primer (ILT1,+62 to +29; ILT2/4,+69 to +36), which was complementary to the first exon of each ILT gene, was synthesized and end-labeled with α-[32P]ATP. Then 100 μg of total RNA and the primer were annealed for 2 h at 70–75°C in the Primer Extension Buffer (Promega). Next, the primer was extended for 1 h at 42°C with 1 μl of AMV reverse transcriptase. The reaction products were loaded onto an 8% acrylamide gel under denaturing conditions, followed by autoradiography. A sequencing ladder of the ILT genome was made using the T7 Sequencing Kit (U.S. Biochemical Corp., Cleveland, OH) with the reporter plasmid as a template.

EMSAs

Nuclear extracts were prepared as previously described (12). Oligonucleotides corresponding to position 1 of ILT2 (−155 to −126), position 1 of ILT4 (−178 to −149), position 2 of ILT2 (−117 to −97)/ILT4 (−118 to −98), positions 5–6 of ILT1 (−74 to −49), position 6 of ILT1 (−63 to −49), and position 7 of ILT1 (−34 to −14) were generated and used as probes. Mutations were included in the same manner as for the reporter constructs. Nuclear extract (5 μg) was incubated with 50 fmol of 32P end-labeled probes at room temperature for 20 min. For competition assays, a 100-fold molar excess of cold oligonucleotides was also added to the reaction mix. In supershift experiments, nuclear extracts were preincubated with 2 μl of Ab for 20 min at 4°C before adding the labeled probe. The polyclonal Abs and Sp1 consensus and mutant oligonucleotides were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Heat shock transcription factor (HSF) competitor oligonucleotides were previously described (13). DNA binding reactions were separated on 5% native polyacrylamide gels at 150 V for 60–240 min.

Chromatin immunoprecipitation assays (ChIP)

The ChIP procedure was performed according to the manufacturer’s protocols (Upstate Biotechnology, Lake Placid, NY) with slight modifications. After the equivalent of 2 × 10⁶ THP-1, U937, C1R, and 721.221 cells were cross-linked with 1% formaldehyde, cells were lysed in SDS buffer with sonicated on ice. The length of the DNA fragments averaged 250–500 bp. Aliquots of equal volume from each sample were used as input controls. After intensive preclearing, immunoprecipitation was performed using specific Abs against acetylated histones H3 (AcH3) and H4 (AcH4) (Upstate Biotechnology) and PU.1 (Santa Cruz Biotechnology; 10 μg was used for each precipitation). After purification, DNA pellets were dissolved in 50 μl of H2O, and 1 μl of serial 3-fold dilutions thereof was used for PCR amplification with 1 μl (α-32P)dCTP (30 cycles: 94°C for 30 s, 60°C for 30 s, 72°C for 30 s) after denaturation for 3 min at 94°C. The core promoter regions of ILT2 (−159 to −10) and ILT4 (−161 to −10) and upstream regions of the core promoter in ILT2 (−688 to −493) and ILT4 (−671 to −497) were amplified with specific sets of primers. PCR products were electroeluted on 8% polyacrylamide gels.

Treatment of cells with 5-aza-2′-deoxycytidine (AZA), trichostatin A (TSA), and heat shock stimulation

Nuclear extracts were prepared from heat-shocked NIH-3T3 cells or THP-1 cells at 42°C for the indicated time and subjected to EMSA. For luciferase assays, 1 day after transfection of reporter plasmids, THP-1 cells were heat-shocked for 1 h at 42°C and allowed to recover at 37°C for 0, 2, 4, and 6 h, respectively, before making lysates. For RT-PCR analysis, before total RNA extraction cells were treated with 0, 1, and 5 μM AZA (Sigma-Aldrich, St. Louis, MO) for 48 h or 100 ng/ml TSA (Sigma-Aldrich) for 15 h or were heat-shocked for 1 h at 42°C, followed by 2-h recovery at 37°C.

HCMV infection

The AD169 strain of HCMV was used for infection, THP-1 and U937 were infected at multiplicities of infection of 100–1000 PFU/cell for 6 h at 37°C and maintained in RPM1 1640 with 10% FCS. After 48-h incubation, total RNA was extracted and subjected to RT-PCR analysis.

Results

Genomic organization of ILT promoters

According to the database (GenBank accession no. AC009892, AC010518, NM006866, AF004230, and XM 008961) and some previous reports (7, 9), ILT2 and ILT4 have a 5′-untranslated exon like other ILTs, whereas translation of ILT1 is started in the first exon (Fig. 1A). To determine the precise transcription initiation sites, primer extension was performed (Fig. 1B). Alignments of nucleotides with genomic sequences indicated that the major transcription initiation site was a C residue at −75 nt upstream of the ATG initiation codon in ILT1 and a G residue at −600 nt upstream of the ATG in ILT2 and ILT4 (Fig. 1B, large arrow), whereas several minor transcription initiation sites were observed (small arrow). We set the major transcription initiation sites as +1.

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Cis-elements in ILT2 and ILT4 promoters

To identify regulatory elements, we performed luciferase assays with sets of ILT2 and ILT4 promoter-containing constructs. As shown in Fig. 2A, the ~1-kb 5′-flanking region of ILT2/4 demonstrated significant transcriptional activities in the myeloid cell line THP-1, exhibiting 50- to 150-fold activities compared with the promoterless Basic. In contrast, the epithelial cell line 293 and the T cell line Jurkat conferred very little or no transcriptional activity. A deletion up to position ~160 resulted in no significant alteration; however, truncation up to ~100 led to a drastic reduction of promoter activities in THP-1 (Fig. 2A and B). These findings indicate that the proximal ~160 bp is the core promoter for ILT2/4.

According to our database search (http://www.cbrc.jp/research/db/TFSEARCHJ.html and http://tfbind.ims.u-tokyo.ac.jp), there are several transcription factor binding sites in the core promoter region of ILT2/4 (Fig. 1A). Position 1 is a GC-box known to interact with Sp1 family proteins, positions 2 and 3 have MZF-1-binding motifs, and position 4 has a GATA family-binding motif. To identify the cis-elements precisely, site-directed mutants of each position as well as 5′-deletion mutants were generated and tested in three myeloid cell lines (Fig. 2B). Although the effects of mutations varied in the cell lines tested, mutation or complete deletion of position 2 consistently led to a large reduction in transcriptional activities of both ILT2 and ILT4 genes. The relative light units (RLU) are shown after normalization against activity of the pRL-TK internal control vector. The RLU of promoterless pGL3-Basic are shown (□). The mean ± SD from quadruplicates are indicated. B, Analysis of minimal regions of ILT2 and ILT4 promoters. THP-1, U937, and K562 cells were transfected with reporter constructs of 5′-deletion or substituted mutants as illustrated, and luciferase activities were measured (RLU ± SD).
or 4 had no consistent or significant effect on promoter activities. Taken together, these data suggest that position 2 is the essential cis-element for the basal transcription of ILT2/4 genes, whereas position 1 has less effect, especially in ILT2.

**Nuclear factors involved in ILT2/4 trans-activation**

ILT2/4 position 2, the most important cis-element, is thought to interact with MZF-1, which is present as two splice variants, MZF-1A and MZF-1B (14). However, even using overexpressed nuclear extracts, we failed to detect binding of MZF-1 to position 2 (data not shown). Because position 2 also has an ets family transcription factor-binding motif, GGA(A/T) (Fig. 1A), we performed supershift EMSA with anti-Ets family Abs (Fig. 3A). In THP-1 and U937, nuclear proteins formed large complexes with the position 2 probe, but not with a mutant probe (Mu2). Most complexes were supershifted by anti-Pu.1 (lane 4). Nuclear extracts from Jurkat formed few bands, which were weakly supershifted by anti-Ets1/2 (lane 2). Next, to examine the proteins binding to ILT2/4 position 1, EMSA was performed using THP-1 nuclear extracts (Fig. 3B). ILT2 position 1 nucleotides formed several bands, and high m.w. bands were supershifted by anti-Sp1 and -Sp3 (left columns, lanes 2 and 3), and most bands were totally eliminated by adding anti-Sp1 and -Sp3 together (lane 4) or in the presence of an excess amount of Sp1 consensus oligos (lane 5). ILT4 position 1 nucleotides showed similar binding specificities, although they were less clear (right columns, lanes 1–6). Consid-ering that the sequence of position 1 was slightly different in ILT2 and ILT4 (Fig. 1A), and position 1 of ILT2 was more influential than the same position in ILT4 (Fig. 2B), some differences in binding properties must exist. For this reason we further tested competition and supershift EMSA using the ILT2 position 1 probe with a 100-fold excess of cold ILT4 position 1 nucleotides and vice versa (Fig. 3B, lanes 7–12). Despite competition with excessive ILT4 position 1 nucleotides, significant amounts of Sp1 family proteins were still bound to the ILT2 position 1 probe, as shown by supershifts with anti-Sp1, anti-Sp3, or both (left columns, lanes 8–10) and competition with Sp1 consensus oligos (lane 11). In contrast, ILT4 position 1 probe did not show any binding of Sp1 family proteins in competition with ILT2 position 1 nucleotides (right columns, lanes 7–12). These findings suggest that the affinity of ILT2 position 1 to Sp1 family proteins was much higher than that of ILT4 position 1.

Next, cotransfection studies were conducted. When Pu.1 was introduced into Pu.1-negative Jurkat cells, a large trans-activation of ILT2/4 promoters was observed (except for mutants at position 2 (Mu2); Fig. 3, C and D). MZF-1 and Sp1 essentially failed to trans-activate the ILT4 promoter (Fig. 3C), whereas Sp1 cotransfection induced a slight increase in ILT2 promoter activity and had additive effects to Pu.1 on trans-activation in Jurkat (Fig. 3D, left panel). Cotransfection of Sp1 was as effective as Pu.1 in THP1 cells, which possess endogenous Pu.1 (Fig. 3D, right panel). Together the data show that Pu.1 functioned as a potent trans-activator of ILT2/4 genes, but Sp1 had a weak and selective role. To further confirm the importance of the former, Pu.1 dominant-negative mutants (Pu.1-DN) were transfected into THP-1 cells (Fig. 3E). Pu.1-DN caused a large reduction of ILT2/4 promoter activity by competition with endogenous Pu.1.

**Promoter analysis of ILT1**

Transcription of ILT1 is initiated at the corresponding first intronic region of ILT2/4, whereas the 5′-upstream region of ILT1 has a sequence highly homologous to exon 1 of ILT2/4 (Fig. 1A). It is noteworthy that the genomic sequence of ILT1 distal from −525 is completely different. As shown in Fig. 4A, a series of constructs with the 5′-flanking region of ILT1 truncated up to −73 demonstrated significant transcriptional activity in THP-1, but very little in 293 and Jurkat. Moreover, constructs containing −663 to −484 or −763 to −484 corresponding to ILT2/4 promoter regions had no promoter activity, corroborating the evidence that ILT1 does not have a 5′-untranslated exon. Thus, the ILT1 core promoter exists within the proximal −73 bp located at the intronic region of ILT2/4. According to a database search, there are three putative transcription factor binding sites, which we designated positions 5, 6, and 7 (Fig. 1A). They were predicted to interact with the Ets, Runx, and HSF families of transcription factors, respectively. Luciferase assays were performed with 5′-deletion and site-directed mutants introduced into the ILT1 core promoter (Fig. 4B). In all cell lines tested, mutation or deletion of position 5 caused profound defects in promoter activity. Mutation of position 6, adjacent to position 5, also led to a large decrease. In contrast, mutation of position 7 led to a 50% reduction in THP-1 and K562, but no reduction in U937. These findings indicate that positions 5 and 6 are the essential cis-elements in the ILT1 promoter, whereas position 7 functions only in certain cells.

**Transcription factors that activate the ILT1 promoter**

EMSA was conducted to identify transcription factors acting in ILT1. Because positions 5 + 6 are in close proximity, oligonucleotides consisting of both positions were used as a probe (Fig. 5A, lanes 1–4). A large amount of nuclear proteins formed complexes with the position 5+6 probe and were supershifted by anti-Pu.1 or anti-Runx1 (small arrow, lanes 2 and 3). Relatively low m.w. bands were all shifted and disappeared by adding anti-Pu.1 (lanes 1 and 2), whereas Runx1-containing complexes were adsorbed in the high density bands and could not be detected before the supershift (lanes 1 and 3). To confirm the Runx1 binding to position 6, probes with a mutation at position 5 (Mu5+6) or containing only position 6 were generated to eliminate position 5 binding proteins and were then subjected to supershift assays. Relatively high m.w. bands were clearly erased and supershifted by anti-Runx1 (lanes 5–8). It must also be noted that by adding anti-Pu.1 and anti-Runx1 together to the position 5+6 probe, most binding complexes were further supershifted to the highest position (large arrow, lane 4), suggesting that the majority of Pu.1 and Runx1 formed complexes with one another.

Position 7 is a heat shock element with an NGAAN motif (Fig. 1A). EMSA revealed that a high m.w. complex was formed with the position 7 probe in a heat shock-dependent manner (Fig. 5B). In NIH-3T3 cells, complexes were totally supershifted with anti-HSF1 and partially supershifted with anti-HSF2 (lanes 5, 6, 8, and 9). In contrast, in THP-1, the HSF2-containing complex already formed, albeit in small amounts, with nuclear extracts from non-heat-shocked samples (small arrow, lane 13). This may explain the results of luciferase assays indicating that mutation of position 7 halved the promoter activity without heat shock treatment of THP-1 (Fig. 4B). After heat shock, a large amount of heat-responding proteins, recognized by anti-HSF1 Ab and competed against by specific competitor oligonucleotides, overwhelmed and replaced the HSF2-containing complex (Fig. 5B, lanes 14–17).

To define the significance of these transcription factors, we performed cotransfection and heat shock experiments. Cotransfection of Pu.1 or Runx1 separately with the ILT1 −73 reporter plasmid resulted in a small increase (∼1.3-fold) over background activity, but double transfection of Pu.1 and Runx1 together had a marked effect (Fig. 5C). In addition, expression of Pu.1-DN induced a remarkable decrease in luciferase activity. Cotransfection did not modulate the activity of the mutant at position 5 (Mu5) or of Basic.
FIGURE 3. A, EMSA of ILT2/4 position 2. ILT2/4 position 2 and mutant (Mu2) probes were labeled and incubated with nuclear extracts from THP-1, U937, and Jurkat cells. Anti-Ets1/2 (lanes 2 and 6), anti-Elk (lanes 3 and 7), and anti-PU.1 (lanes 4 and 8) were used for supershift assays. Reactions were separated on gels for 90 min (THP-1, U937) or 70 min (Jurkat). B, EMSA of ILT2 position 1 and ILT4 position 1. Nuclear extracts of THP-1 were used. Anti-Sp1 (lanes 2 and 8), anti-Sp3 (lanes 3 and 9), or both (lanes 4 and 10) were added for supershift, and Sp1 consensus (lanes 5 and 11) or mutant oligonucleotides (lanes 6 and 12) were added for competition. In addition, a 100-fold excess of cold ILT4 position 1 oligonucleotides was mixed with the labeled ILT2 position 1 probe for competition and vice versa (lanes 7–12). Gels were run for 70 min. C, Trans-acting candidates -PU.1, Sp1, MZF-1A, and MZF-1B were cotransfected into Jurkat with ILT4 reporter constructs. The relative light units (RLU) of transfectants with reporter plasmids and backbone vectors was set at 1, and fold increases in transcription factor transfectants are shown (mean ± SD). D, Reporter assays of ILT2 with transcription factors. Sp1, PU.1, or both were cotransfected into Jurkat (left panel) and THP-1 (right panel) with ILT2 constructs. The fold increase in RLU against backbone vector transfectants is shown. E, Effect of PU.1 dominant negative forms. PU.1ΔAD and PU.1-Ets were generated and used for cotransfection assay. RLU with only reporter plasmids was set at 1, and relative activities of cotransfectants are shown (mean ± SD).
were measured (RLU) of the ILT1 minimal promoter as illustrated, and luciferase activities—deletion or substituted mu-
U937, and K562 cells were transfected with 5 quadruplicate determinations. B, Core promoter analysis of ILT1. THP-1, Jurkat, and 293 cells were transfected with a series of constructs containing the 5′-upstream region of ILT1. In the same manner as described in Fig. 2A, THP-1, Jurkat, and 293 cells were transfected with a series of constructs containing the 5′-upstream region of ILT1. −663 to −484 and −763 to −484, corresponding to promoter regions of ILT2/4, are shown as light gray bars. Results are expressed as relative light units (RLU) ± SD of quadruplicate determinations. B, Core promoter analysis of ILT1. THP-1, U937, and K562 cells were transfected with 5′-deletion or substituted mu-
tants of the ILT1 minimal promoter as illustrated, and luciferase activities were measured (RLU ± SD).

Together with EMSA analysis, these results suggested that PU.1 and Runx1 functioned cooperatively to trans-activate the ILT1 promoter. Immediately after heat shock, luciferase activities were reduced to one-third because protein synthesis was shut off (Fig. 5D, upper panel). However, luciferase activity of ILT1 −73 rose and reached a maximum after 2 h recovery at 37°C, showing a 1.5-fold increase compared with the untreated control. This rapid increase was not observed in the position 7 mutant (Mu7). We also investigated the effect of heat shock by quantitative, real-time PCR (Fig. 5D, lower panel). After 1-h heat shock treatment and 2-h recovery, only ILT1 increased significantly by ~3-fold in the mes-
sage level.

Chromatin modification involved in ILT transcription

Because cis- and trans-interaction in the core promoter is not the sole element controlling gene expression, the B cell lines C1R and 721.221 did not express ILT4 despite possessing endogenous PU.1 (Fig. 6A, upper panel). Even after overexpression of PU.1 by transfection, ILT4 was still negative in C1R and Jurkat (lower panel). The discrepancy between reporter gene assays and endog-
enous transcription of ILT4 suggested that transcriptional activa-
tion was controlled by a higher order chromatin structure. Eukary-
otic gene activation is dependent on chromatin modifications that facilitate access of trans-factors to cognate DNA binding sites. The amino-terminal tails of core histones are subjected to a large num-
ber of covalent modifications, including phosphorylation, acetyla-
tion, methylation, and ubiquitination. Histone acetylation, the best-
described of these modifications, is positively correlated with transcrip-
tional activation. In genomic DNA, the most relevant modification is cytosine methylation at CpG dinucleotides. Meth-
ylation of the promoter region CpG islands is associated with tran-
scriptional silencing of imprinted genes. To examine the involve-
ment of chromatin remodeling in ILT gene expression, THP-1 and C1R cells were treated with AZA (which promotes demethylation of CpG sites) or TSA (which inhibits histone deacetylases) and then subjected to RT-PCR analysis. As shown in Fig. 6A, a slight increase in ILT3 expression was observed in THP-1 after AZA treatment, but significant effects of AZA on the expression of the other ILTs were not observed, and ILT4 was still negative in C1R.

In contrast, the ILT4 mRNA was markedly elevated after TSA stimulation in THP-1, although the message level of ILT1 to -3 did not increase greatly in either cell line (Fig. 6C). Surprisingly, after TSA treatment C1R now did express ILT4, albeit at very low levels (Fig. 6C, lower panel). To confirm that histone acetylation controls chromatin accessibility and transcription of ILT4, chromatin immunoprecipitation experiments were performed (Fig. 6D). After immunoprecipitation with anti-PU.1, -AcH3, and -AcH4, purified DNA was amplified by PCR to detect the core promoter regions of ILT2 (−159 to −10) and ILT4 (−161 to −10) and also the upstream regions of the core promoter in ILT2 (−688 to −493) and ILT4 (−671 to −497). The ILT2 core promoter locus (−159 to −10) was amplified comparably from precipitates of all four cell lines. In contrast, the ILT4 core promoter locus (−161 to −10) was markedly amplified in precipitates from the myeloid cell lines THP-1 and U937, but very little or not at all in the B cell lines C1R and 721.221. These results demonstrated that high levels of histone acetylation and chromatin opening at the ILT2 core promoter locus were present in both myeloid and B cell lines, but at the ILT4 core promoter locus these were largely lim-
lited to myeloid cell lines. In contrast, when immediate upstream regions of the core promoter locus were amplified from anti-AcH4 precipitates, no major differences were observed between myeloid cell lines and B cell lines or between ILT2 (−688 to −493) and ILT4 (−671 to −497). Thus, the ILT4 core promoter locus was hypoacetylated in nonmyeloid cell lines, but the lower levels of acetylation were not present across the entire ILT4 locus. Taken together, these findings indicate that ILT4 transcription is strictly regulated by histone acetylation that facilitates chromatin accessibility of the core promoter locus.
HCMV strongly trans-activates ILT2 and ILT4 expression

It was recently reported that increased expression of ILT2 was evident in patients who developed HCMV disease after lung transplantation. This elevation was observed several weeks before virus DNA could be detected in serum and might be an early identification of HCMV disease (11). In addition, it was demonstrated that murine Ig superfamily gp49B inhibitory receptors, homologues of KIR, were up-regulated on NK cells after murine CMV infection (15). HCMV IE proteins, IE1 and IE2, either independently or synergistically, are reported to activate the transcription of many cellular genes by direct binding to DNA or interaction with transcription factors such as Sp1, TBP, p300, c-Jun, and PU.1 (16–20). Therefore, the expression of ILTs could also be modulated by HCMV infection through IE-mediated trans-activation. As previously reported, THP-1 and U937 could not be productively infected, but successfully expressed IE genes 48 h after virus exposure (Fig. 6E). After infection, significantly higher levels of ILT2 and ILT4 expression were observed. At the same time, the effect of HCMV infection on ILT1, ILT3, or β-actin expression was far less, if any. To clarify the mechanism of HCMV-induced up-regulation of ILTs, we performed luciferase assays with cotransfection of HCMV IE expression vectors (Fig. 6F). IE1 alone slightly up-regulated all promoter activities, whereas IE2 alone did so less effectively. However, IE1 and IE2 synergistically trans-activated ILT2 and ILT4 promoters to a significant degree, but this was not observed in ILT1. These results were compatible with RT-PCR analysis, supporting the evidence that HCMV largely enhanced transcriptional activity of ILT2 and ILT4 genes through IE expression.

Discussion

In the present study we have demonstrated the importance of PU.1 in trans-activating all ILT promoters investigated. PU.1 (also termed Spi-1) is a hemopoietic-specific ets family transcription factor required for the development of both myeloid and lymphoid lineages (21–25). PU.1 expression is detectable even in multipotent hemopoietic stem cells and is up-regulated during myeloid and B cell differentiation. Considering that there are a number of genes

FIGURE 5. A, EMSA of ILT1 position 5 and position 6. THP-1 nuclear extracts were incubated with a probe of ILT1 positions 5 + 6 (lanes 1–4), Mu5 + 6 (substituted mutation at position 5; lanes 5 and 6), or position 6 (lanes 7 and 8). Supershift EMSA was performed using anti-PU.1 (lanes 2 and 4) and anti-Runx1 (lanes 3, 4, 6, and 8). Gels were run for 90 min. B, EMSA of ILT1 position 7. NIH-3T3 and THP-1 cells were heat-shocked for the indicated period, and nuclear extracts were subjected to EMSA. Anti-HSF1 (lanes 2, 5, 8, 12, and 16) and anti-HSF2 (lanes 3, 6, 9, 13, and 17) were used for supershift, and oligonucleotide binding to HSF were used for competition (lanes 11 and 15). Samples were separated on gels for 70 min (NIH-3T3) and 240 min (THP-1), respectively. C, Expression vectors of PU.1, Runx1, and PU.1-DN were cotransfected into THP-1 cells with reporter plasmids. Relative activities of cotransfectants against backbone vector transfecants are shown (mean ± SD). D, One day after transfection with ILT1 reporter constructs, THP-1 cells either were heat-shocked for 1 h, followed by recovery at 37°C in the time course, or were not heat-shocked. The relative light units (RLU) of untreated samples was set at 1, and the relative activity of heat-shocked samples is shown (mean ± SD; upper panel). After 2-h recovery from heat shock, RNA was extracted from THP-1, and quantitative, real-time PCR was performed. The quantity of specific gene product was normalized to GAPDH, and the differences are expressed as fold increases over untreated samples (lower panel).
FIGURE 6. Other factors that modulate ILT transcription. A, RT-PCR analysis. ILT2, ILT4, and PU.1 were amplified from cDNA of THP-1 (lane 1), U937 (lane 2), C1R (lane 3), and 721.221 (lane 4). Amplified β-actin is shown as a control (upper panel). C1R and Jurkat transfected with PU.1 were also subjected to RT-PCR (lower panel). B, THP-1 and C1R cells were incubated in the presence of 0, 1, or 5 μM AZA for 48 h, and RT-PCR analysis was performed. ILT1 to -4 genes were amplified from synthesised cDNA that was serially diluted. C, THP-1 and C1R cells were either treated with TSA (100 ng/ml) for 15 h or left untreated, followed by RT-PCR analysis. D, ChIP assay. Abs to PU.1, AcH3, and AcH4 were used for chromatin immunoprecipitation from THP-1, U937, C1R, and 721.221. PCR was performed to amplify the core promoter regions of ILT2 (−159 to −10) and ILT4 (−161 to −10) and their upstream regions in ILT2 (−688 to −493) and ILT4 (−671 to −497) from serially diluted samples. Input corresponded (Figure legend continued)
regulated by PU.1 in myeloid and B cells (26), it is not surprising that PU.1 plays a crucial role in ILT transcription. To a lesser extent, Sp1 family transcription factors also affected trans-activation of ILT2/4, especially ILT2. Because Sp1 is ubiquitously expressed and regulates a large number of genes (27), this may explain the wide tissue distribution of ILT2. In contrast, it is intriguing that transcription of the activating receptor ILT1 is regulated by a unique mechanism. In addition to PU.1, Runx1 (also termed AML1) was important for the transcriptional activity of ILT1. Runx1 is a member of the runt family of transcription factors, which is absolutely required for hematopoiesis and is involved in leukemogenesis (28–30). Runx1 functions in numerous gene promoters and is known to interact with ets family transcription factors Ets-1 and PU.1 to cooperate in the regulation of TCR 

This corresponds to our findings that Runx1 and PU.1 bound to the ILT1 core promoter in close proximity and cooperated. Furthermore, the Runx1 binding motif TGTGGT was carried only by ILT1, but not by ILT2/4 (TGTGGC; Fig. 1A), supporting the idea that ILT1 alone uses this region as a promoter. The ILT1 core promoter also possesses a heat shock element. Among the NKG2 family of NK receptors, the stimulatory NKG2D receptor recognizes the stress-inducible ligands, MHC class I-related chain A and MHC class I-related chain B (4). As the ligand for ILT1 has not been identified yet, it may possibly be a stress- or heat shock-related protein.

Chromatin remodeling is largely involved in modulation of the immune system, such as controlling Th1 and Th2 deviation or V(DJ) recombination (34–36). Open chromatin is characterized by hyperacetylation of associated histones as well as the increased accessibility of transcription factors (37). In our study treatment of cells with TSA enhanced the expression of ILT4, and high levels of histone acetylation and chromatin opening at the ILT4 core promoter locus were observed only in myeloid cells. Thus, chromatin remodeling by histone acetylation contributes to the strictly limited expression of ILT4 within myeloid lineage cells only. The effects of histone acetylation appear to be different between KIR family and ILT family gene expression, because it was recently reported that KIR expression was controlled by DNA methylation, and TSA treatment did not result in the induction of KIR gene expression (38, 39). The mechanisms controlling chromatin structure of the ILT locus remain to be determined; however, there may exist regions that recruit chromatin remodeling factors and affect the transcription of ILT genes in distant locations. In many genes distal regulatory elements, designated locus control regions, can cooperate with proximal promoters by opening local chromatin and maintaining a transcriptionally competent domain that confers tissue-specific and physiological levels of transcription.

A wealth of information has now been accumulated on genomics, structure, distribution, and biochemical and functional characteristics of ILT family receptors. Recently, CDS\(^7\)CDS\(^8\) alloantigen-specific T suppressor cells were reported to up-regulate ILT3 and ILT4 (40). Although ILTs are certain to modulate the function of myeloid cells, their physiological significance is still largely unknown. HCMV is a major pathogen in immunocompromised individuals, and an active infection usually leads to mortality. Although primary infection elicits an immune response through the production of various inflammatory cytokines, the response is insufficient to eliminate the virus, and life-long infection persists (16, 41). HCMV has strategies to subvert the immune system, for example, down-regulation of HLA class I molecules and expression of UL-18 to escape from recognition of infected cells by virus-specific T cells and NK cells, respectively (42–45). Myeloid cells, such as monocytes and tissue macrophages, play prominent roles in the host response to HCMV infection and are also an important reservoir during latent infection. HCMV infection of monocytes results in synthesis only of the IE and early gene products, which are critical factors in immunosuppression and have a dramatic influence on monocyte function (46–48). Our experiments showed that HCMV selectively trans-activated the ILT2 and ILT4 promoters through the expression of IE1 and IE2 proteins. These results present a potential new mechanism of immune evasion by HCMV to suppress the function of myeloid cells through up-regulation of inhibitory ILTs.

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**References**

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to PCR products containing 0.05–0.5% of the total amount of chromatin. *E. THP-1 and U937 cells were infected with the AD169 strain of HCMV. After 48-h virus exposure, RNA was extracted, and RT-PCR was performed. The fold increase in quantity as measured by real-time PCR is indicated under the band for each gene. *F. HCMV IE expression vectors pEQ274 (IE1), pEQ276 (IE1+IE2), pEQ326 (IE2), and control promoter vector (pEQ336) were cotransfected with ILT reporter constructs into THP-1 cells. The fold increase in cotransfectants is shown after normalization to relative light units (RLU) of reporter constructs alone (mean ± SD).*


