

TABLE 2 Genotype of IKBLp and HLA-A, -B, and -DRB1 loci of B-lymphoblastoid cell lines

Name	IHWC number	IKBLp ^a	HLA-A	HLA-B	HLA-DRB1	HLA homozygosity ^b
TUBO	IHW 9045	*01/*01	*0216/*0301	*5101	*1104/*1201	HLA-A, DRB1 heterozygote
BM16	IHW 9038	*01/*01	*0201	*1801	*1201	yes
RML	IHW 9016	*01/*01	*0204	*5101	*1602	yes
SA	IHW 9001	*02/*02	*2402	*0702	*0101	yes
HHKB	IHW 9065	*02/*02	*0301	*0702	*1301	yes
MGAR	IHW 9014	*02/*02	*2601	*0801	*1501	yes
Akiba	IHW 9286	*03/*03	*2402	*5201	*1502	yes
HY969	not applicable	*03/*03	*2402	*5201	*1502	yes
TOK	not applicable	*03/*03	*2402	*5201	*1502	yes
BM21	IHW 9043	*05/*05	*0101	*4101	*1101	yes
TAB069	IHW 9066	*05/*05	*0201/*0207	*4601	*0803	HLA-A heterozygote
KAS116	IHW 9003	*05/*05	*2402	*5101	*0101	yes

^a Genotype of IKBLp was confirmed by nucleotide sequencing.

^b All the cell lines were derived from HLA-B homozygotes. They were also homozygous for HLA-A and DRB1 loci otherwise mentioned.

Culture of Genotype-Defined Cells and Quantification of *NFKB1* Transcript

HLA-defined B lymphoblastoid cell lines homozygous for four of five IKBLp alleles (Table 2) were cultured in RPMI medium 1640 supplemented with 10% fetal bovine serum under 5% CO₂. Cells were collected at growing phase and approximately 10⁷ cells were subjected to total cellular RNA preparation by RNeasy mini kit (Qiagen). One microgram of RNA was used to synthesize cDNA by Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instruction. Quantitative real-time PCR used iCycler iQ Real-Time PCR Detection System (Bio-Rad) and iQ SYBR Green Supermix kit (Bio-Rad) to measure relative amount of mRNA. Primers for quantification of *NFKB1* and *GAPDH* transcripts are listed in Table 1E. Relative amount of *NFKB1* transcript was divided by that of *GAPDH*, a well-known housekeeping gene, for standardization.

RESULTS

Polymorphisms of *NFKB1* Locus

Our previous study mapped one of the susceptibility loci for TA around the 70-kb-long interval between the *TNFA* and the *MICB* loci of the HLA region [2], which was identical to the susceptibility locus for RA [6]. There are six functional genes in this interval; *TNFA*, *LTA*, *NFKB1*, *ATP6G*, *BAT1*, and *MICB*. We then searched for polymorphisms of *NFKB1* gene as a candidate genetic risk for TA, because an *NFKB1* polymorphism was associated with the susceptibility to RA [8]. By the screening of 96 healthy Japanese individuals by SSCP and subsequent sequencing analysis, we could not find polymorphism in the protein coding sequence, but there were four polymorphic sites in the 5'-flanking region of the gene which were registered in the dbSNP

database with the ID numbers in parentheses: eight or nine consecutive thymidines (T)₈/(T)₉ at position -422 (rs3219186, rs9279341), C/G at position -325 (rs3219185), A/G at position -263 (rs3219184), and T/A at position -63 (rs2071592). Sequencing of 669 (or 670)-nucleotide-long inserts of PCR-derived clones that contained all four sites revealed that the combinations of four SNPs fell into only five types of 16 possible combinations. We designated these five types of *NFKB1* promoter alleles IKBLp*01 through IKBLp*05 (IKBLp*01, co-occurrence of -422(T)₈, -325C, -263A, and -63T; IKBLp*02, co-occurrence of -422(T)₉, -325C, -263A, and -63T; IKBLp*03, co-occurrence of -422(T)₈, -325C, -263G, and -63T; IKBLp*04, co-occurrence of -422(T)₈, -325G, -263A, and -63A; and IKBLp*05, co-occurrence of -422(T)₈, -325C, -263A, and -63A) (Figure 1).

Development of RSCA-Based Typing Method for *NFKB1* Promoter

Because the alleles of *NFKB1* promoter were defined as a combination of the four SNPs, we attempted to develop a typing method to determine the alleles directly by RSCA. The sensitivity of sequence discrimination upon RSCA is dependent on the sequence of the reference strand so that it can be improved empirically by mutagenesis of the reference strand. Generally, a mispairing caused by insertion/deletion mutation or mismatches in two or more consecutive positions causes a greater effect on the mobility of the heteroduplex than that caused by a single base substitution. Therefore, we expected that introducing a mutation at or near the SNP site would improve the allele discrimination. When we used a native IKBLp*04 sequence as a reference to make a duplex with cloned DNA, mobility of each duplex was similar to the others except for the heteroduplex with IKBLp*02

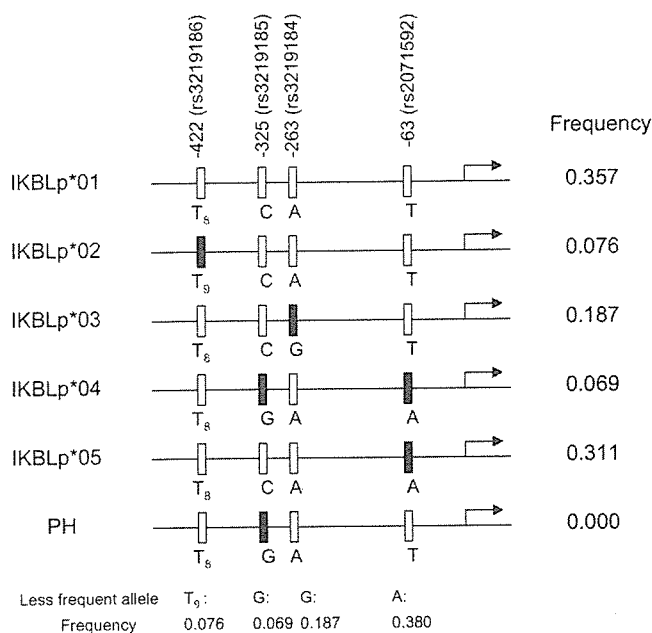


FIGURE 1 Schematic illustration of the *NFKB1* promoter alleles. Five of 16 possible combinations of four SNPs were commonly found in Japanese, and designated IKBLp*01 through *05. Frequency of each combination in the general population is at the right of the scheme. Ref SNP ID and frequency of less frequent allele for each SNP are also presented. A molecular clone corresponding to the bottom one (PH), which has not yet been identified, was made artificially to test the performance of the typing method.

which possessed a mismatch of one base insertion/deletion (Figure 2A, left). An insertional mutation of G at -325 G of IKBLp*04 produced remarkable changes in the mobility of duplexes with the -325 C alleles (Figure 2A, center). In addition, insertion of A at -63 brought an enhancement in discrimination of IKBLp*05 from the others (Figure 2A, right). Similarly, IKBLp*03 was mutagenized at -263 . During the isolation of reference DNA in the plasmid, an unexpected base change took place at -422 which helped to separate the homoduplex signal and heteroduplex signals (Figure 2B). The final set of the reference strands was "Ref-1" with a deletion of T at -422 and an A to G change at -262 introduced in IKBLp*03 sequence and "Ref-2" with an insertion of G at -325 and an insertion of A at -63 . With Ref-1, the IKBLp*01 and IKBLp*03 could be uniquely distinguished from the other alleles (Figure 2B, right), whereas the discriminations of IKBLp*04 and IKBLp*05 were apparent with Ref-2. Using these two reference strands in combination, all five alleles can be identified without ambiguity.

An advantage of RSCA over the individual SNP genotyping was an ability to resolve "haplotypic" or *cis-trans* ambiguities. For example, when the genotype of these four SNPs is homozygous for (T)₈ at -422

((T)₈/(T)₈), heterozygous at -325 (C/G), homozygous for A at -263 (A/A), and heterozygous at -63 (T/A), one cannot determine whether the haplotypic combination of these SNPs is heterozygous for IKBLp*01 ((T)₈-C-A-T) and IKBLp*04 ((T)₈-G-A-A) or heterozygous for IKBLp*05 ((T)₈-C-A-A) and a possible haplotype (T)₈-G-A-T ("PH" in Figure 1, so far not identified in Japanese). The electrophoretic patterns with these two genotypes were clearly different as demonstrated in Figure 2C.

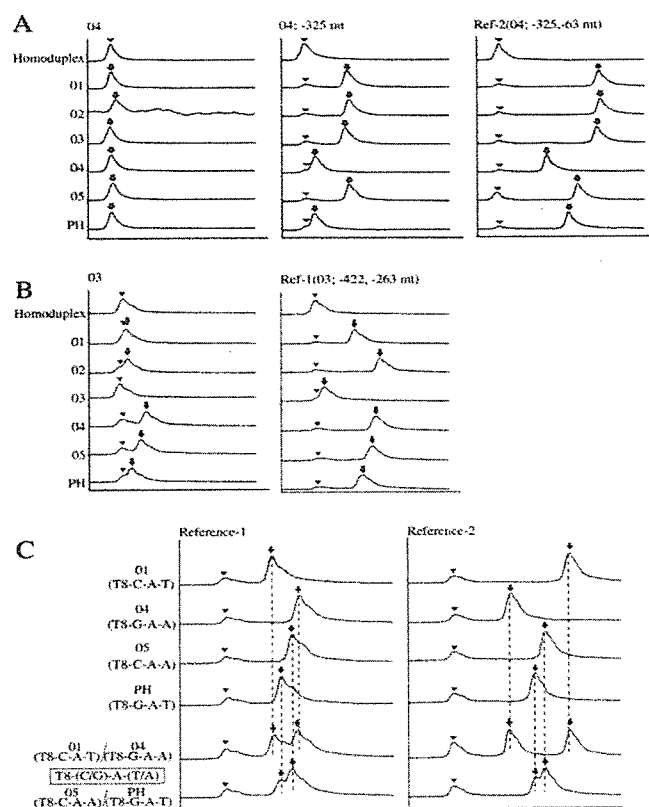


FIGURE 2 Effect of mutations of the references on the allele discrimination of RSCA. Different reference strands were tested for discrimination of the *NFKB1* promoter alleles. PCR products derived from plasmid clones of five common alleles and an unidentified combination (T)₈-G-A-T, tentatively assigned to PH, were examined. (A) Native IKBLp*04 (left), a mutant at position -325 (middle), and a double mutant at positions -325 and -63 (right, named Ref-2) were examined. Homoduplex of the reference is indicated by arrow head, and heteroduplex is indicated by arrow. (B) Native IKBLp*03 (left) and a double mutant at positions -422 and -263 (right, named Ref-1). (C) Resolution of *cis-trans* ambiguity of compound heterozygote for SNPs. PCR products were mixed to mimic heterozygote samples, *01/*04 and *05/PH. RSCA clearly resolved these genotypes, which fall on the same combination of individual SNP genotype: (T)₈/(T)₈-C/G-A/A-T/A.

TABLE 3A Association of IKBL polymorphism to Takayasu's arteritis

IKBLp allele	Carriers in patients (n = 84)	Carriers in control (n = 217)	Odds ratio (95% CI)	p	pc
IKBLp*01	38 (45.2%)	126 (58.1%)	0.60 (0.36–1.00)	n.s. ^a	n.s.
IKBLp*02	8 (9.5%)	33 (15.2%)	0.59 (0.26–1.33)	n.s.	n.s.
IKBLp*03	48 (57.1%)	76 (35.0%)	2.47 (1.45–4.14)	0.00065	0.0032
IKBLp*04	10 (11.9%)	30 (13.8%)	0.84 (0.39–1.81)	n.s.	n.s.
IKBLp*05	42 (50.0%)	109 (50.2%)	0.99 (0.60–1.64)	n.s.	n.s.

^a Not significant; p or pc > 0.05.

Analysis of *NFKB1L1* Promoter Alleles in TA

We examined 84 TA patients. The frequency of IKBLp*03 was significantly increased in the patients compared to that in the controls, 57.1% vs 35.0%, odds ratio of risk (OR) = 2.47, pc = 0.0032, indicating that this allele was strongly associated with the susceptibility to TA (Table 3A). Because -263G SNP is unique to IKBLp*03 among the five common alleles, these findings suggest that -263G SNP is a promising candidate for the responsible polymorphism controlling the susceptibility to TA.

Analysis of *NFKB1L1* Promoter Alleles in RA

We have previously reported that -63T SNP was strongly associated with RA in Japanese [8]. To examine the association further at the allele level, we used the RSCA-based typing method to analyze 120 patients with RA. As shown in Table 3B, the frequency of IKBLp*01 was increased in the RA patients; 73.3% vs 58.1%, OR = 1.99, pc = 0.032, whereas the frequencies of the other -63T-bearing *NFKB1L1* alleles, IKBL*02 and IKBLp*03 (Figure 1), were not increased. Although the results were consistent with -63T being strongly associated with RA, it was suggested that not the presence of the -63T SNP alone but the specific haplotypic combination of *NFKB1L1* promoter polymorphisms may confer the susceptibility to RA.

Linkage Disequilibrium of *NFKB1L1* Promoter Alleles with HLA

Because significant extent of LD between alleles of the genes in the HLA region had been observed, we next examined LD between *NFKB1L1* alleles and other polymorphisms in the HLA region in the control population.

We found that several alleles of genes in the HLA region manifested significant LD with five common *NFKB1L1* alleles as listed in Table 4. It was of note that significant LD was observed in a rather expanded region of the genome encompassing over a few megabases (from HLA-A to HLA-DPB1) when D' was used as an index of LD. We considered that this was due to the multiallelic nature of HLA loci and microsatellite markers and that the observations were in accordance with the concept of "HLA ancestral haplotypes" [21]. The frequencies of alleles were generally low so that LD could be captured by the analysis with D', whereas another index for LD, r², was not necessarily high between alleles of distant loci.

With regard to the TA-associated alleles, microsatellite alleles TNFd*134, C1_2_A*238, TNFa*119, and MICA(GCT)n*A6 were in strong LD with IKBLp*03, which was in good accordance with the TA-associated *NFKB1L1* allele being identified in our previous study [2] as a genetic variation linked to C1_2_A*238. B*5201 was also in strong LD with IKBLp*03 (D' = 0.828, r² = 0.464) with an estimated haplotype frequency of 0.119, which was the most frequent *NFKB1L1*-HLA-B haplotype in Japanese. Because of strong LD between IKBLp*03 and B*5201, one may assume that either of them was truly associated with TA and that the other had no effect on the disease susceptibility but merely increased in frequency as the carriers of *NFKB1L1*-HLA-B haplotype were increased in patients. Then, we analyzed the interaction between the disease-associated alleles by stratifying the population by the presence or absence of the alleles at risk (Table 5). IKBLp*03 increased the risk from OR of 2.54 to 6.98 in

TABLE 3B Association of IKBL polymorphism to rheumatoid arthritis

IKBLp allele	Carriers in patients (n = 120)	Carriers in control (n = 217)	Odds ratio (95% CI)	p	pc
IKBLp*01	88 (73.3%)	126 (58.1%)	1.99 (1.22–3.23)	0.0065	0.032
IKBLp*02	16 (13.3%)	33 (15.2%)	0.86 (0.45–1.63)	n.s. ^a	n.s.
IKBLp*03	36 (30.0%)	76 (35.0%)	0.80 (0.49–1.28)	n.s.	n.s.
IKBLp*04	8 (6.7%)	30 (13.8%)	0.45 (0.20–1.00)	0.049	n.s.
IKBLp*05	52 (43.3%)	109 (50.2%)	0.76 (0.48–1.19)	n.s.	n.s.

^a Not significant; p or pc > 0.05.

TABLE 4 Linkage disequilibria between IKBL polymorphisms and alleles of genetic markers in the HLA region

Markers	IKBLp*01	IKBLp*02	IKBLp*03	IKBLp*04	IKBLp*05
HLA-A			*2402 (0.460,0.073)	*3303 (0.764,0.542)	*0207 (0.791,0.063)
C3_2_11		*227 (0.799,0.326)	*207 (0.548,0.246)	*197 (0.782,0.158)	
C2_4_4	*251 (0.689,0.044)	*231 (0.854,0.053)	*243 (0.751,0.404)	*259 (0.751,0.300)	*255 (0.575,0.104)
C1_3_1	*279 (0.596,0.063)	*293 (0.623,0.254)	*291 (0.618,0.262)	*291 (0.890,0.184)	*288 (0.768,0.214)
C1_2_5	*192 (0.685,0.048)	*200 (0.619,0.169)	*208 (0.752,0.383)	*218 (0.799,0.425)	*194 (0.692,0.036)
HLA-B	*3901 (0.819,0.032)	*0702 (0.911,0.637)	*5201 (0.828,0.464)	*4403 (0.887,0.759)	*1501 (0.665,0.092)
	*5401 (0.694,0.068)		*6701 (0.999,0.066)		*4601 (0.876,0.130)
	*3501 (0.821,0.073)				
C1_4_1	*213 (0.396,0.040)	*225 (0.678,0.380)	*217 (0.672,0.104)	*217 (0.706,0.039)	
	*221 (0.354,0.060)				
	*229 (0.750,0.066)				
MIB	*344 (0.741,0.046)	*336 (0.644,0.227)	*326 (0.528,0.124)	*336 (0.870,0.372)	*326 (0.216,0.040)
	*346 (0.624,0.166)				
MICA (GCT)n	A4 (0.677,0.152)	A5.1 (0.798,0.297)	A6 (0.685,0.283)	A6 (0.883,0.160)	A5 (0.542,0.287)
	A9 (0.458,0.039)				
C1_2_A	*242 (0.521,0.152)	*242 (0.628,0.103)	*238 (0.771,0.294)	*236 (0.854,0.114)	*236 (0.696,0.414)
	*250 (0.611,0.035)				
	*256 (0.487,0.047)				
TNFA	*95 (0.999,0.043)	*115 (0.750,0.244)	*119 (0.892,0.731)	*105 (0.921,0.121)	*105 (0.839,0.570)
	*97 (0.965,0.261)				
	*113 (0.793,0.124)				
LTA A252G	252A (1.000,0.338)	252A (0.999,0.051)	252A (0.925,0.119)	252G (0.905,0.106)	252G (1.000,0.717)
TNFA promoter	*B (0.966,0.287)	*A (0.999,0.048)	*A (1.000,0.130)	*A (0.999,0.042)	*A (0.853,0.169)
	*C (1.000,0.036)				
	*D (0.943,0.330)				
TNFD	*130 (0.519,0.264)	*130 (0.925,0.123)	*134 (0.782,0.206)	*132 (0.910,0.361)	*134 (0.487,0.154)
					*138 (0.929,0.129)
D6S273		*132 (0.648,0.151)	*136 (0.522,0.194)	*136 (0.834,0.169)	*134 (0.267,0.065)
HLA-DRB1	*0405 (0.604,0.094)	*0101 (0.842,0.614)	*1502 (0.763,0.461)	*1302 (0.909,0.674)	*0803 (0.395,0.038)
HLA-DQA1	*0302 (0.294,0.075)	*0101 (0.842,0.614)	*0103 (0.499,0.171)	*0102 (0.789,0.341)	
DQ-CARII		*201 (0.703,0.461)	*205 (0.436,0.141)	*207 (0.842,0.502)	*203 (0.999,0.034)
HLA-DQB1	*0401 (0.593,0.088)	*0501 (0.809,0.589)	*0601 (0.479,0.166)	*0604 (0.952,0.683)	*0302 (0.474,0.040)

Alleles of genetic markers in the HLA region that showed significant linkage disequilibria (LD) with each IKBL promoter allele are shown. LD values (D' and r^2) are indicated in parentheses. Allele definitions follow those in the literature [2,29].

the presence of B*5201, although this was not statistically significant because both patients and controls in columns were decreased by stratification. It was also observed for the risk conferred by B*5201 in the presence of IKBLp*03 (OR increase from 2.91 to 3.36), and in this case statistical significance was obtained. Accordingly, both IKBL*03 and B*5201 were suggested to be truly associated with TA.

Functional Analysis of the *NFKB1L1* Promoter Polymorphism at -263

Because the polymorphism of *NFKB1L1* was found in the upstream sequence, the risk for TA or RA would be attributed to the difference in the regulation of gene expression. Thus the promoter activity was examined by the luciferase reporter system. Since IKBLp*03 was unique at

TABLE 5 Stratification analysis to access the HLA-B-linked and IKBLp-linked risks for Takayasu's arteritis

Population	Allele at risk	Carriers/noncarriers		OR (95% CI)	<i>p</i>
		In patients	In controls		
Unstratified	IKBLp*03	48/35	74/137	2.54 (1.51–4.27)	0.00058
	B*5201	42/41	55/156	2.91 (1.71–4.93)	0.00010
B*5201 carriers	IKBLp*03	41/1	47/8	6.98 (0.84–58.18)	n.s. ^a
B*5201 noncarriers	IKBLp*03	7/34	27/129	0.98 (0.39–2.45)	n.s.
IKBLp*03 carriers	B*5201	41/7	47/27	3.36 (1.32–8.54)	0.012
IKBLp*03 noncarrier	B*5201	1/34	8/129	0.47 (0.06–3.92)	n.s.

^a Not significant: $p > 0.05$.

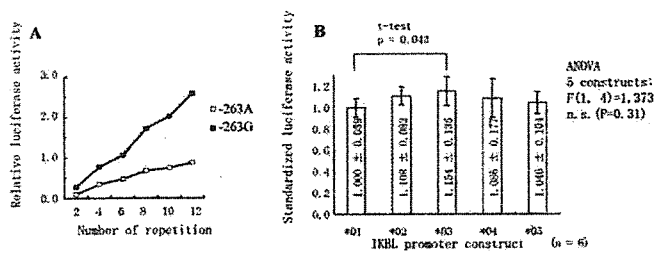


FIGURE 3 Functional analysis of *NFKBIL1* promoter. (A) A series of enhancer test constructs with variable numbers of 20-bp repeats containing -263 SNP were made by inserting the multimer of oligonucleotide upstream of adenoviral E1B basal promoter and tested for the transcriptional activity. The firefly luciferase activity was measured and standardized by *Renilla* luciferase activity driven by HSV thymidine kinase promoter. Numbers on x axis indicate the repetition of 20-bp sequence. (B) Nucleotide sequence encompassing -1182 to +23 of five alleles (IKBLp*01 through *05) were examined for transcriptional activity. Bar graph shows mean ± standard error of the mean ($n = 6$). A test with one-way ANOVA did not demonstrate unevenness of activity among five constructs with statistical significance ($F(1,4) = 1.37, p = 0.31$).

-263, we focused on this point. We compared transcriptional enhancer activity of -263G and -263A SNP alleles. For this purpose, a series of multimers of sequence containing either -263G or -263A was inserted upstream of E1B TATA promoter and the resultant plasmids were transfected to Raji B lymphoblastoid cells, in which *NFKBIL1* gene was expressed [our unpublished observation]. The transfectants were assayed for luciferase activity. As shown in Figure 3A, -263G, characteristic SNP of IKBLp*03, showed higher driving activity on E1B TATA promoter consistently, suggesting that IKBL*03 may manifest higher transcriptional activity than the other alleles.

To further corroborate the difference in transcriptional activity, we compared the promoter activity of the region spanning from -1182 to +82 of all five kinds of IKBLp alleles (Figure 3B). Although one-way ANOVA indicated that the experiment failed to demonstrate the significant unevenness in the transcriptional activity among five promoter alleles ($F(1,4) = 1.37, p = 0.31$), only the comparison between IKBLp*01 and IKBLp*03 constructs reached statistical significance ($p = 0.043$) by Student's *t*-test among ten possible pairwise comparisons. These results raised the possibilities that the augmented expression of *NFKBIL1* gene or IKBL protein may be involved in the predisposition to TA and that the lower expression may be involved in the predisposition to RA.

Effect of NFKBIL1 Promoter Polymorphism on the Amount of Transcripts

The difference in promoter activity may lead to the difference in the amount of transcript in the cells. We

examined the steady state mRNA levels of *NFKBIL1* in B lymphoblastoid cell lines derived from homozygotes of IKBLp*01, ILBLp*02, ILBLp*03, and ILBLp*05 (Table 2, Figure 4). The amount of *NFKBIL1* mRNA standardized by GAPDH mRNA among 12 different cell lines varied significantly (one-way ANOVA, $F(1,11) = 10.82, p = 0.0072$), while the variation within each of four groups of three cell lines with the same genotype was not significant. Thus, one of the major factors for the variation was the difference in *NFKBIL1* genotype. Post hoc analysis of comparison between genotypes revealed the consistent tendency of lower expression level in ILBLp*01 compared to levels in the others. The results were in good concordance with functional studies and reinforced the notion that the augmentation and attenuation of *NFKBIL1* gene expression may contribute to the susceptibility for TA and RA, respectively.

DISCUSSION

In this study, we developed an RSCA-based typing method for *NFKBIL1* gene and applied it to the associ-

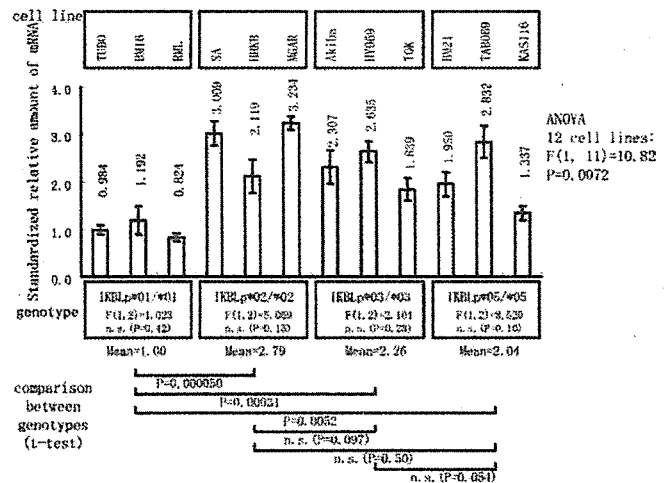


FIGURE 4 Quantification of *NFKBIL1* mRNA. Bar graph shows mean ± standard error of the mean of standardized relative amount of *NFKBIL1* mRNA ($n = 3$). Genotype of each cell line is listed in Table 2. The values were obtained by dividing the relative amount of *NFKBIL1* mRNA by the relative amount of *GAPDH* mRNA in the same sample and normalizing the mean value of three IKBLp*01 homozygotes to be 1.00. One-way ANOVA demonstrated that the amount of RNA among 12 cell lines was different ($F(1,11) = 10.82, p = 0.0072$), although every three cell lines with the same genotype did not vary. $F(1,2)$ values were 1.013, 5.073, 2.106, and 8.520 which corresponded to p values of 0.42, 0.15, 0.28, and 0.10, respectively, for IKBLp*01/*01, *02/*02, *03/*03, and *05/*05. Post hoc analysis of comparison between mean standardized relative amounts of mRNA of respective genotypes revealed that the smaller amount of *NFKBIL1* mRNA associated with IKBLp*01/*01 genotype.

ation analysis of the susceptibility to TA and RA. RSCA had been shown to be useful for determining genotypes of complicated polymorphic loci such as the HLA genes [9]. In the course of the development, we found that introducing insertion/deletion mutation at or near the polymorphic nucleotide to be determined facilitated the allelic discrimination by the mobility shift of heteroduplex DNA. This finding would be helpful in designing a new RSCA assay for genotyping of other gene polymorphisms. The RSCA-based typing of *NFKBIL1* was accurate because we did not see any discrepancy between RSCA typing data and SNP typing data of 116 RA samples which had been used in the previous study [8] or between RSCA typing data and SSCP typing data of 96 controls described above. RSCA is also useful for the discovery of polymorphism because any difference in the sequence has a possibility to result in the molecular conformation change as in the SSCP analysis. In fact, during the association study of TA, we found an individual with aberrant pattern of RSCA who turned out to have a novel SNP of C for G at +63.

We observed an association of IKBLp*01 with RA. This was in part a confirmative result of the association between RA and -63T of *NFKBIL1*. However, since IKBLp*02 and IKBLp*03, both of which carried -63T, did not show the association, our findings clearly indicated that -63T solely could not confer the susceptibility to RA. On the other hand, the observed association of IKBLp*03 allele with TA was in a good accordance with our previous observation of the association between TA and a microsatellite allele C1_2_A*238, because IKBLp*03 and C1_2_A*238 were in strong LD in the Japanese population. In addition to HLA-B*5201, HLA-B*3902 and HLA-B*6701 were associated with TA [1]. Although the number of samples was not sufficient to demonstrate the statistical significance for B*3902, these three HLA-B alleles were commonly in LD with IKBLp*03. So it is possible that the association of HLA-B alleles with TA may reflect a LD between these alleles and IKBLp*03. However, it was not the case because the stratification analysis demonstrated that both IKBLp*03 and HLA-B alleles separately contributed to the risk at least for HLA-B*5201 (see Results).

IKBLp*03 carries a unique sequence of G at -263, suggesting that this SNP would be responsible for the susceptibility to TA. We could demonstrate the difference in promoter activity with the SNP by luciferase reporter assays. TFSEARCH program (<http://mbs.cbrc.jp/research/db/TFSEARCH.html>) revealed that the sequence around -263A forms a putative E2F binding site [22], whereas that around -263G matched a putative c-Rel binding site [23] at default setting of threshold (85%). Thus transcriptional regulation of the alleles might be different by change in sequence with

-263A/G polymorphism. Recently, several reports on disease association of *NFKBIL1* promoter polymorphism were published to support the notion that *NFKBIL1* was a regulator gene for autoimmunity; *NFKBIL1* promoter with -263G and -63T, that is IKBLp*03, was associated with the resistance to type I diabetes [24].

Tissue distribution of *NFKBIL1* mRNA, data for which was not presented here, was somewhat different from that of mouse homologue [20]. *NFKBIL1* mRNA was detected in a wide variety of organs in both species but not found in mouse spleen, in which the expression level was highest in human [our unpublished observation]. Although the name of *NFKBIL1* or IKBL was originally used because of the structural similarity to I kappa B protein, a cytosolic protein which binds to and inhibits NF kappa B, the function of IKBL remains unknown. We tried to but could not demonstrate either augmenting or inhibitory effect on the kappa B enhancer-dependent transcription by overexpression of IKBL protein [unpublished observation]. It was reported that the expression of *NFKBIL1* was rather inversely regulated to that of other members of the I kappa B family [25]. The similarity between IKBL and I kappa B proteins appeared so limited to only a part of ankyrin-like domain which is a well-known motif of protein-protein interaction [26]. Because IKBL protein is localized in the nucleus [27, and our unpublished observation], which is totally different from the cytosolic distribution of I kappa B [28], the function of IKBL may be quite different from that of I kappa B. We are currently in search of the binding molecule(s) of IKBL protein, which may exist in the nucleus, to reveal genuine function(s) of IKBL protein in the cell.

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Apolipoprotein B mRNA–Editing Enzyme, Catalytic Polypeptide–Like 3G: A Possible Role in the Resistance to HIV of HIV-Exposed Seronegative Individuals

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Apolipoprotein B mRNA–editing enzyme, catalytic polypeptide–like 3G (APOBEC3G), a human cytidine deaminase, is a potent inhibitor of HIV replication. To explore a possible role of this protein in modulating in vivo susceptibility to HIV infection, we analyzed APOBEC3G expression in HIV-exposed seronegative individuals, HIV-seropositive patients, and healthy control subjects. The results showed that the expression of APOBEC3G is significantly increased in peripheral blood mononuclear cells (PBMCs)—mainly CD14⁺ cells—and in cervical tissues of HIV-exposed seronegative individuals. Higher APOBEC3G expression correlated with a reduced susceptibility of PBMCs to in vitro infection with the HIV-1_{Ba-L} R5 strain. APOBEC3G could be important in modulating in vivo susceptibility to sexually transmitted HIV infection.

Apolipoprotein B mRNA–editing enzyme, catalytic polypeptide–like 3G (APOBEC3G) belongs to a family of at least 10 other proteins with broad antiretroviral activity. After the initiation of the reverse transcription of the HIV RNA genome into DNA, the cytidine deaminase activity of APOBEC3G cat-

alyzes the conversion of cytosine to uracil in negative-strand viral cDNA, resulting in the reduction of viral fitness [1, 2]. The viral infectivity factor (Vif) protein of HIV counteracts the activity of APOBEC3G, inducing its degradation by a ubiquitin-proteasome pathway [3].

Susceptibility to HIV is widely different among humans [4, 5]. To verify whether APOBEC3G is involved in modulating susceptibility to HIV infection, we analyzed the expression of this protein in peripheral blood mononuclear cells (PBMCs) and cervical biopsy samples from individuals who, in spite of repeated exposure to HIV, do not become infected (HIV-exposed seronegative individuals). APOBEC3G expression was also evaluated in in vitro HIV infection assays performed on PBMCs from healthy control subjects and HIV-exposed seronegative individuals.

Methods. This study was approved by the institutional review boards of the S. M. Annunziata Hospital; written, informed consent was obtained from all patients. Blood samples were collected from 30 HIV-exposed seronegative individuals. Inclusion criteria were a history of multiple unprotected sexual episodes for ≥ 4 years at the time of the enrollment, with at least 8 episodes of at-risk intercourse within the 4 months before study entry and an average of 30 (range, 18 to >100) reported unprotected sexual contacts per year. Thirty age-matched HIV-infected patients and 30 age-matched healthy control subjects without any known risk factor for HIV infection were also included in the study. All HIV-seropositive patients were undergoing highly active antiretroviral therapy. All women underwent gynecologic and laboratory evaluation that did not reveal any concomitant infectious or gynecological problems. Cervical biopsy samples from 7 HIV-exposed seronegative individuals, 5 HIV-infected patients, and 7 healthy control subjects were collected in vials containing 1 mL of RNAlater (Ambion). Multiple PBMC samples (mean, 3 samples; range, 2–4 samples) but only 1 cervical biopsy sample was collected from each patient over a period of 3 months, to minimize sampling errors. The final results were expressed as mean values.

For PBMC and CD4⁺, CD8⁺, and CD14⁺ cell isolation and

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culture, whole blood was collected by venipuncture in Vacutainer tubes containing EDTA (Becton-Dickinson), and PBMCs were separated on lymphocyte separation medium (Organon Teknica). CD4⁺ and CD8⁺ lymphocyte isolation was done with the RosetteSep kit (StemCell Technologies), in accordance with the manufacturer's instructions. CD8⁺ and CD4⁺ cell purity was assessed by flow cytometry and ranged between 89% and 97% and between 85% and 94%, respectively. Monocytes were isolated with the Monocyte Isolation Kit (Miltenyi Biotec). Purity (range, 88%–94%) was evaluated by flow cytometry. Cells were cultivated in medium alone (RPMI 1640 and 20% fetal bovine serum [FBS]) or in the presence of 400 U/mL recombinant human interferon (IFN)– α (R&D) for either 4 (mRNA extraction) or 8 (protein extraction) h.

For real-time polymerase chain reaction (PCR), total RNA was extracted from cells or cervical biopsy samples with RNeasy lysis reagent (Qiagen) and were retrotranscribed as described elsewhere [6]. cDNA quantification for APOBEC3G and GAPDH was performed by real-time PCR (DNA Engine Opticon 2; MJ Research). Reactions were performed using a SYBR Green PCR mix (Finnzymes), as described elsewhere [6]. Primer sequences were designed to distinguish among the highly homologous sequences of cytidine deaminases (for APOBEC3G, 5'-CCGTC-TGGCTGTGCTACGAA-3' [forward] and 5'-GCTTCCTCCA-CTTGCTGAACCA-3' [reverse]); for GAPDH, 5'-CGGATTG-GTCGTATTGGG-3' [forward] and 5'-GCTTCCCGTTCTC-AGCCTTG-3' [reverse]). Results were expressed as $\Delta\Delta Ct$ (where "Ct" is the cycle threshold) and presented as ratios between the target gene and the GAPDH housekeeping mRNA.

For quantification of APOBEC3G protein by ELISA, total proteins extracted with an M-Per reagent and Halt Protease Inhibitor Cocktail (Pierce) were quantified by a BCA assay (Pierce). Ten micrograms of sample was coated on a 96-well plate overnight at 4°C. After blocking with 5% bovine serum albumin in Tris-buffered saline, the samples were incubated with 5 μ g/mL chicken anti-APOBEC3G antibody, produced and characterized in our laboratory (Clivio A. et al; manuscript in preparation), for 2 h at 37°C. After a wash step, an anti-IgY–horseradish peroxidase conjugate (diluted 1:8000; Promega) was added. The coloring reaction was performed with the TMB Microwell Peroxidase Substrate (KPL).

For IFN- α receptor 1 (IFNAR1) analysis, PBMCs were stained for 1 h at 4°C in the dark with an anti-human IFN- α/β receptor 1 biotinylated antibody (R&D Systems), anti-CD4–phycoerythrin (PE), and CD8-PE-Cy5 or with biotinylated anti-human IFN- α/B receptor 1 and anti-CD14–PE–Cy5 (Caltag Laboratories). Cytometric analysis was performed using an FC500 flow cytometer (Beckman Coulter).

For the in vitro challenge assay, PBMCs (2×10^6 cells/mL) were cultured for 2 days at 37°C and 5% CO₂ in RPMI 1640 containing FBS (20%), phytohemagglutinin (5 μ g/mL), and

interleukin (IL)–2 (10 ng/mL). After viability assessment and CD4⁺ and CD8⁺ cell percentage evaluation, done as described elsewhere [7], 3×10^6 cells were resuspended in medium containing 0.05 ng of HIV-1_{Ba-L} p24 viral input and incubated for 3 h at 37°C. Cells were then washed and resuspended in 3 mL of complete medium with IL-2 (10 ng/mL). Cells were plated in 24-well tissue culture plates and incubated at 37°C and 5% CO₂. PBMCs were then divided into 3 wells to be analyzed on days 2, 3, and 5. Cultures were refed with complete medium plus IL-2 on day 3, and supernatants were collected for batched ELISA of p24 antigen. Absolute levels of p24 were measured using the Alliance HIV-1 p24 ELISA Kit (PerkinElmer), and APOBEC3G protein expression was evaluated by ELISA.

Statistical analyses were performed using SPSS (SPSS). Differences between the groups were assessed using nonparametric analyses (Mann-Whitney *U* test). The standard *t* test was used for comparing different conditions within the same group. All *P* values are two-tailed.

Results. APOBEC3G mRNA expression in unstimulated PBMCs was significantly augmented in HIV-exposed seronegative individuals, compared with that in healthy control subjects (*P* = .042). IFN- α stimulation resulted in a 3-fold increase in APOBEC3G mRNA levels in PBMCs from HIV-exposed seronegative individuals (*P* = .001) but had only a modest effect in HIV-infected patients and healthy control subjects. As a result, APOBEC3G mRNA levels in IFN- α –stimulated PBMCs were significantly augmented in HIV-exposed seronegative individuals, compared with those in both healthy control subjects (*P* = .042) and HIV-infected patients (*P* = .026) (figure 1A).

ELISA results confirmed that the highest levels of APOBEC3G protein were detected in PBMCs from HIV-exposed seronegative individuals, both in basal conditions (*P* = .006, vs. HIV-infected patients) and after IFN- α stimulation (*P* = .001, vs. HIV-infected patients; *P* = .031, vs. healthy control subjects) (figure 1B).

IFN- α stimulation of CD14⁺ cells resulted in a 20-, 9-, and 6-fold increase in APOBEC3G mRNA levels in HIV-exposed seronegative individuals, healthy control subjects, and HIV-infected patients, respectively (*P* = .42, for HIV-exposed seronegative individuals vs. healthy control subjects) (figure 1C). A weaker effect was seen for CD4⁺ cells (6-, 4-, and 3-fold increase in HIV-exposed seronegative individuals, HIV-infected patients, and healthy control subjects, respectively), whereas IFN- α –induced modulation of APOBEC3G mRNA levels was minimal (<2-fold increase) in CD8⁺ cells from all individuals included in the study (figure 1C). ELISA analyses of APOBEC3G protein in CD14⁺ cells confirmed these results by showing higher levels of APOBEC3G in CD14⁺ cells from HIV-exposed seronegative individuals than in healthy control subjects and HIV-infected patients, both in basal condition and after IFN- α stimulation (figure 1D).

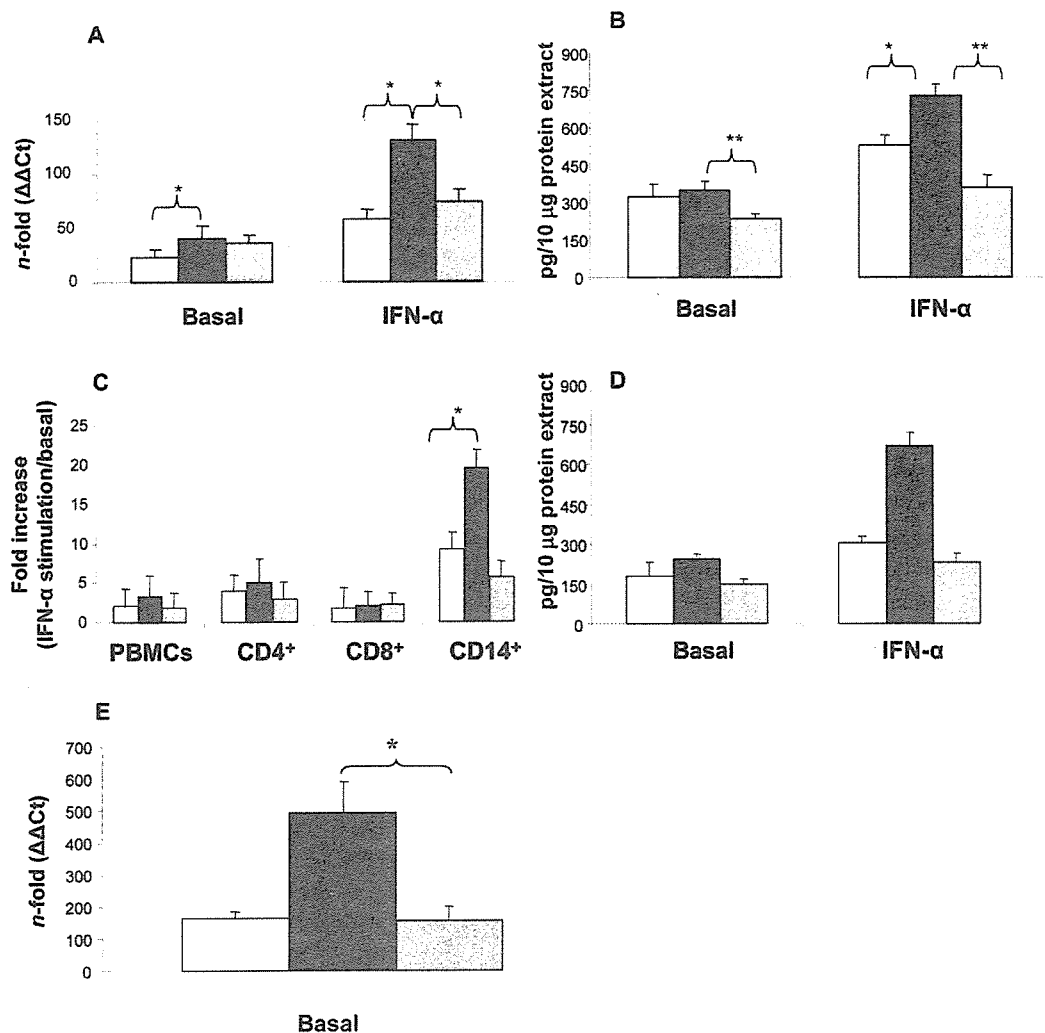


Figure 1. A, Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) mRNA expression in basal conditions and after stimulation with 400 U/mL interferon (IFN)- α in peripheral blood mononuclear cells (PBMCs) from healthy control subjects (*white bars*), HIV-exposed seronegative individuals (*black bars*), and HIV-infected patients (*grey bars*). B, APOBEC3G protein quantification by ELISA in PBMCs from healthy control subjects (*white bars*), HIV-exposed seronegative individuals (*black bars*), and HIV-infected patients (*grey bars*) in basal conditions and after IFN- α stimulation. C, Changes in APOBEC3G mRNA expression after IFN- α stimulation of PBMCs and CD4⁺, CD8⁺, and CD14⁺ cells isolated from healthy control subjects (*white bars*), HIV-exposed seronegative individuals (*black bars*), and HIV-infected patients (*grey bars*). D, APOBEC3G protein quantification by ELISA in CD14⁺ cells from healthy control subjects (*white bars*), HIV-exposed seronegative individuals (*black bars*), and HIV-infected patients (*grey bars*) in basal conditions and after IFN- α stimulation. E, APOBEC3G mRNA expression in cervical biopsy samples from healthy control subjects (*white bars*), HIV-exposed seronegative individuals (*black bars*), and HIV-infected patients (*grey bars*). Results are mean \pm SE values. Ct, cycle threshold. * $P < .05$; ** $P < .01$.

Flow cytometry analysis (mean fluorescence intensity) showed that IFNAR1 expression was significantly higher in CD14⁺ cells (mean \pm SE percentage of IFNAR1-positive cells, 8.2% \pm 3.8% for HIV-exposed seronegative individuals, 7.9% \pm 3.6% for HIV-infected patients, and 8.3% \pm 3.1% for healthy control subjects) than in both CD4⁺ cells (2.9% \pm 0.6% for HIV-exposed seronegative individuals, 3.5% \pm 1.3% for HIV-infected patients, and 3.0% \pm 1.4% for healthy control subjects) and CD8⁺ cells (3.7% \pm 1.1% for HIV-exposed seronegative individuals, 4.5% \pm 2.6% for HIV-infected patients, and 4.2% \pm 1.9% for healthy control subjects), providing a

possible explanation for the enhanced responsiveness of monocytes to IFN- α . Surface levels of IFNAR1 on CD14⁺ cells were, nevertheless, comparable among the 3 groups of individuals.

APOBEC3G mRNA levels were significantly increased in cervical biopsy samples from HIV-exposed seronegative individuals, compared with those in HIV-infected patients and healthy control subjects ($P = .024$, vs. HIV-infected patients) (figure 1E). Unfortunately, protein analyses could not be performed because of a limitation in the amount of available material.

In vitro HIV infection of PBMCs from HIV-exposed seronegative individuals resulted in a lower amount of p24 after 2,

3, and 5 days, compared with that in PBMCs from healthy control subjects. This result was not likely to be due to quantitative differences in CD4⁺ and CD8⁺ cells, because the median CD4⁺ (45% ± 6% for HIV-exposed seronegative individuals and 44% ± 5% for healthy control subjects) and CD8⁺ (20% ± 3% for HIV-exposed seronegative individuals and 19% ± 4% for healthy control subjects) T cell percentage was comparable between the 2 groups. Quantification of APOBEC3G protein by ELISA showed that PBMCs from HIV-exposed seronegative individuals produced higher levels of APOBEC3G on days 2, 3, and 5 than did those from healthy control subjects. Differences were statistically significant ($P < .05$) at day 5 (table 1).

Discussion. In this study, we investigated whether the reduced susceptibility to HIV infection that characterizes HIV-exposed seronegative individuals could be related to differential expression of APOBEC3G and whether the expression of this protein could be differently modulated by exposure to IFN- α . The results showed that APOBEC3G mRNA levels were significantly increased in PBMCs from both HIV-exposed seronegative individuals and HIV-infected patients, compared with those in PBMCs from healthy control subjects. In contrast, levels of APOBEC3G protein were significantly augmented only in PBMCs from HIV-exposed seronegative individuals. The chronic exposure to HIV taking place in HIV-infected patients and HIV-exposed seronegative individuals could explain why APOBEC3G mRNA expression is stimulated in both. The Vif protein of HIV induces the degradation of APOBEC3G by a ubiquitine-proteasome pathway. By definition, HIV infection does not take place in HIV-exposed seronegative individuals [8]: in these individuals, the absence of Vif would prevent APOBEC3G degradation, possibly justifying the discrepancy between mRNA and protein levels.

After IFN- α stimulation, both APOBEC3G mRNA and protein levels increased in the 3 groups studied, with a more pronounced effect in HIV-exposed seronegative individuals than in both HIV-infected patients and healthy control subjects. Although IFN- α -induced modulation of APOBEC3G levels could seem relatively modest (3-fold), it has been demonstrated that even slight changes in the *in vitro* expression of APOBEC3G result in the reduction of HIV infectivity. These results, thus, suggest that population-level variation in APOBEC3G and Vif levels are likely to deeply influence the outcome of infection with HIV [9]. To verify this hypothesis, we performed *in vitro* infection assays on PBMCs from HIV-exposed seronegative individuals and healthy control subjects. We decided to use a macrophagotropic HIV-1 strain (R5) to approach the situation seen in *in vivo* primary HIV infection during sexual transmission [10]. Interestingly, the kinetics of changes in p24 and APOBEC3G levels were different in the 2 groups examined. Thus, compared with what was observed in healthy control

subjects, *in vitro* HIV infection of PBMCs from HIV-exposed seronegative individuals resulted in a slower increase in p24 concentration and a much more rapid up-regulation of APOBEC3G. A working hypothesis stemming from these data is that, once exposed to the virus, exposed seronegative individuals would respond to IFN- α production with a faster and more robust increase in the expression of APOBEC3G and a consequent reduced susceptibility to HIV infection.

The present data show that monocytes are exquisitely responsive to IFN- α -induced up-regulation of APOBEC3G. Monocyte-derived type I IFNs are responsible for the earliest phase of the immune responses against pathogens, and their synthesis is directly triggered by viruses [11]. The higher amounts of APOBEC3G proteins seen in IFN- α -stimulated monocytes from HIV-exposed seronegative individuals could result in an amplification of these early, innate defensive immune mechanisms. Presumably, the peculiar responsiveness to IFN- α and up-regulation of APOBEC3G in CD14⁺ cells observed in HIV-exposed seronegative individuals would be seen not only in monocytes but, more generally, in nonlymphoid cells such as macrophages and Langerhans cells. It is tempting to speculate that the higher quantity of APOBEC3G produced by monocyte-derived cells, including Langerhans cells and macrophages, that cluster in peripheral tissues at mucosal sites could constitute an important barrier to HIV penetration during sexual exposure. This hypothesis is reinforced by the observation that APOBEC3G mRNA expression was indeed increased in cervical biopsy samples from HIV-exposed seronegative individuals.

Recent findings have demonstrated that APOBEC3G can ex-

Table 1. p24 concentration in supernatants and apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) protein expression level in peripheral blood mononuclear cells from HIV-exposed seronegative individuals and healthy control subjects.

Day, protein	Exposed/seronegative (n = 10)	Healthy controls (n = 10)
Day 2		
p24, pg/mL	4 ± 0.8	21 ± 7.2
APOBEC3G, HIV challenge/basal	1.43 ± 0.15	0.98 ± 0.23
Day 3		
p24, pg/mL	96 ± 23.0	357 ± 79.5
APOBEC3G, HIV challenge/basal	2.91 ± 0.63	1.17 ± 0.32
Day 5		
p24, pg/mL	4315 ± 114.3	16613 ± 175.6
APOBEC3G, HIV challenge/basal	4.01 ± 0.54 ^a	2.03 ± 0.85

NOTE. Data are mean ± SE values. Cultures were infected with limiting amounts (0.05 ng of p24 viral input) of the HIV-1_{BasL} R5 strain.

^a $P < .05$.

ist into 2 different forms: an active one (low molecular weight [LMW]) and an inactive one (high molecular weight [HMW]) [12, 13]. It will be interesting to evaluate whether the prevalent localization of APOBEC3G in LMW and HMW complexes differs in HIV-exposed seronegative individuals. Similarly, we will evaluate in our cohort the presence of the C40693T *APOBEC3G* gene sequence variant recently associated with an increased risk of infection [14].

Given that, worldwide, the vast majority of HIV infections are sexually acquired [15], a potent APOBEC3G-mediated systemic and mucosal antiviral response could offer a formidable barrier against HIV infection. The present results could play an important role in the design of new therapeutic and vaccine strategies.

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MHC Class I-Like MILL Molecules Are β_2 -Microglobulin-Associated, GPI-Anchored Glycoproteins That Do Not Require TAP for Cell Surface Expression¹

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MILL (MHC class I-like located near the leukocyte receptor complex) is a family of MHC class I-like molecules encoded outside the MHC, which displays the highest sequence similarity to human MICA/B molecules among known class I molecules. In the present study, we show that the two members of the mouse MILL family, MILL1 and MILL2, are GPI-anchored glycoproteins associated with β_2 -microglobulin (β_2 m) and that cell surface expression of MILL1 or MILL2 does not require functional TAP molecules. MILL1 and MILL2 molecules expressed in bacteria could be refolded in the presence of β_2 m, without adding any peptides. Hence, neither MILL1 nor MILL2 is likely to be involved in the presentation of peptides. Immunohistochemical analysis revealed that MILL1 is expressed in a subpopulation of thymic medullary epithelial cells and a restricted region of inner root sheaths in hair follicles. The present study provides additional evidence that MILL is a class I family distinct from MICA/B. *The Journal of Immunology*, 2006, 177: 3108–3115.

Classical MHC class I molecules, also known as class Ia, are heterodimeric glycoproteins made up of a transmembrane-type H chain and β_2 -microglobulin (β_2 m).³ They bind small peptides primarily derived from cytosolic proteins in a groove comprised of the $\alpha 1$ and $\alpha 2$ domains and present them to CD8⁺ T cells, thereby enabling the immune system to destroy abnormal cells that synthesize viral or other foreign proteins (1). Class Ia molecules are almost ubiquitously expressed and their H chains exhibit an extraordinary level of polymorphism (2).

By contrast, class I molecules, collectively called nonclassical class I or class Ib, are usually oligomeric or monomeric, and do not necessarily bind peptides (3–5). Many class Ib molecules have a more restricted tissue distribution than class Ia molecules. Although the majority of class Ib molecules form complexes with β_2 m, MICA/B (MHC class I-related chains A and B) (6), zinc- $\alpha 2$ -glycoprotein (7), the endothelial protein C receptor (8), and the RAE-1 (retinoic acid early inducible-1) family of class Ib mole-

cules (9) are not associated with β_2 m. Furthermore, a significant proportion of class Ib genes (the genes coding for the H chains of class Ib molecules) are located outside the MHC region (5). Accumulated evidence indicates that class Ib molecules have diverse functions ranging from specialized Ag presentation (10–12) to the activation of NK cells (13, 14), transport of IgG (15), pheromone detection (16, 17), and lipid mobilization and catabolism (18).

Recently, we identified a new family of class Ib genes designated *Mill* (MHC class I-like located near the leukocyte receptor complex) in mice (19) and rats (20). The two members of the *Mill* family, *Mill1* and *Mill2*, are located close to the leukocyte receptor complex, thus outside the MHC. *Mill1* and *Mill2* show only limited levels of polymorphism and are transcribed at low levels in most adult tissues. RT-PCR analysis showed that *Mill1* is transcribed in selected tissues such as neonatal thymus and skin whereas *Mill2* is transcribed more ubiquitously at low levels. Predicted MILL1 and MILL2 molecules are glycoproteins with three extracellular domains ($\alpha 1$ to $\alpha 3$), but their $\alpha 1$ and $\alpha 2$ domains lack many of the residues essential for the docking of peptides, suggesting that MILL molecules do not bind peptides. Phylogenetically, MILL1 and MILL2 are most closely related to MICA/B among known class I molecules. Because rodents lack the MICA/B family and conversely, humans do not have the MILL family, we suggested previously that MILL might be a functional substitute for MICA/B (19).

In the present study, we show that MILL1 and MILL2 are GPI-anchored glycoproteins associated with β_2 m. Consistent with the absence of critical residues required for the docking of peptides (19), cell surface expression of MILL1 and MILL2 did not require TAP molecules. Immunohistochemical analysis revealed that MILL1 is expressed in a subpopulation of thymic medullary epithelial cells and a restricted region of inner root sheaths in hair follicles. The ability to form complexes with β_2 m, anchorage to the membrane by GPI, and unique expression patterns all provide further evidence that MILL is a class I family distinct from MICA/B.

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³ Abbreviations used in this paper: β_2 m, β_2 -microglobulin; MILL, MHC class I-like located near the leukocyte receptor complex; PI-PLC, phosphatidylinositol-specific phospholipase C; PNGase F, peptide:N-glycosidase F.

Materials and Methods

Cell lines and Abs

The mouse T lymphoma cell line RMA (H2^b-positive) and its TAP2-deficient mutant RMA-S (H2^b-negative) (21) were obtained from Dr. Kärre (Karolinska Institute, Stockholm, Sweden). Cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated FBS at 37°C and 5% CO₂.

Anti-FLAG mAb M2 (F3165) was purchased from Sigma-Aldrich. Goat polyclonal Ab to mouse β_2m (sc-8361) was purchased from Santa Cruz Biotechnology. Anti-human pan-cytokeratin mAb AE1/AE3 (M1590) and anti-human hair shaft cytokeratin mAb AE13 (ab16113) were purchased from DakoCytomation and Abcam, respectively. Mouse anti-H2-K^b mAb (clone AF6-88.5) and anti-CD45 mAb (clone 30-F11) were from BD Pharmingen. The Abs used as secondary reagents were as follows: FITC-labeled goat anti-mouse IgG, F(ab')₂ fragment (IM0819; Beckman Coulter). FITC-labeled swine anti-rabbit Ig, F(ab')₂ fragment (F0054; DakoCytomation), HRP-conjugated sheep anti-mouse IgG (NA931; Amersham Biosciences), HRP-conjugated donkey anti-rabbit IgG (NA934; Amersham Biosciences), HRP-conjugated donkey anti-goat IgG (sc-2056; Santa Cruz Biotechnology), Alexa Fluor 594-conjugated goat anti-rabbit IgG (A11072; Molecular Probes), and Alexa Fluor 488-conjugated goat anti-mouse IgG (A11001; Molecular Probes). Isotype-matched mouse IgG1 Ab (PP100) and pooled normal rabbit serum (CL1000) were purchased from Chemicon International Inc. and Cedarlane Laboratory Ltd., respectively.

Production of polyclonal Ab against mouse MILL molecules

The $\alpha 1$ - $\alpha 3$ domains of MILL1 and MILL2 with 6 \times His tags at their N termini were expressed in *Escherichia coli* strain M15 using the pQE30 expression vector following the instructions of the manufacturer (Qiagen). Briefly, the DNA fragments encoding the $\alpha 1$ - $\alpha 3$ domains of mouse MILL molecules were amplified by PCR using the BALB/c-derived *Mill* plasmid cDNA (19) as templates. The primer sequences were 5'-TTGCGAGCTC CACTCTGCGCTATGACCT-3' (with a *Sac*I site at its 5'-end) and 5'-CCCAAGCTTATATTGTGGTTGCCGTGCTT-3' (with a *Hind*III site at its 5'-end) for MILL1 and 5'-GTGGATCCACCCACTCTGCGC TATAA-3' (with a *Bam*HI site at its 5'-end) and 5'-CCCAAGCTTCATC CTGACTGTCTCAGCA-3' (with a *Hind*III site at its 5'-end) for MILL2. PCR products digested with *Sac*I/*Hind*III for MILL1 and *Bam*HI/*Hind*III for MILL2 were ligated into *Sac*I/*Hind*III- and *Bam*HI/*Hind*III-digested pQE30, respectively. After transformation into M15, recombinant proteins were induced by adding isopropyl-1-thio- β -D-galactopyranoside to a final concentration of 1 mM. *E. coli* cells were harvested and lysed in buffer B (100 mM NaH₂PO₄, 10 mM Tris-HCl, 6 M guanidine hydrochloride, pH 8.0), and lysates were centrifuged at 10,000 \times g for 20 min at room temperature. Ni-NTA acid resins were added to supernatants and mixed gently by shaking. Resin-lysate mixtures were loaded into an empty column and washed twice with buffer C (100 mM NaH₂PO₄, 10 mM Tris-HCl, 6 M guanidine hydrochloride, pH 5.9). Recombinant proteins were eluted by buffer D (100 mM NaH₂PO₄, 10 mM Tris-HCl, 6 M guanidine hydrochloride, pH 4.5), separated by preparative SDS-PAGE, eluted and concentrated. Purified recombinant proteins (200 μ g per rabbit) were mixed with CFA and injected into rabbits. After 2, 4, and 6 wk, the animals were boosted with the same amount of recombinant proteins mixed with IFA. Whole bloods were collected and antiserum prepared 1 wk after the last boost.

Construction of mammalian expression plasmids

Mouse MILL molecules have an insertion of amino acids between the leader peptide and the $\alpha 1$ domain (19). The coding regions of mouse MILL1 and MILL2 excluding this inserted sequence and the leader peptide were obtained by PCR using the *Mill* plasmid cDNA (19) as templates. The primer sequences were 5'-CCAAGCTTGAACCCACACTCTGCGC TA-3' (with a *Hind*III site at its 5'-end) and 5'-GTGGATCCCTACCAA CACTGTAGAAAAGAGC-3' (with a *Bam*HI site at its 5'-end) for MILL1 and 5'-CCAAGCTTACCCACTCTGCGCTATAA-3' (with a *Hind*III site at its 5'-end) and 5'-GTGGATCCCTCAGTTGGCTCTGGCCAGTG-3' (with a *Bam*HI site at its 5'-end) for MILL2. After digestion with *Hind*III/*Bam*HI, the PCR products were ligated to the *Hind*III/*Bam*HI-digested pFLAG-CMV-3 expression vector carrying a preprotrypsin leader sequence (Sigma-Aldrich). These constructs, designated MILL1-pFLAG-CMV-3 and MILL2-pFLAG-CMV-3, respectively, enabled the expression of MILL molecules with an N-terminal FLAG tag. In all cases, the integrity of expression constructs was verified by sequencing. DNA for transfection was isolated with the plasmid purification kit purchased from Qiagen.

Establishment of stable transfectants

To establish stable cell lines expressing MILL molecules, RMA and RMA-S cells were transfected with linearized MILL1-pFLAG-CMV-3 or MILL2-pFLAG-CMV-3 plasmids by electroporation at 250 V, 950 μ F with Gene Pulser II according to the instructions of the manufacturer (Bio-Rad). Neomycin-resistant cells were selected by treatment with G418 (600 and 800 μ g/ml for RMA and RMA-S, respectively) and clones exhibiting high levels of MILL expression were expanded: expression of MILL proteins was monitored by flow cytometry and immunoblotting with anti-FLAG and anti-MILL Abs.

Flow cytometric analysis

For cell surface staining, single cell suspensions (1×10^6 cells) were washed with ice-cold PBS (pH 7.4) and incubated in 100 μ l of PBS (pH 7.4) containing 0.1% NaN₃ with 1 μ g of mAb or isotype controls for 30 min on ice. After washing with ice-cold PBS (pH 7.4), cells were incubated in 100 μ l of PBS (pH 7.4) containing 0.1% NaN₃ with the FITC-conjugated F(ab')₂ fragment of goat anti-mouse IgG or F(ab')₂ fragment of swine anti-rabbit Ig (1:200 dilution). Subsequently, cells were washed with ice-cold PBS (pH 7.4) and analyzed by EPICS ALTRA (Beckman Coulter). Data were analyzed with EXPO32 software (Beckman Coulter).

Immunoprecipitation and glycosidase digestion

For purification of FLAG-tagged MILL proteins, RMA-MILL1 and RMA-MILL2 stable transfectants (1×10^8 cells) were solubilized by 1 ml of ice-cold lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 20 μ M leupeptin, 1 μ M pepstatin, pH 7.5). After incubation for 30 min at 4°C, cell lysates were centrifuged at 13,000 \times g for 10 min at 4°C to remove cell nuclei and insoluble proteins. Cleared lysates were incubated with protein G-Sepharose beads (Amersham Biosciences) at 4°C for 1 h. Supernatants were incubated with anti-FLAG mAb-coupled protein G-Sepharose beads at 4°C for 1 h. After washing 4 times with lysis buffer, immunoprecipitated proteins were eluted by 0.1 M glycine-HCl (pH 3.0), and immediately neutralized by adding 0.1 M Tris-HCl (pH 9.0). Eluted proteins were denatured and treated with 500 U/ μ l peptide:N-glycosidase F (PNGase F; New England Biolabs) at 37°C for 18 h.

Immunoblotting

To detect MILL proteins and β_2m , samples were incubated in 1 \times SDS sample buffer at 95°C for 10 min. Denatured proteins were separated on 12% SDS-PAGE and transferred to Hybond-P polyvinylidene difluoride membranes (Amersham Biosciences) using a semidry blotter (Bio-Rad) at 15 V for 45 min. The blotted membranes were incubated with 5% skim milk or 3% BSA in PBS (pH 7.4) containing 0.1% Tween 20 (PBST) at room temperature for 60 min and then incubated with 1/500 diluted antiserum or 1 μ g/ml of Ab in PBST at room temperature for 60 min. After washing twice with PBST, the membranes were incubated with 1/25,000 diluted HRP-conjugated anti-mouse, rabbit or goat IgG Abs. After washing three times with PBST, positive bands were visualized using the ECL-Plus (Amersham Biosciences) or the Super Signal West Dura detection system (Pierce).

Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment

RMA-MILL and RMA-S-MILL cells were washed with PBS (pH 7.4) and treated with 1 U/ml PI-PLC (Sigma-Aldrich) in PBS (pH 7.4) at 37°C for 1 h. Subsequently, cells were washed with ice-cold PBS (pH 7.4) and used for flow cytometric analysis.

Coimmunoprecipitation of cell surface MILL molecules

Cell surface MILL proteins expressed on the RMA-MILL stable transfectants were purified by PI-PLC treatment and immunoprecipitation with anti-FLAG Ab-coupled protein G-Sepharose beads. Immunoprecipitates were subjected to immunoblotting using anti-FLAG and anti-mouse β_2m Ab.

Refolding of bacterially expressed MILL ectodomains

cDNA encoding the ectodomains of MILL1 and MILL2 were amplified by PCR using the *Mill* plasmid cDNA (19) as templates. Primers used were 5'-CATTAAATGGACAACCAAGACTGGTG-3' (sense) and 5'-TCC CCGGGGGCAGCAGGTTCAATTGATA-3' (antisense) for MILL1, and 5'-CCATATGTCCAGCATCCAAGGAACC-3' (sense) and 5'-AAAAG TACTGACAGCTGTCTGCATGATG-3' (antisense) for MILL2. These

primers contained *AseI*, *SmaI*, *NdeI*, or *ScaI* restriction enzyme sites indicated by underlines. The PCR-generated cDNA fragments of MILL1 and MILL2 were cloned into the bacterial expression vector pET3cN-bio, which was designed to express a recombinant protein with an N-terminal enzymatic biotinylation signal (22), to construct MILL1-pET3cNbio and MILL2-pET3cNbio, respectively. Rosetta (DE3) strain of *E. coli* (Novagen, Merck) was transformed with MILL1-pET3cNbio or MILL2-pET3cNbio. Expression of soluble MILL1 or MILL2 was induced with 1 mM isopropyl-1-thio- β -D-galactopyranoside, and MILL proteins were refolded from the purified inclusion bodies by dilution as described previously (22). To examine effects of β_2m on refolding, C57BL/6-derived β_2m (β_2m^b), similarly expressed in *E. coli*, was included in the refolding mixture at the molar ratio of 1:2 (MILL/ β_2m). Refolded soluble MILL1 and MILL2 proteins were purified by anion-exchange column chromatography and gel-filtration chromatography. In anion-exchange chromatography on a UNO Q-6 column using 20 mM Tris-HCl buffer (pH 8.5) as a mobile phase, soluble MILL1 or MILL2 refolded in the presence of β_2m was eluted in the approximately 250 mM Cl⁻ fraction by a 0–500 mM NaCl gradient. The gel-filtration column chromatography was performed on a Superdex 75 10/30 column (Amersham Biosciences) equilibrated with 25 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl at the flow rate of 0.5 ml/min. The column was calibrated with gel-filtration standards from Bio-Rad.

Immunohistochemistry

For immunostaining, frozen sections prepared from 3-day-old, 10-day-old, and 6-wk-old male BALB/c mice were fixed using cold acetone for 5 min, washed with PBS, stained by the standard method (23), and then mounted in fluorescent mounting medium (DakoCytomation). Immunofluorescence was detected using a fluorescence microscope (ECLIPSE E600; Nikon). To evaluate the specificity of staining, the antiserum against MILL1 was diluted 1/40 with PBS to a final volume of 1 ml and absorbed with 5×10^7 RMA-MILL1 or RMA cells at 4°C overnight. The preabsorbed antiserum was diluted 1/80 with PBS and used for immunostaining. All experiments using animals have been reviewed and approved by the institutional review committee of Hokkaido University Graduate School of Medicine.

Isolation of thymic stromal cells

Thymi were dissected from 4-wk-old C57BL/6 and β_2m -deficient mice. Breeding pairs of the β_2m -deficient strain, B6.129P2-*B2m^{mtUscfJ}* (stock no. 002087), were purchased from The Jackson Laboratory, and their progenies were produced at Kinki University School of Medicine. Thymic stromal cells were enriched as described (24). Briefly, thymic fragments were digested in RPMI 1640 medium containing collagenase D and DNase I

(both obtained from Roche) at 37°C for 15 min. After repeating this procedure 3 times, cells were pooled and stained with mAb for CD45. CD45-negative fractions containing stromal cells including thymic epithelial cells were subjected to flow cytometric analysis.

Results

Establishment of stable cell lines expressing N-terminally FLAG-tagged MILL molecules and generation of rabbit antisera specific for MILL molecules

To facilitate biochemical analysis, we transfected FLAG-tagged expression plasmids into the mouse T lymphoma cell line RMA and established stable cell lines, RMA-MILL1 and RMA-MILL2, expressing N-terminally FLAG-tagged MILL1 and MILL2 molecules, respectively (Fig. 1A). Cell surface expression of MILL1 and MILL2 was confirmed by flow cytometry using the anti-FLAG Ab as well as the rabbit antisera generated against bacterially expressed MILL1 and MILL2 molecules. The specificity of our rabbit antisera was further confirmed by Western blot analysis of whole cell lysates (Fig. 1B). The anti-FLAG Ab detected two major bands of 48 and 41 kDa in RMA-MILL1 cells. The band of 41 kDa was nonspecific because it was detected in untransfected RMA cells. A major band of 48 kDa and a minor band of 44 kDa were detected by the anti-MILL1, but not anti-MILL2, antiserum (Fig. 1B). In RMA-MILL2 lysates, the anti-FLAG Ab detected bands of 43 and 41 kDa (Fig. 1B, top), which were also detected with the anti-MILL2, but not anti-MILL1, antiserum (Fig. 1B, bottom). Thus, the band of 41 kDa detected by the anti-FLAG Ab in RMA-MILL2 cells presumably represents doublets containing both specific and nonspecific signals. We also expressed MILL1 and MILL2 molecules on RMA cells using their endogenous signal peptides and performed cytometric analysis using the MILL-specific rabbit antisera. We obtained staining patterns similar to those shown in Fig. 1A (data not shown).

Deduced MILL1 and MILL2 molecules have three potential N-linked glycosylation sites, respectively (19). To examine glycosylation status, we isolated MILL molecules from the stable transfectants by immunoprecipitation with the anti-FLAG Ab, removed

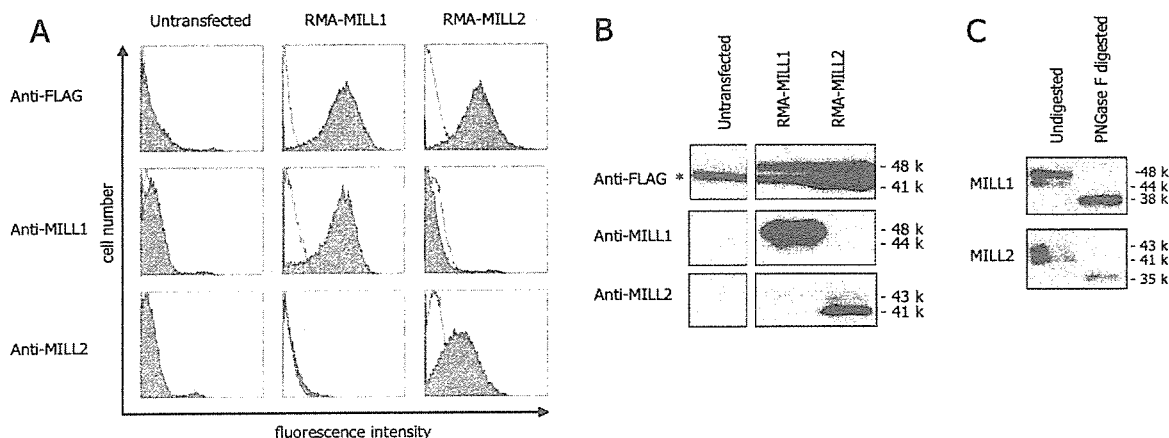


FIGURE 1. MILL1 and MILL2 are cell surface glycoproteins with N-linked sugars. **A**, Untransfected RMA cells and the transfected cell lines, RMA-MILL1 and RMA-MILL2, which stably express MILL1 and MILL2, respectively, were incubated with anti-FLAG mAb and FITC-conjugated goat anti-mouse IgG, anti-MILL1 antiserum (1/500 dilution) and FITC-labeled swine anti-rabbit Ig, or anti-MILL2 antiserum (1/500 dilution) and FITC-labeled swine anti-rabbit Ig (from the top to the bottom, shaded histograms). Negative control staining (open histograms) was obtained using an isotype-matched control Ab (top three panels) or normal rabbit serum (all other panels). Stained cells were analyzed by flow cytometry. **B**, Whole cell lysates of RMA-MILL1 and RMA-MILL2 were separated on 12% SDS-PAGE and subjected to immunoblotting using anti-FLAG mAb (top), anti-MILL1 antiserum (middle), or anti-MILL2 antiserum (bottom). Signals were detected by HRP-conjugated secondary Ab and ECL-Plus reagents. Nonspecific bands are indicated by asterisks. **C**, MILL1 and MILL2 proteins were immunoprecipitated with anti-FLAG mAb-coupled protein G-Sepharose beads from RMA-MILL1 and RMA-MILL2 cell lysates, respectively. After digestion with PNGase F at 37°C for 18 h, samples were separated on 12% SDS-PAGE and subjected to immunoblotting. MILL1 was detected by the rabbit anti-MILL1 antiserum and MILL2 by the rabbit anti-MILL2 antiserum. Signals were detected by HRP-conjugated secondary Ab and ECL-Plus reagents.

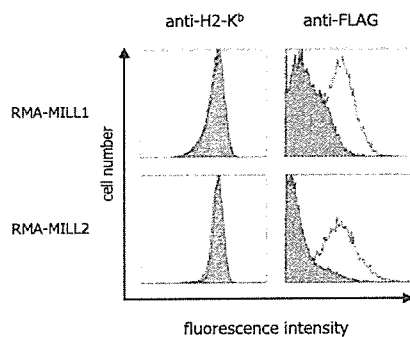


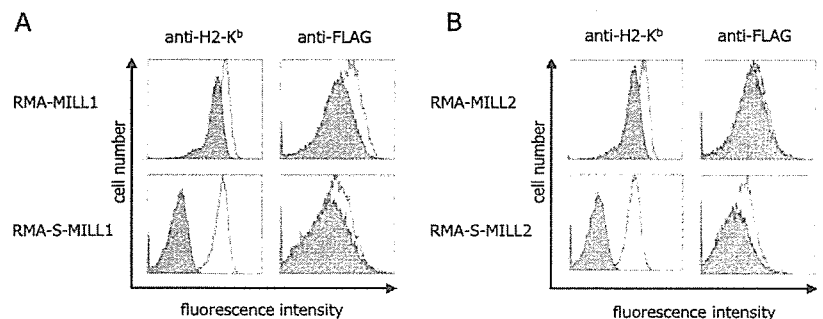
FIGURE 2. MILL1 and MILL2 are GPI-anchored proteins. RMA-MILL1 and RMA-MILL2 cells (top and bottom, respectively) were incubated with 1 U/ml PI-PLC (shaded histograms) or PBS (open histograms). Subsequently, cells were stained with anti-H2-K^b (left) or anti-FLAG (right) mAb. An FITC-conjugated F(ab')₂ fragment of goat anti-mouse IgG was used as a secondary Ab. Stained cells were analyzed by flow cytometry.

N-linked glycans with PNGase F and performed immunoblot analysis with the MILL-specific antisera (Fig. 1C). We obtained two bands of 44 and 48 kDa for non-treated MILL1, and a single band of 38 kDa for PNGase F-treated MILL1 (Fig. 1C, top). Similarly, we obtained two bands of 41 and 43 kDa for non-treated MILL2, and a single band of 35 kDa for PNGase F-treated MILL2 (Fig. 1C, bottom). The expression constructs used for stable transfection predicted *M_r* of 39280.83 and 35013.86 for the protein moieties of N-terminally flagged MILL1 and MILL2 molecules, respectively. Thus, the sizes of deglycosylated products agreed well with theoretical expectations. These results indicate that MILL1 and MILL2 are cell surface glycoproteins with *N*-linked sugars.

MILL1 and MILL2 are GPI-anchored proteins

We initially assumed that MILL1 and MILL2 were transmembrane proteins (19, 20). However, different prediction algorithms yielded inconsistent results concerning the presence or absence of transmembrane regions. Subsequent sequence analysis using the software 'big-PI Predictor' (25) suggested that MILL1 and MILL2 were likely GPI-anchored proteins. To examine this possibility, RMA-MILL1 and RMA-MILL2 cells were treated with PI-PLC, stained with the anti-FLAG Ab and examined by flow cytometry. In both RMA-MILL1 and RMA-MILL2 cells, cell surface staining was reduced markedly by PI-PLC treatment (Fig. 2, right panel). By contrast, cell surface staining with the H2-K^b Ab was not affected by similar treatment (Fig. 2, left panel), consistent with the fact that H2-K^b is an integral membrane protein. These results indicate that MILL1 and MILL2 are GPI-anchored cell surface proteins.

FIGURE 3. Cell surface expression of MILL molecules does not require functional TAP molecules. *A*, RMA-MILL1 and RMA-S-MILL1 cells were cultured at 25°C (open histograms) or 37°C (shaded histograms) for 18 h. Cells were incubated with anti-H2-K^b (left) or anti-FLAG (right) and then treated with FITC-conjugated F(ab')₂ fragments of goat anti-mouse IgG. Stained cells were analyzed by flow cytometry. *B*, RMA-MILL2 and RMA-S-MILL2 cells were treated in the same manner as in *A*, and cell surface expression of MILL2 was monitored by flow cytometry.



Cell surface expression of MILL molecules is TAP-independent

RMA-S is a variant derived from RMA cells (21) that lacks functional TAP molecules because of a defective TAP2 subunit (26, 27). At 37°C, classical class I molecules are barely expressed on the surface of RMA-S cells because empty class I molecules (class I molecules without peptides) are thermodynamically unstable. However, RMA-S cells express empty class I molecules when they are cultured at lower temperatures (28). To examine whether surface expression of MILL requires TAP, we transfected RMA-S cells with MILL expression plasmids and established stable transfectants. These cells were cultured at 25°C or 37°C and stained with the anti-FLAG or anti-H2-K^b Ab. As expected, endogenous H2-K^b molecules were expressed on RMA-S cells at the level comparable to that expressed on RMA cells when these cells were cultured at 25°C (Fig. 3, *A* and *B*, left panel, open histograms). However, expression of H2-K^b on RMA-S cells was reduced markedly when the cells were cultured at 37°C (Fig. 3, *A* and *B*, left panel, shaded histograms). By contrast, the expression levels of MILL1 and MILL2 detected by the anti-FLAG Ab were nearly the same regardless of whether the RMA-S cells were cultured at 25°C or 37°C (Fig. 3, *A* and *B*, right panel). These results indicate that cell surface expression of MILL molecules is TAP-independent.

Cell surface-expressed MILL1 and MILL2 molecules are associated with β_2m

To examine whether MILL molecules are associated with β_2m *in vivo*, we performed coimmunoprecipitation analysis. After treatment of RMA-MILL1 and RMA-MILL2 cells with PI-PLC, the MILL molecules released into the supernatants were immunoprecipitated with the anti-FLAG Ab and subjected to immunoblotting analysis using the anti-FLAG and anti-mouse β_2m Ab (Fig. 4). We found that β_2m was coimmunoprecipitated with both MILL1 and MILL2, indicating that MILL molecules are associated with β_2m on the cell surface.

β_2m facilitates the refolding of MILL molecules

To examine whether MILL1 and MILL2 can associate with β_2m *in vitro*, we expressed the extracellular domains of MILL1 and MILL2 in *E. coli* and refolded them in the presence or absence of mouse β_2m . MILL1 could be successfully refolded only in the presence of β_2m (Fig. 5A, top panel), and MILL1 and β_2m were eluted in the same fractions in gel filtration chromatography as revealed by SDS-PAGE analysis (Fig. 5B, bottom half, top panel). Although MILL2 was able to form soluble proteins when it was refolded in the absence of β_2m , β_2m appeared to improve the efficacy of refolding, consistent with our other results (Fig. 5A, bottom panel). MILL2 refolded in the presence of β_2m was eluted earlier in gel filtration chromatography than that refolded in the absence of β_2m (Fig. 5A, bottom panel), indicating that MILL2

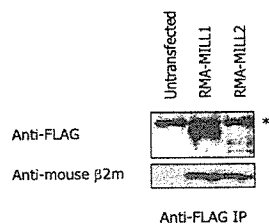


FIGURE 4. Cell surface-expressed MILL1 and MILL2 molecules are associated with β_2m . RMA-MILL1 and RMA-MILL2 cells were treated with PI-PLC and soluble MILL proteins were purified by immunoprecipitation with anti-FLAG mAb-coupled protein G-Sepharose beads (right). Precipitated samples were separated on 12% (top) or 14% (bottom) SDS-PAGE and subjected to immunoblotting analysis. MILL1 and MILL2 were detected by anti-FLAG mAb (top) whereas mouse β_2m was detected by anti-mouse β_2m Ab (bottom). Signals were detected by HRP-conjugated secondary Ab using the Super Signal West Dura kit. An asterisk indicates mouse IgG H chains.

molecules refolded in the presence of β_2m were associated with β_2m , which was further confirmed by SDS-PAGE analysis (Fig. 5B, bottom panel). MILL1 and MILL2 refolded in the presence of β_2m were purified by anion-exchange chromatography followed by gel filtration chromatography. The purified MILL1 and MILL2 proteins contained β_2m as a subunit (Fig. 5C). These results indicate that efficient refolding of MILL1 and MILL2 requires β_2m as a subunit. The molecular masses of MILL1/ β_2m and MILL2/ β_2m complexes estimated by gel filtration chromatography (Fig. 5A) and the relative intensities (3:1) of the MILL1 and MILL2 bands to the β_2m bands in the purified MILL1/ β_2m and MILL2/ β_2m complexes (Fig. 5C) indicate that the MILL1 or MILL2 polypeptide and β_2m bind at a 1:1 ratio.

MILL1 is expressed in a subpopulation of thymic medullary epithelial cells and a restricted region of inner root sheaths in hair follicles

To determine the tissue distribution of MILL1 and MILL2 molecules, we first performed Western blot analysis using the antisera for MILL1 and MILL2 against a panel of tissues isolated from adult and neonatal mice. These experiments yielded no bands in any tissues, presumably because the expression levels of MILL1 and MILL2 are low (data not shown). Our previous RT-PCR analysis (19) indicated that *Mill1* was transcribed in selected tissues including neonatal thymus and skin. We therefore examined expression of MILL1 in these tissues (Fig. 6). Staining was observed in a subpopulation of medullary epithelial cells in the neonatal thymus (Fig. 6A). These MILL1-positive cells were also detectable in the thymus of adult mice (data not shown). In the skin of 3-day-old mice, cells stained with the anti-MILL1 antiserum were found in the hair follicle (Fig. 6B, left). However, these cells became undetectable in the skin of 10-day-old (Fig. 6B, right) or 6-wk-old (not shown) mice. To more precisely address the locations of cells stained with the anti-MILL1 antiserum, we performed immunohistochemical staining of hair shafts and outer root sheaths (Fig. 6C). Cells stained with the anti-MILL1 antiserum were located outside the hair shaft (stained green with AE13 mAb), but inside the outer root sheath (stained green with AE1/AE3 mAb). Thus, positively stained cells are located in the inner root sheath. Because not all regions of inner root sheaths were stained with the antiserum, MILL1 seems to be expressed in a restricted region of the inner root sheath. To confirm the specificity of staining, we prepared anti-MILL1 antiserum preabsorbed with RMA-MILL1 or RMA cells. Preabsorption of the antiserum with RMA-MILL1 cells almost eliminated staining in thymic epithelial cells and hair

follicles whereas staining was retained when the antiserum was preabsorbed with RMA cells (data not shown). *Mill2* is transcribed almost ubiquitously at low levels (19). We stained several tissues including neonatal thymus and skin as well as adult aorta, uterus, heart, kidney and spleen with the antiserum for MILL2 (1/200 dilution). Although this antiserum, when used at this dilution, was capable of staining RMA-MILL2 cells grown in vivo in C57BL/6 mice, we were unable to obtain any positive staining for MILL2 in any of the tissues (data not shown).

Cell surface expression of MILL1 on thymic epithelial cells requires β_2m

To examine whether cell surface expression of MILL1 requires β_2m , we isolated thymic stromal cells from 4-wk-old β_2m -deficient mice and stained with the anti-MILL1 antiserum (Fig. 7). Cell surface expression of MILL1 was almost completely abrogated in the β_2m -deficient mice compared with the adult C57BL/6 mice, indicating that cell surface expression of MILL1 is β_2m -dependent.

Discussion

MILL is the latest addition to the growing list of mammalian MHC class I families encoded outside the MHC region. Our previous work has revealed several unique features of this class I family (19, 20). First, not all mammalian species have the MILL family; although mice and rats have this family, it is absent in humans. Because MILL apparently arose before the radiation of mammals, humans seem to have lost this class I family. Second, unlike all other class I genes, the genes coding for mouse MILL have an exon between those coding for the signal peptide and the $\alpha 1$ domain. Third, the MILL family is phylogenetically most closely related to the MICA/B family among known class I families. Because the MILL family is absent in humans, and conversely, mice and rats lack the MICA/B family, we suggested that MILL might serve as a functional substitute of MICA/B in rodents (19). Fourth, deduced MILL molecules lack most of the residues required for the docking of peptide termini, suggesting that they are unlikely to bind peptides. Fifth, RT-PCR analysis indicated that the members of the MILL family are poorly transcribed in most adult tissues, suggesting a role other than conventional Ag presentation. Sixth, sequence comparison of rat and mouse MILL molecules revealed that *Mill* is one of the most rapidly evolving class I gene families, and that, in both *Mill1* and *Mill2*, non-synonymous substitutions occur more frequently than synonymous substitutions in the $\alpha 1$ domain whereas the opposite is the case in the $\alpha 2$ and $\alpha 3$ domains, suggesting that the $\alpha 1$ domain may be under positive selection (20). Taking all of these points into consideration, we suggested that MILL may perform specialized immune functions required only in certain species or some redundant functions, part of which are executed by other molecules (20).

In the present study, we set out to perform a biochemical characterization of mouse MILL molecules. Consistent with the absence of key residues required for the docking of peptides, we found that cell surface expression of MILL1 and MILL2 does not require functional TAP molecules (Fig. 3). Furthermore, the extracellular domains of MILL1 and MILL2 expressed in *E. coli* could be efficiently refolded in the absence of peptides under standard class I refolding conditions when β_2m was added into the mixture (Fig. 5). This is in contrast to the fact that refolding of recombinant class Ia molecules isolated from purified bacterial inclusion bodies requires the presence of a peptide ligand and is reminiscent of the behaviors of certain class Ib molecules, the refolding of which is ligand-independent (29–31). Taken together, it

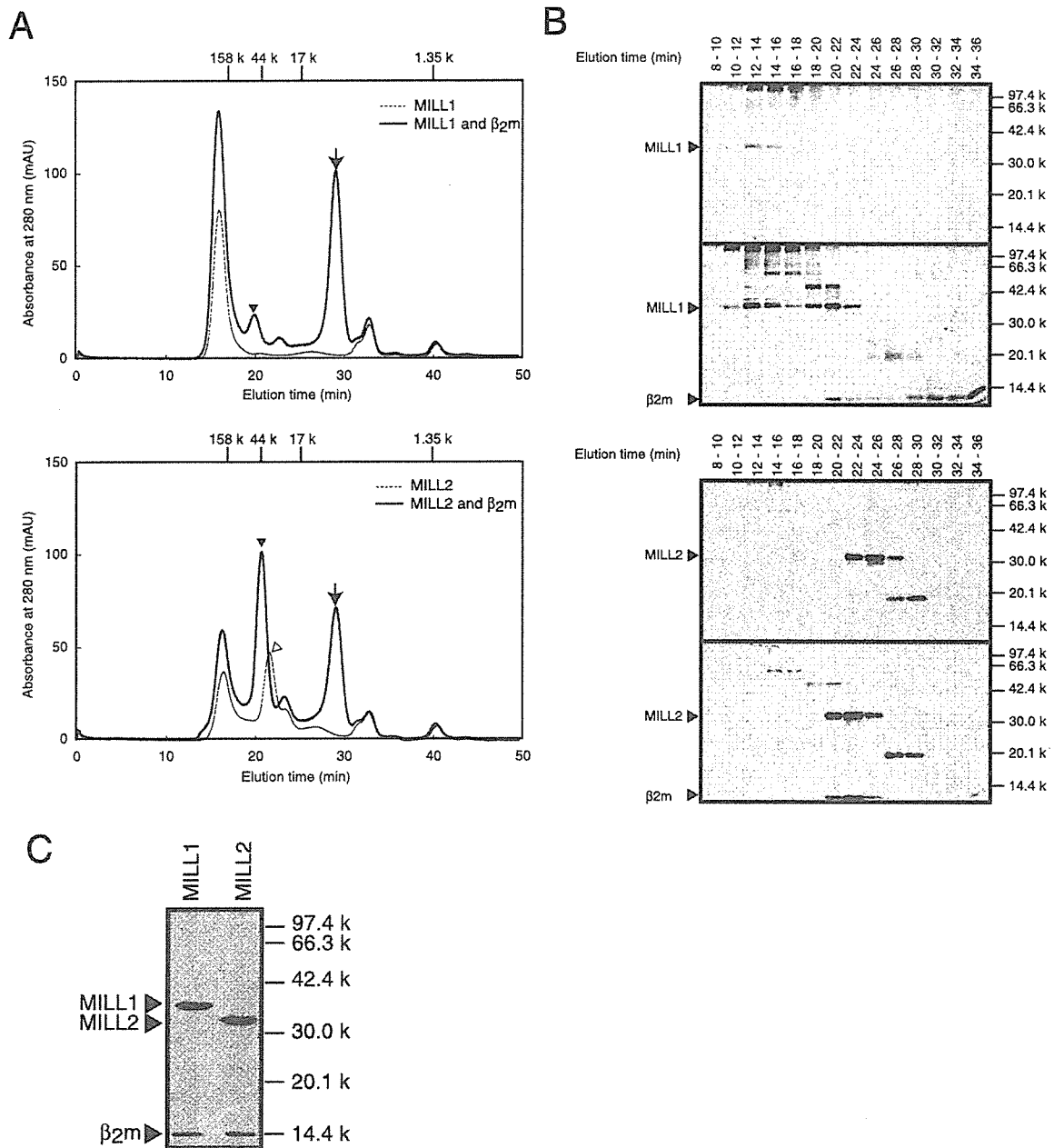


FIGURE 5. Refolding of MILL1 and MILL2 requires β_2m . **A**, Bacterially expressed extracellular domains ($\alpha 1$ – $\alpha 3$) of MILL1 and MILL2 were refolded in the presence (continuous line) or absence (broken line) of β_2m and subjected to gel filtration chromatography on Superdex-75. Filled arrowheads indicate the peaks of MILL1 and MILL2 proteins associated with β_2m . Arrows indicate the peaks of free β_2m . An open arrowhead indicates the peak of MILL2 refolded in the absence of β_2m . **B**, The fractions from gel filtration chromatography were analyzed on SDS-PAGE, and the gels were stained with silver staining. The top and bottom halves of each panel indicate fractionation of the samples refolded in the absence and presence of β_2m , respectively. **C**, Coomassie brilliant blue-stained SDS-PAGE gel of *in vitro* refolded MILL1 and MILL2 molecules purified by sequential chromatography.

is likely that the MILL family of class I molecules performs functions other than the presentation of peptides.

Two observations made in this work were rather unexpected. First, we initially assumed that, like most other class I family members, MILL1 and MILL2 were integral membrane proteins with a transmembrane region (19, 20). Contrary to this assumption, MILL1 and MILL2 turned out to be GPI-anchored proteins (Fig. 2). The occurrence of GPI anchors is not unprecedented for class I molecules because most if not all members of RAE-1 and ULBP families as well as a large proportion of Qa-2 molecules are GPI-anchored (32–35). Like other GPI-anchored proteins (36, 37), MILL may be primarily located in lipid rafts. Second, we assumed that MILL1 and MILL2 were unlikely to be associated with β_2m

because they lack many of the residues known to interact with β_2m in classical class I molecules (19). Our present work demonstrates that both MILL1 and MILL2 are associated with β_2m on the cell surface (Fig. 4). A similar unexpected association with β_2m was previously observed for MR1; this class Ib molecule lacks many of the phylogenetically conserved motifs implicated in β_2m association in class Ia molecules (38), yet biochemical studies have revealed that it associates with β_2m (38, 39). We also found that β_2m promoted refolding of bacterially produced MILL ectodomains *in vitro* (Fig. 5). Hence, β_2m appears to constitute an integral component of MILL class I molecules. Consistent with this, cell surface expression of MILL1 on thymic stromal cells was almost completely abrogated in β_2m -deficient mice, indicating that cell

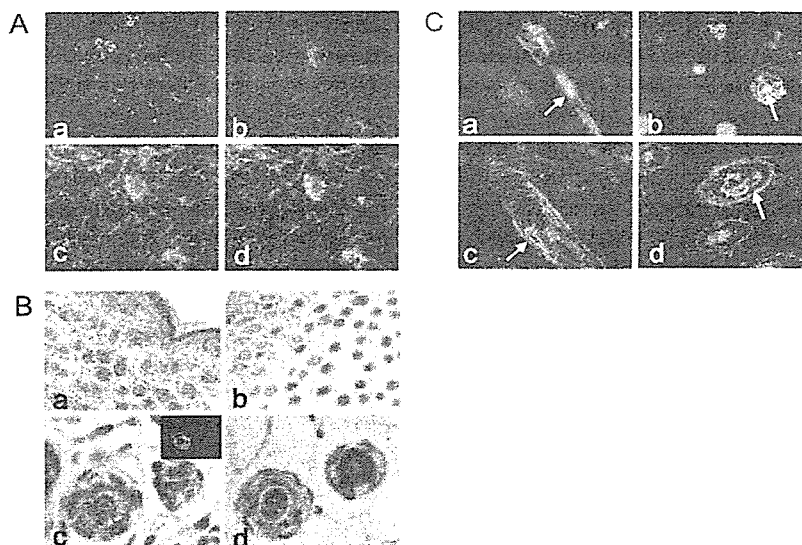


FIGURE 6. MILL1 is likely expressed in thymic medullary epithelial cells and hair follicles. *A*, Thymic tissue sections obtained from 3-day-old mice were blocked by incubation with normal goat serum (1/500 dilution), reacted with AE1/AE3 and anti-MILL1 (1/400 dilution) followed by staining with Alexa Fluor 488-conjugated goat anti-mouse IgG (1/300 dilution) and Alexa Fluor 594-conjugated goat anti-rabbit IgG (1/300 dilution). *Aa*, a low-power photo micrograph of the thymic medulla (original magnification $\times 100$); *Ab–Ad*, a high-power magnification (original magnification $\times 400$). Images for MILL1 (stained red, *Ab*) and AE1/AE3 (stained green, *Ac*) as well as the merged image (*Ad*) were obtained with a Nikon ECLIPSE E600 microscope. *B*, Skin tissues of 3-day-old (*Ba* and *Bc*) and 10-day-old (*Bb* and *Bd*) mice. *Upper panels*, H&E (original magnification $\times 100$). *Lower panels*, H&E (original magnification $\times 400$). The inset in *Bc* shows staining with the anti-MILL1 antiserum (original magnification $\times 100$). Staining was done as described in the legend to *C*. *C*, MILL1 is likely expressed in cells of the inner root sheaths. In *Ca* and *Cb*, tissue sections were blocked by incubation with normal goat serum (1/500 dilution), reacted with AE13 (1/1000 dilution) and anti-MILL1 (1/400 dilution) and stained with Alexa Fluor 488-conjugated goat anti-mouse IgG (1/300 dilution) and Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:300 dilution). In *Cc* and *Cd*, AE13 was substituted by AE1/AE3. MILL1 is stained red. The hair cortex (*Ca* and *Cb*) and outer root sheaths (*Cc* and *Cd*) are stained green. In *Ca* and *Cc*, hair shafts were sectioned parallel to the long axis. *Cb* and *Cd* show cross sections of hair shafts. Arrows in *Ca* and *Cb* indicate the hair cortex, whereas those in *Cc* and *Cd* indicate outer root sheaths. Original magnification $\times 400$.

surface expression of MILL1 requires β_2m (Fig. 7). Given the overall structural similarity of MILL1 and MILL2 (19), and their shared biochemical properties (Figs. 2–5), it seems reasonable to assume that MILL2 also requires β_2m for cell surface expression. Because the refolding experiments showed that MILL2, but not MILL1, was able to form soluble proteins in the absence of β_2m , albeit much less efficiently than in the presence of β_2m (Fig. 5), β_2m might not be an absolute requirement for cell surface expression of MILL2. Human CD1d molecules, normally associated with β_2m , can be expressed on the surface of intestinal epithelial cells in a β_2m -independent manner (40, 41), indicating that the requirement for β_2m can differ depending on tissues. Therefore, it will be necessary to identify cells or tissues where MILL2 is physiologically expressed to determine whether cell surface expression of MILL2 requires β_2m in vivo, and if it does, whether β_2m is absolutely required.

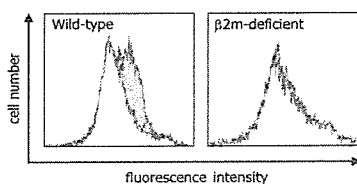


FIGURE 7. Cell surface expression of MILL1 requires β_2m . Thymic stromal cells isolated from C57BL/6 (*left panel*) and β_2m -deficient (*right panel*) mice were stained with normal rabbit serum (open histograms) or the rabbit anti-MILL1 antiserum (shaded histograms). An FITC-labeled swine anti-rabbit Ig was used as a secondary Ab. Stained cells were analyzed by flow cytometry.

Immunohistochemical analysis showed that MILL1 is expressed in a subpopulation of thymic medullary epithelial cells and a restricted region of inner root sheaths in hair follicles (Fig. 6). Expression in the thymus is suggestive of an immunological role for MILL1. Totally unexpected was the observation that some inner root sheath cells in 3-day-old, but not 10-day-old or 6-wk-old mice were stained with the antiserum for MILL1, although we cannot rule out the possibility that our anti-MILL1 antiserum cross-reacts with epitopes on unrelated molecules in hair follicles. Hair follicles have been proposed to enjoy immune privilege (42, 43). Thus, MILL1 may somehow be involved in the establishment and maintenance of immune privilege in hair follicles. On the other hand, we have thus far been unable to identify cells expressing MILL2 proteins despite the fact that the *Mill2* gene is ubiquitously transcribed at low levels. Thus, expression of MILL2 proteins might be translationally regulated or MILL2 proteins might be expressed at detectable levels only in highly specialized cells as recently demonstrated for certain class I molecules (16, 17). It is also possible that expression of the MILL family is enhanced by certain stimuli or under pathologic conditions. To fully understand the expression patterns of the MILL family, more detailed analysis is required.

In conclusion, this study highlights the biochemical differences between the MILL and MICA/B families of class I molecules. MILL1 and MILL2 are TAP-independent, β_2m -associated glycoproteins attached to the cell surface by GPI anchors. In contrast, MICA and MICB are TAP-independent, transmembrane proteins that do not associate with β_2m (44). These two families of class I molecules also differ in their expression patterns. MICA and MICB are stress-inducible class I molecules usually not expressed

on the surface of normal cells (44). In contrast, expression of *Mill1* or *Mill2* mRNA is not inducible by heat shock (our unpublished observation), and the expression in hair follicles seems unique to the MILL family. Furthermore, our preliminary work indicates that NK cells are not stained with MILL tetramers. All of these observations argue against the possibility that MILL is a functional substitute of MICA/B in rodents. Generation of knockout mice may provide a clue for understanding the biologic function of the MILL family.

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Disclosures

The authors have no financial conflict of interest.

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