resembled that of constant light (Fig. 1) during the day phase. Given that the wave form observed in light/dark cycles results from a combination of circadian regulation and the light induction of *cab2-luc* (*11*), aberrant rhythms in the *elf3* mutant could be due to a defect in one or both of these regulatory pathways.

To further test the hypothesis that a circadian clock remains functional in *elf3-1* mutants, we assayed *cab2-luc* expression under constant dark conditions. Expression in constant dark of *cab2-luc* in populations of wild type or *elf3-1* was similar (Fig. 3, A and B) (*12*). Although the first peak of *cab2-luc* expression observed in constant dark conditions could result from entrainment in light/dark cycles, a second peak in *cab2-luc* bioluminescence was detectable in both wild type and *elf3-1* mutants, indicating the presence of a dampened, long-period rhythm. This observation was confirmed by the use of a mutant background that results in overexpression of *cab2-luc* (*12*). Rhythmic *cab2-luc* transcription was observed in constant dark in both *supernova* (*snv*) and *elf3-1 snv* mutant seedlings (Fig. 3, C and D). *elf3-1* mutants therefore contain a functional circadian clock in darkness but are arrhythmic in continuous white light (LL).

The conditionality of arrhythmic *cab2-luc* expression in the *elf3-1* mutant, with a lack of rhythm observed in constant light but not in constant dark, together with increasingly altered expression of *cab2-luc* in lengthening photoperiods, suggests that a longer daylength—or the lack of a dark period—results in the loss of circadian regulation in the absence of *ELF3* function. An alteration in two distinct circadian responses suggests that the *elf3-1* mutation causes a global defect in circadian regulation. The retention of rhythmicity in *elf3-1* mutant seedlings grown in constant dark suggests that *elf3* is not a simple null mutation of an oscillator component itself. The previously described defect in light inhibition of hypocotyl elongation in *elf3* mutant seedlings is indicative of *ELF3* function being required for light perception or signaling. We favor a model in which the *ELF3* gene product functions on a light-input pathway to the circadian oscillator, and that the aberrant coordination of light and circadian regulatory pathways contributes to the altered flowering time and photoperiodic insensitivity observed in *elf3* mutants.

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5. The KUJATA imaging system recorded cotyledon position every 20 min. Individual traces were analyzed to identify rhythmic behavior and provide a measurement of the period and intensity of rhythmic patterns. Significance levels were taken as 5%. Because the strength of rhythmicity is inversely proportional to relative amplitude, robust rhythmicity was defined as any 19- to 30-hour period with relative amplitude equal to or less than the wild-type mean relative amplitude plus one arithmetic standard deviation. Circadian rhythmicity was defined as robust rhythmicity with a period of 22 to 27 hours. Less than 100% of wild-type seedlings showed robust rhythmicity of leaf movements because of technical difficulties inherent in the assay. Period values are arithmetic means ± SD.
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12. Traces were analyzed as described (*5*). The mean wild-type period (±SD) was 28.6 ± 0.61 hours on a sample size of six, all of which showed rhythmic behavior. The mean *elf3-1* period was 28.8 ± 1.9 hours on a sample size of 18, 16 of which showed rhythmic behavior. The *supernova* (*snv*) mutation was isolated in a screen for alterations in *cab2-luc* expression (*13*). *SNV* maps to a locus distinct from the *cab2-luc* transgene, and the *snv* mutation has no effect on the period, phase, or relative amplitude of circadian regulation of *cab2-luc* expression (*13*). The mean *snv* period was 25.2 ± 2.3 hours on a sample size of 26, 16 of which showed rhythmic behavior. The mean *elf3-1 snv* period was 25.3 ± 1.7 hours on a sample size of 40, 21 of which showed rhythmic behavior.
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29 April 1996; accepted 27 August 1996

Protection from Natural Killer Cell–Mediated Lysis by HLA-G Expression on Target Cells

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The outermost layer of the human placenta is devoid of classical class I human leukocyte antigens (HLA-A, HLA-B, and HLA-C) and class II proteins (HLA-DR, HLA-DQ, and HLA-DP). Although this prevents recognition by maternal T lymphocytes, the lack of class I molecules leaves these cells susceptible to attack by natural killer (NK) cells. However, trophoblast cells directly in contact with the maternal tissues express the class I molecule HLA-G, which may be involved in protecting the trophoblast from recognition by NK cells. Here evidence is provided that expression of HLA-G is sufficient to protect otherwise susceptible target cells from lysis by activated NK1 and NK2 cell lines and clones that are specific for distinct groups of HLA-C alleles. The receptors on NK cells that recognize HLA-G are also identified.

During mammalian pregnancy, hemiallogeneic fetal cells invade the uterine structures and survive without immunological rejection. The outermost extravillous cytotrophoblast cells of the human placenta lack classical major histocompatibility complex (MHC) molecules (*1*). However, the presence of an unusual class I molecule has long been demonstrated (*2, 3*). This molecule was shown to be the product of the HLA-G gene (*4*), originally detected as an intact class I gene not expressed in any cell or tissue examined (*5*). HLA-G is 86% homologous to the HLA-A, -B, -C consensus sequence, but lacks the cytoplasmic tail (*5*). Although reduced expression of this molecule is associated with some abnormalities during pregnancy (*6*), its func-

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tion, if any, remains elusive. One recognized role of NK cells is to eliminate cells that have lost MHC class I surface expression because of malignant transformation or viral infection (*7, 8*). The human trophoblast, except for the presence of some HLA-C molecules during the first trimester (*9*), is devoid of classical HLA molecules. Thus, there must be mechanisms protecting these cells from NK cell–mediated lysis. One widely discussed possibility is that HLA-G expression serves to provide this protection (*10, 11*). Human and rodent decidua contain a large accumulation of cells that morphologically resemble NK cells (*12*). In humans, these have been shown to be $CD56^+$ (*13*). Two previous studies investigated the role of HLA-G in inhibiting NK cell function. One of these used unfractionated decidual cells, of which only 70 to 80% were $CD56^+$ and whose functional activity could have been altered by extended contact with trophoblasts (*14*). The other study used functionally uncharacterized $CD56^+$ $CD3^-$ clones established from decidua and peripheral blood (*15*). Both of these studies were able to demonstrate only a limited protective effect attributable to the expression of the HLA-G molecule on target cells.

Although originally NK cells were assumed to detect the lack of MHC class I molecules on their targets, it is now clear that at least a subpopulation of these cells shows alloreactive properties, recognizing the presence or absence of well-defined alleles. In this case, HLA molecules act as ligands recognized by specific inhibitory receptors (*16, 17*). Receptors composed of two immunoglobulin superfamily domains [p58: NK inhibitory receptor 1 (NKIR1) and NKIR2] enable NK cells to discriminate between two dimorphic groups of HLA-C molecules (*18, 19*) whereas others, with three immunoglobulin domains (p70/NKB1: NKIR3) recognize supertypic public determinants within the HLA-B locus (*20, 21*). Unlike T and B cells, which carry antigen-specific recognition molecules that are clonally distributed, NK cells usually express several different receptors of various specificities at the same time (*19, 22*). An important feature of the biology is that cross-linking of any single inhibitory receptor is sufficient to inactivate NK cell activity against all possible targets (*22, 23*).

If the function of HLA-G is to provide protection against NK cell–mediated lysis of cells that are unprotected by classical HLA class I molecules, then the expression of this molecule alone should be sufficient to inhibit NK cells of various specificities. To test this hypothesis, NK1- and NK2-specific $CD3^-$, $CD4^-$, and $CD56^+$ cell lines and clones were established from multiple donors, and their ability to kill the HLA class I–negative cell line 721.221 and its transfectants with HLA-Cw6, HLA-Cw7, or HLA-G (Fig. 1) was tested.

NK cell lines were obtained from donors homozygous for HLA-Cw4 or -Cw5 (to yield NK1-specific effectors) or for HLA-Cw7 or -Cw9 (to generate NK2 effectors). These exhibited the expected functional phenotype, with NK1-specific effectors exclusively killing 721.221 cells transfected with HLA-Cw7 whereas targets expressing HLA-Cw6 were protected. NK2-specific effectors showed the reciprocal behavior. The expression of HLA-G conferred resistance to the 721.221 transfectant irrespective of the specificity of the effector cells used (Fig. 2). This protection was observed with all tested donors over a wide range of effector: target (E:T) ratios and was reproducible with NK cells obtained from repeated bleedings of the same donors.

To investigate this inhibition further, a number of NK clones were established and were tested with the 721.221 transfectants (Table 1). All clones, which were functionally NK1- or NK2-specific, were inhibited to background by the expression of HLA-G on the target cells. When these clones were used, the expression of the appropriate inhibitory HLA-C locus allele on the target cells also resulted in complete protection, which suggests that once an inhibitory NK receptor is engaged either by the appropriate HLA-C molecule or by HLA-G, the NK

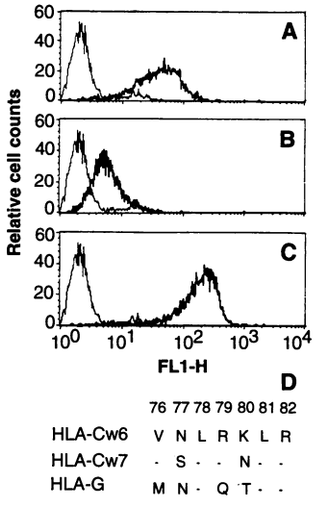

Fig. 1. Expression of HLA molecules on the transfectants of the 721.221 cell line detected by cytofluorometry. (**A**) HLA-Cw6. (**B**) HLA-Cw7. (**C**) HLA-G. (**D**) Amino acid sequences of the transfected HLA molecules in the region of the class I molecule that interacts with the NK cell inhibitory receptors. Abbreviations for the amino acid residues are as follows: K, Lys; L, Leu; M, Met; N, Asn; Q, Gln; R, Arg; S, Ser; T, Thr; and V, Val. The left trace in each FACS profile is the 721.221 parental cell line. The cloning and expression of the HLA-C locus alleles is described elsewhere (*27*). The HLA-G cDNA molecule in the expression vector pBJ5 (*31*) was obtained from S. A. Ellis (*4, 32*) and was further modified by insertion of the neomycin resistance gene between the *amp*^{r} and the SV40 *ori* to aid the selection of transfectants. FACS staining was carried out with the pan–HLA class I antibody W6/32. The transcription of the HLA-G mRNA is driven by the strong SRα promoter, resulting in the much brighter expression. The transfectants were also stained with two other mAbs (BB7.7 and PA2.6) with identical results (*26*). According to FACS staining with the same antibodies, the HLA-G–positive choriocarcinoma cell line JEG-3 expresses HLA-G at approximately the same level as does the HLA-G transfectant (*26*).

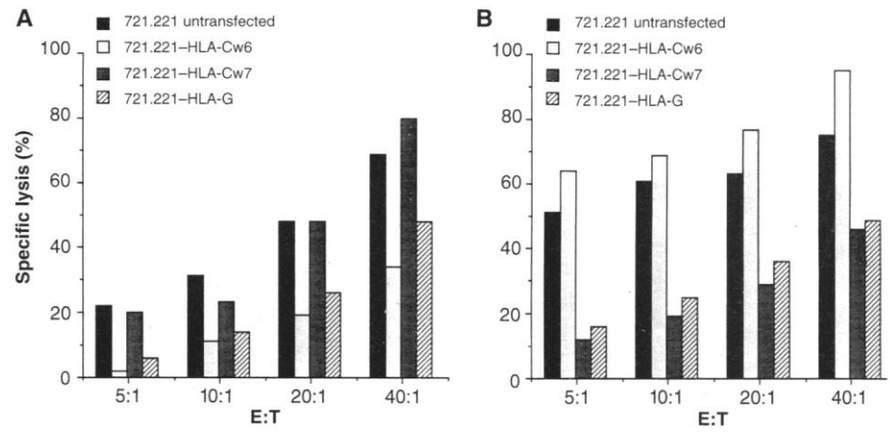

Fig. 2. Cytotoxicity of NK cell lines against various transfectants of the 721.221 cell line. NK cell lines were generated as previously described (*27*). (**A**) The NK1 specific cell line was obtained from donor MV (HLA-A2, -A11, -B18, -B44, and -Cw5). (**B**) NK2 specific cells from donor HTR (HLA-A1, -A2, -B7, -B8, and -Cw7). Cytotoxicity assays were carried out with ^{35}S methionine–labeled target cells, and the results were calculated as described (*27*). These experiments were repeated with NK cells obtained from six different donors, giving the same pattern of protection. In all the experiments presented, the assays were done in triplicates. The standard error of the mean of the triplicates was below 5%, and the spontaneous release never exceeded 25% of the maximum release.

killing is abolished completely.

The 721.221/HLA-G transfectant was also tested with the NK cell tumor cell line NK92 (*24*). These naturally arising human lymphoma cells show the phenotype of activated NK cells and have the capacity to lyse 721.221 cells. This killing was inhibited by the expression of HLA-Cw7 but not HLA-Cw6 molecules on the target cells, indicating that NK92 cells express NK2-specific receptors. The fact that their effector function was also inhibited by the presence of HLA-B*2705 on target cells suggests that the tumor cells also carry a three-domain receptor. These functional data were confirmed by polymerase chain reaction analysis (*25*). Similarly to NK cell clones established from peripheral blood, the lysis of the HLA-G–bearing transfectant by NK92 tumor cells was reduced to near background levels. The degree of protection provided by HLA-Cw7 and HLA-G was comparable (Table 1).

To show that the inhibition of NK lysis was due to the presence of specific HLA class I molecules on the cell surface, cytotoxicity assays were performed in the presence or absence of antibodies that interact with nonpolymorphic determinants on HLA molecules, including HLA-G. The addition of either of the two pan–class I monoclonal antibodies used completely reversed the HLA class I–mediated inhibition of NK cell activity, restoring the killing of the transfected target cells (Fig. 3). The expression of the HLA-G molecule on the transfected 721.221 cells was much higher than that of the HLA-Cw6 or -Cw7 molecules (Fig. 1, A through C). This high expression level was required for the protective effect described above. During extended culture in the absence of selective antibiotics, the HLA-G expression level of the transfected cell line decreased gradually. With this decrease in expression, the protective effect attributed to HLA-G expression disappeared. After the brightest 5% of the cell population was isolated by fluorescence-activated cell sorting (FACS), the transfectants regained their ability to inhibit NK cell killing (*26*). It seems plausible that the limited success of previous efforts to demonstrate the inhibition of NK cell lysis by HLA-G transfection could be due to the lower expression level of the 721.221 transfectants generated with the HLA-G genomic DNA clone (*14*, *15*). These findings indicate that the affinity of the HLA-G protein to the two-domain inhibitory receptors may be lower than that of the HLA-C locus products. This assumption fits well with the fact that HLA-C expression on most cells is relatively low, whereas HLA-G appears to be present on trophoblast cells and on some choriocarcinoma cell lines at high levels.

Given the well-defined allospecificity of the NK cell lines and clones used in our studies, it was likely that the HLA-G molecule would interact with both types of the two-domain p58 NK cell inhibitory receptors (NKIR1 and NKIR2), thus inhibiting both NK1- and NK2-specific effectors. However, it was also possible that a separate receptor existed on both NK1- and NK2- specific killers and that this reacted only with HLA-G but not with the classical HLA-C locus products. In this latter case, the HLA-C– and HLA-G–mediated inhibition would use two different recognition pathways. To distinguish between these possibilities, saturating amounts of NK1- or NK2-specific monoclonal antibodies (mAbs) to p58 were added to the effector cells. When NK1-specific cells were treated with NKIR1-reactive mAbs, the killing of HLA-Cw6– and HLA-G–expressing targets was restored, whereas an NKIR2-specific antibody had no effect on these effectors. On the other hand, the NKIR2-specific antibody restored the lysis of HLA-Cw7– and HLA-G–positive targets by NK2-specific killers; again, inappropriate NKIR1-specific antibodies had no effect (Fig. 3). These experiments prove that HLA-G has the capacity to interact with both types of the p58 receptor, thus identifying the NKIR1 and NKIR2 molecules as receptors for HLA-G on NK cells.

These experiments show conclusively

Table 1. Specific cytotoxic activity of NK1- and NK2-specific clones and the NK lymphoma cell line NK92. NK cell clones were established by limiting dilution of NK lines as described (*27*). In the absence of other suitable markers, clonality was assumed on a statistical basis, with less than one-third of the wells showing growth on the plates. NK1-specific clones were derived from donor NQ (HLA-A1, -A29, -B35, -B51, and -Cw4), and NK2-specific clones were derived from donors HTR and ABH (HLA-A1, -A3, -B8, -B62, and -Cw9). Cytotoxicity assays were performed at the indicated E:T ratios. All clones were tested at least twice with the indicated targets.

NK clone	E:T	Specific lysis (%)			
		721.221 target cells transfected with the indicated HLA molecule			
		None	HLA-Cw6	HLA-Cw7	HLA-G
NK1-specific clones					
NQ 13/1	3:1	29	1	32	1
NQ 13/2	5:1	36	2	34	6
NQ 14	5:1	56	0	39	0
NK2-specific clones					
HTR 2/1	3:1	30	27	1	5
HTR 2/2	5:1	56	44	1	1
HTR 20/1	5:1	41	26	1	1
HTR 20/2	5:1	47	48	0	0
ABH 10.12	5:1	58	56	14	9
NK92 lymphoma					
NK92	10:1	38	33	12	6

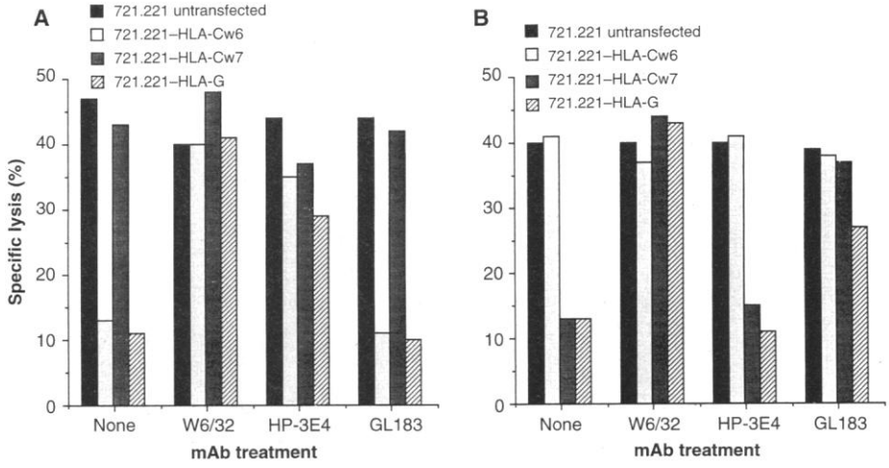

Fig. 3. Effects of mAb treatment on the cytotoxicity of specific NK cell lines. Cytotoxicity assays were set up with the indicated targets and (**A**) NK1-specific (from donor MV) and (**B**) NK2-specific (from donor HTR) effectors. The antibodies were added to the assay at the following concentrations: W6/32 (pan–HLA class I), 20 μg/ml; HP-3E4 [p58 receptor, NK1-specific (*33*)], 1:50 dilution of ascites; GL183 [p58 receptor, NK2-specific (*34*)], 10 μg/ml. Another pan–HLA class I antibody, PA2.6, and another antibody to p58, the NK1-specific EB6 (*34*), gave identical results to those obtained with W6/32 and HP-3E4, respectively. Isotype-matched control antibodies had no effect on the cytotoxicity. The reversal of HLA-G–induced inhibition by either HP-3E4 (A) or GL183 (B) was incomplete. Similar partial reversal could be obtained with an mAb to CD94 (HP-3D9). However, the addition of the mAb to CD94 together with HP-3E4 and GL183 resulted in complete reversal of inhibition (*26*).

that membrane-bound HLA-G molecules are able to inhibit alloreactive NK cells with both NK1 and NK2 specificity. The basis for the discrimination between HLA-C locus alleles is a dimorphism involving residues 77 and 80 in the α1 domain of the molecule (*16*), and recent work has pinpointed the importance of amino acid residue 80 for the specificity of this recognition (*27*). The combination of Asn^{77}-Thr^{80} that is present in HLA-G can be found in some HLA-Bw4 but not HLA-C locus alleles. The fact that HLA-G binds to HLA-C–specific inhibitory receptors whereas HLA-B locus alleles do not indicates that other polymorphisms must influence HLA class I–NKIR interactions. Within this region, Met^{76} (Val^{76} in C locus and Glu^{76} in B locus alleles) and Gln^{79} (Arg^{79} in B and C locus alleles) are unique to HLA-G (Fig. 1D). It is possible that this unusual combination of amino acids enables the molecule to interact with both NK1- and NK2-specific receptors. HLA-G is more polymorphic than originally thought (*28*, *29*), but none of the reported amino acid changes involve the above-described segment of the α1 domain, which perhaps indicates the importance of this region.

The data presented here prove that at a functional level HLA-G is able to protect target cells from destruction by NK1- and NK2-specific effector cells. Whether HLA-G can also react with the p70-NKB1 family of NK cell receptors is unclear. Given that on average 14% of the $CD56^+$ cells are $NKB1^+$ (*21*) and that the overwhelming majority of these cells will also express at least one HLA-C–specific inhibitory receptor (*23*, *30*), this interaction may not be necessary to protect trophoblast cells from NK cell–mediated lysis. If a surrogate molecule replacing classical class I MHC molecules can interact with one major subclass of NK receptors, it will inactivate the majority of NK cells with which it comes into contact. Undoubtedly, the expression of HLA-G alone can fulfill these requirements, and thus molecule can function to protect cytotrophoblast cells from destruction by maternal NK cells.

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35. Supported by NHI research grant CA-47554. L.P. is the recipient of a postdoctoral fellowship from the Arthritis Foundation, O.M. is a European Molecular Biology Organization fellowship scholar. H.T.R. is supported by a Wellcome Trust International Prize Travelling Research Fellowship. We thank S. A. Ellis for the HLA-G cDNA clone and B. Rybalov for technical assistance.

15 May 1996; accepted 23 August 1996

Decreased Resistance to Bacterial Infection and Granulocyte Defects in IAP-Deficient Mice

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Granulocyte [polymorphonuclear leucocyte (PMN)] migration to sites of infection and subsequent activation is essential for host defense. Gene-targeted mice deficient for integrin-associated protein (IAP, also termed CD47) succumbed to *Escherichia coli* peritonitis at inoccula survived by heterozygous littermates. In vivo, they had an early defect in PMN accumulation at the site of infection. In vitro, $IAP^{-/-}$ PMNs were deficient in β_3 integrin–dependent ligand binding, activation of an oxidative burst, and Fc receptor–mediated phagocytosis. Thus, IAP plays a key role in host defense by participating both in PMN migration in response to bacterial infection and in PMN activation at extravascular sites.

PMNs are terminally differentiated cells essential for host defense against infectious diseases. Genetic abnormalities in PMN effector functions prove that they are essential for normal host defense (*1*, *2*). At the same time, the highly destructive potential of PMNs must be strictly controlled to avoid nonspecific tissue damage. Not surprisingly, a large array of regulatory steps governs the emigration and activation of these cells, ensuring full activation of antibacterial effector functions at a site of infection, while maintaining a quiescent, inactive state in the blood stream. PMNs come into contact with extracellular matrix (ECM) when they have extravasated but not while they are in the circulation. Thus, ECM-binding integrins are ideally suited to mediate PMN activation signals specific to extravascular sites.

IAP (CD47) is an immunoglobulin (Ig) family member with a multiply membrane-spanning domain and a short cytoplasmic tail (*3*, *4*). It is physically and functionally

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