200 were generated by PCR and expressed as fusion proteins with GST, and the proteins were purified on glutathione-sepharose and clipped with thrombin as described above. The antisense oligonucleotides used for PCR were TATGAATTCTCACCGCTCCTCCTGCACGGCTTC (130–209); TATGAATTCTCAGGCTTCCCGCTTCATGCCCAT (30–204); TATGAATTCTCACATGCCCATGGCCAGGCACTT (130–200).
22. RXR DBD 130–204 was folded correctly as determined by 2D NMR analysis (M. S. Lee and P. E. Wright, unpublished results).
23. T. E. Wilson, R. E. Paulsen, K. A. Padgett, J. Milbrandt, *Science* **256**, 107 (1992). When placed into the equivalent region of NGFI-B, the 12–amino acid segment from RXRβ blocks binding to the NGFI-B recognition sequence (5′-AAAGGTCA-3′) and promotes binding to a DR-1–type motif (other spacing options were not examined). These data are consistent with a potential role for this region in dimer formation.
24. RXR DBD mutants K201T,R202A and R209A were generated by PCR and expressed as fusion proteins with GST, and the proteins were purified on glutathione-sepharose and clipped with thrombin as described above. The antisense oligonucleotides used for PCR were TATGAATTCTCACCGCTCCTCCTGCACGGCTTCCGCCGTCATGCCCAT (K201T, R202A) and TATGAATTCTCACGCCTCCTCCTGCACGGCTTCCCG (R209A).
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28. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
29. We thank M. Pique for assistance with computer graphics and U. Genick, D. Sem, G. Gippert, D. Case, K. Umesono, T. Perlmann, D. Mangelsdorf, and J. Zhou for discussion. This work was supported by the Jane Coffin Childs Memorial Fund for Medical Research (S.A.K.), NIH (P.E.W. and R.M.E.), the National Cancer Institute (R.M.E.), and a computing grant from Cray Research (P.E.W.). R.M.E. is an Investigator of the Howard Hughes Medical Institute at the Salk Institute for Biological Studies.

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Generation of Allospecific Natural Killer Cells by Stimulation Across a Polymorphism of HLA-C

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The cytotoxicity of human natural killer (NK) cells is modulated by the major histocompatibility complex human leukocyte antigen (HLA)–C molecules on the surface of the target cell. Alloreactive NK cells specific for the NK-1 alloantigen could be reproducibly generated from individuals that were homozygous for HLA-C with asparagine at residue 77 and lysine at residue 80 [HLA-C(Asn^{77}, Lys^{80})] by stimulation with target cells that were homozygous for HLA-C(Ser^{77}, Asn^{80}); the reciprocal stimulation yielded NK cells specific for the NK-2 alloantigen. However, neither homozygous target cell stimulated the generation of alloreactive NK cells from heterozygous individuals. Thus, these data reveal an unanticipated difference between human NK alloreactivity defined by this system and murine "hybrid resistance."

Natural killer cells make up a subpopulation of about 10 to 15% of peripheral blood lymphocytes (PBLs) that lyse tumor and virus-infected cells independently of antigen presentation by the major histocompatibility complex (MHC) molecules (*1*). Some NK cells can specifically recognize and lyse normal allogeneic cells (*2*, *3*). The alloantigen NK-1 on target cells is controlled by HLA-C or by a closely linked gene (*4*–*6*). Target cells that are susceptible to allospecific lysis are homozygous for a two–amino acid polymorphism of HLA-C, namely, Ser^{77} and Asn^{80} [HLA-C(Ser^{77}, Asn^{80})] in the α1 domain (shared by HLA-Cw1, -Cw3, -Cw7, -Cw8, and by some of the -Cw blank alleles). In addition, a second alloantigen (NK-2) correlates with homozygosity for a different pair of amino acids at the same positions [HLA-C(Asn^{77}, Lys^{80})] (shared by HLA-Cw2, -Cw4, -Cw5, -Cw6, and some other -Cw blank alleles). Cells heterozygous for these pairs of amino acids cannot be lysed by NK clones that recognize NK-1 or NK-2.

The requirement for both HLA-C alleles of the target to be of the same type for lysis by a given NK clone to occur (recessive susceptibility) implicates the alternative HLA-C allele as having an inhibitory function on that NK clone (dominant suppression). It also provides a rationale for the reproducible generation of NK cells with NK-1 and NK-2 allospecificities that have so far only been generated by stimulation between random donors. Assuming a reciprocal inhibitory function for the two types of alleles of HLA-C, NK cell lines that specifically recognize cells homozygous for HLA-C(Ser^{77}, Asn^{80}) or for HLA-C (Asn^{77}, Lys^{80}) should be reproducibly established by reciprocal stimulation between cells homozygous for the alternative polymorphisms.

Individuals chosen at random (n = 28) were DNA typed for residues 77 and 80 of HLA-C with sequence-specific oligonucleotides; 12 were homozygous for HLA-C(Ser^{77}, Asn^{80}) (42.9%), 4 were homozygous for HLA-C(Asn^{77}, Lys^{80}) (14.3%), and 12 were heterozygous (42.9%) (Table 1). In the homozygotes Ser^{77} was always linked to Asn^{80}, and Asn^{77} to Lys^{80}, in agreement with the known HLA-C sequences (Fig. 1) (*7*). To generate an NK cell line with specificity for NK-1, we purified CD3$^-$, CD4$^-$, CD8$^-$ lymphocytes from the PBLs of an HLA-C(Asn^{77}, Lys^{80}) homozygous donor (EB) by complement-mediated depletion and cocultured the lymphocytes with γ-irradiated PBLs from an HLA-C(Ser^{77}, Asn^{80}) homozygous donor (HD). After 3 days, interleukin-2 (IL-2) was added to the culture medium, and CD3$^-$, CD4$^-$, CD8$^-$ lymphocytes were expanded four to five times in a 7-day

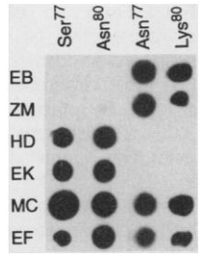

Fig. 1. Oligonucleotide typing of residues 77 and 80 of HLA-C in the blood donors from whom the NK cell lines were derived. Genomic DNA was extracted from PBLs by standard techniques. The second exon of HLA-C was selectively amplified from genomic DNAs by PCR. Primer pairs and reaction conditions for the amplification were previously described (*6*). Amplified fragments were denatured in 0.4 N NaOH and applied to Hybond-N (Amersham) with a slot blot apparatus (Schleicher & Schuell, Inc.). Membranes were hybridized with ^{32}P-labeled oligonucleotides specific for Ser^{77} or Asp^{77} (*6*) and oligonucleotides [CCTGCGGAA(A or C)CTGCGCGGC] (nucleotides 303 to 321) specific for Asp^{80} or Lys^{80}, respectively. Hybridization was carried out for 2 hours at 42°C in 5× Denhardt's solution, 5× standard saline phosphate–EDTA [SSPE; 1× SSPE contains 0.18 M NaCl, 10 mM phosphate (pH 7.4), and 1 mM EDTA] plus 0.5% SDS, and probe at 2 × 10^6 cpm/ml. Membranes were washed in 6× standard saline citrate (SSC)–0.1% SDS for 10 min at room temperature and for 10 min at the calculated t_m (melting temperature) for each oligonucleotide. Similar data were obtained for each of the target cells shown in Table 1.

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culture. Flow cytometry of the cells showed the $CD3^-$, $CD56^+$ phenotype that is characteristic of the NK cells (8); both $CD16^+$ (70%) and $CD16^-$ (30%) cells existed within the NK population (Fig. 2) (9).

The target specificity of this NK cell line (EB1) was tested on PHA-activated T cell blasts from the panel in a standard 4-hour ^{51}Cr-release assay. The NK cell line specifically lysed PHA-activated blasts from the stimulating donor and from all the HLA-C(Ser^{77}, Asn^{80}) homozygous individuals, whereas none of the HLA-C(Asn^{77}, Lys^{80}) homozygous or the heterozygous cells was significantly lysed (Fig. 3A and Table 1). NK cells with the same specificity were also generated from this donor and from another HLA-C(Asn^{77}, Lys^{80}) homozygote (NK cell lines EB2 and ZM1, respectively) by stimulation with PBLs from another HLA-C(Ser^{77}, Asn^{80}) homozygous donor (EK) (Table 1). Thus, culture of NK cells from HLA-C(Asn^{77}, Lys^{80}) with PBLs from HLA-C(Ser^{77}, Asn^{80}) homozygotes leads to the generation of NK cells with NK-1 allospecificity.

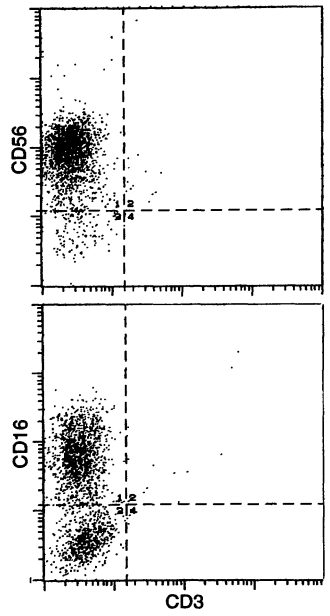

Fig. 2. Expression of CD3, CD16, and CD56 on an alloreactive NK cell line. NK cells were prepared as follows: PBLs were isolated from 50-ml blood samples and depleted of macrophages by plastic adherence for 1 hour at 37°C. Nonadherent PBLs were incubated with saturating quantities of monoclonal antibodies to CD3 (anti-CD3) (50% culture supernatant from the SPV-T3b hybridoma), anti-CD4 (OKT4, 1:200 dilution of ascites fluid), and anti-CD8 (B9.4, 1:200 dilution of ascites fluid) on ice for 30 min; rabbit serum (1:4 Low Tox M rabbit complement, Cedarlane Labs, Ltd.) was added, and incubation was continued for 1 hour at 37°C. Surviving cells were washed and cultured in 96-well U-bottom plates (5×10^4 cells per well) in the presence of γ-irradiated (2000 rad) allogeneic PBLs (1×10^4 cells per well). After 3 days, recombinant IL-2 (Cellular Products, Inc., Buffalo, New York) and IL-2–containing supernatant were added to the culture medium at the final concentration of 100 U/ml and 10%, respectively, and cultures were continued for an additional 7 days. Before cytotoxicity assay, 10^5 effector cells were stained for the surface expression of CD3, CD16, and CD56 with fluorescein isothiocyanate–conjugated anti-CD3, phycoerythrin-conjugated anti-CD16, and phycoerythrin-conjugated anti-CD56 (Becton-Dickinson, Mountain View, California). Events were collected on a FACScan flow cytometer and analyzed with FACScan Research software (Becton-Dickinson).

NK cells specific for NK-2 were also generated by stimulation of NK cells from donor HD or EK [both homozygous for HLA-C(Ser^{77}, Asn^{80})] with irradiated PBLs from donor EB or donor ZM, respectively. The NK cell lines (HD1 and EK1) lysed all of the NK-2 [HLA-C(Asn^{77}, Lys^{80})] homozygous cell samples but none of the targets positive for NK-1 [HLA-C(Ser^{77}, Asn^{80}) homozygous cells]. However, out of nine target cell samples that were heterozygous by DNA typing, both HD1 and EK1 lysed one sample (LP 6-11), and two samples (LP 86 and PK) showed a borderline lysis (Fig. 3B and Table 1).

The few heterozygous cell samples susceptible to NK lysis might be accounted for by the presence of additional new specificities in these NK cell lines. However, because the same heterozygous samples were lysed by independently derived NK cell lines, this possibility is less likely. Lysis of

Table 1. Cytotoxicity mediated by alloreactive NK cell lines at an E/T ratio of 5:1 against the PHA-activated T cells from the random panel. Bold, positive lysis (cytotoxic response >30% at an E/T ratio of 5:1); —, not tested. A cytotoxic response between 16 and 29% was considered borderline. EB1.1 and EB1.2 represent two different NK cell lines from the same responder and stimulator pair, tested on partially overlapping targets. PHA blasts were prepared by culturing of PBLs isolated from blood samples or buffy coats in the presence of PHA (1 μg/ml) and recombinant IL-2 (100 U/ml).

Target cells	HLA-C genotype of NK cell lines									
	HLA-C(Asn^{77}, Lys^{80}) homozygous*				HLA-C(Ser^{77}, Asn^{80}) homozygous†		Heterozygous			
	EB1.1	EB1.2	EB2	ZM1	HD1	EK1	MC1	MC2	EF1	EF2
Homozygous HLA-C(Ser^{77}, Asn^{80}) targets										
EK	—	**68**	**46**	**53**	2	8	4	2	0	3
LP 5-7	—	**73**	**44**	**84**	7	1	—	—	—	—
HD	**80**	**47**	**47**	**51**	0	2	—	—	—	—
DA	**85**	**63**	**38**	**53**	7	0	9	5	0	4
BS	—	**54**	**49**	**51**	0	0	5	2	2	0
CL	**98**	**60**	**38**	**30**	0	0	9	9	0	2
LP 4-10	—	**65**	**34**	**37**	0	11	1	1	12	0
LP 1-17	**100**	**65**	**48**	**64**	0	0	9	12	7	8
LP 3-26	—	**54**	—	**100**	3	10	—	—	—	—
VS	**100**	—	—	**52**	—	1	16	8	0	2
TB	**75**	—	—	—	—	—	13	8	0	5
LP 7-24	—	—	—	—	—	—	10	13	3	7
Homozygous HLA-C(Asn^{77}, Lys^{80}) targets										
ZM	3	4	0	0	**62**	**68**	12	11	25	1
DS	—	2	0	0	**52**	**53**	9	5	11	3
EB	0	2	0	4	**50**	**67**	14	0	9	—
JO	—	—	—	0	—	**61**	—	—	—	—
Heterozygous targets										
PP	—	4	0	0	5	6	9	9	4	16
PK	0	0	0	0	17	22	10	8	4	1
LP 6-26	—	0	0	—	6	0	9	5	3	0
LP 86	—	14	0	12	24	25	14	14	9	4
SP	0	5	0	0	4	4	14	17	4	3
LP 6-11	—	10	0	6	**44**	**32**	6	2	8	0
LP 3-13	—	12	—	—	5	9	12	7	1	1
EF	0	—	—	6	0	0	6	5	0	0
LP 7-8	—	—	2	8	—	13	3	6	3	0
LP 88	—	—	—	—	—	—	15	6	4	2
LP 87	—	—	—	—	—	—	23	18	7	2
MC	0	—	—	—	—	—	4	2	4	0

*Significance of homozygosity for Ser^{77}, Asn^{80} and recognition by these NK cell lines; $P < 10^{-4}$ by Fisher's exact test. †Significance of homozygosity for Asn^{77}, Lys^{80} and recognition by these NK cell lines; $P < 3 \times 10^{-4}$ by Fisher's exact test.

10% of the heterozygous samples was also previously observed with cloned NK cells with NK-2 specificity (6). Alternatively, one or more HLA-C alleles carrying Ser^{77} and Asn^{80} might be unable to inhibit efficiently NK lysis in the heterozygotes. To identify these alleles, we amplified and sequenced the first and the second exon of HLA-C from the present and previous (6) NK-2–positive heterozygous samples (*10*). All of the HLA-C(Ser^{77}, Asn^{80}) alleles in these five heterozygotes were HLA-Cw14, suggesting that this allele may be incapable of NK inhibition. Moreover, none of the nine NK-2–negative heterozygous samples typed as HLA-Cw14 with sequence-specific oligonucleotides (*11*), thus supporting involvement of this particular allele in heterozygote susceptibility.

No alloantibodies specific for HLA-Cw14 are available, reflecting low antigenicity in vivo of this allele, possibly resulting from poor expression on the cell surface (*12*). HLA-Cw14 accounts for a fraction of HLA-C alleles known as HLA-Cw blanks, which could also be incapable of NK inhibition.

"Hybrid resistance" in the mouse (*13*) refers to the rejection of homozygous parental bone marrow grafts by heterozygous F_1 irradiated mice. It is linked to the MHC and is mediated by NK cells. Thus, attempts were made to establish alloreactive NK cell lines with NK-1 and NK-2 specificities from heterozygous individuals (MC and EF) by stimulation with PBLs from HLA-C(Ser^{77}, Asn^{80}) homozygotes or HLA-C(Asn^{77}, Lys^{80}) homozygotes (BS or EB, respectively). None of these four NK cell lines (MC1, MC2, EF1, or EF2) was cytotoxic against the cell targets (Fig. 3, C and D, and Table 1). Thus, an unexpected difference between human NK-1 and NK-2 alloreactivity and murine hybrid resistance was revealed.

The cytotoxicity patterns of these alloreactive NK cell lines are consistent with either the effector inhibition or target masking models (*14, 15*). In the effector inhibition model, HLA-C molecules on the target cells deliver a negative signal to inhibit NK cell activity through an NK cell receptor that is specific for self or related HLA-C alleles. Accordingly, NK cells specific for NK-2 are inhibited by HLA-Cw3, when transfected into the mouse mastocytoma cell line P815 (*16*).

In the masking model, HLA-C molecules mask specific target antigens that are recognized by NK cell receptors and trigger NK lysis. As found in HLA-B27, residues 77 and 80 of HLA-C should be located in the F pocket of the peptide-binding cleft of HLA-C and should hydrogen bond to the COOH-terminal amino acid (P9) of the self peptides (*17*). Thus, the NK target antigen could be a self peptide bound to HLA-C. Target cell sensitivity to NK cells is modulated by peptides bound to other class I molecules (*18–20*).

The prediction that NK cells from heterozygous individuals will lyse homozygous targets, as observed in murine F_1 hybrid resistance, is not fulfilled in our system. Thus, NK alloreactivity could be distinct from murine hybrid resistance. However, this discrepancy might be also explained in two other ways. First, HLA-C might shape the NK repertoire in vivo as well as in vitro, so that heterozygosity for HLA-C alleles with respect to residues 77 and 80 would result in deletion or inhibition of effector cells with NK-1 and NK-2 specificities. Human NK cells were stimulated for 4 days with irradiated homozygous PBLs before addition of IL-2 and expansion for 7 days. The development of alloreactive NK cells from heterozygotes might therefore be prevented in vitro by our experimental conditions. This "preimmunization" does not occur in hybrid resistance, where the rejection of parental bone marrow cells takes place within 24 to 36 hours after the graft (*21*), and moreover, the injection of parental splenocytes 20 days before the graft induced a marked decrease of the phenomenon (*22, 23*) (that is, tolerance was induced). Similarly, NK cells from F_1 hybrids could lyse normal T cell blasts from the parental strains in vitro after the NK cells were expanded in IL-2 without activation in mixed lymphocyte culture, although the specific cytotoxicity was low (below 30% at

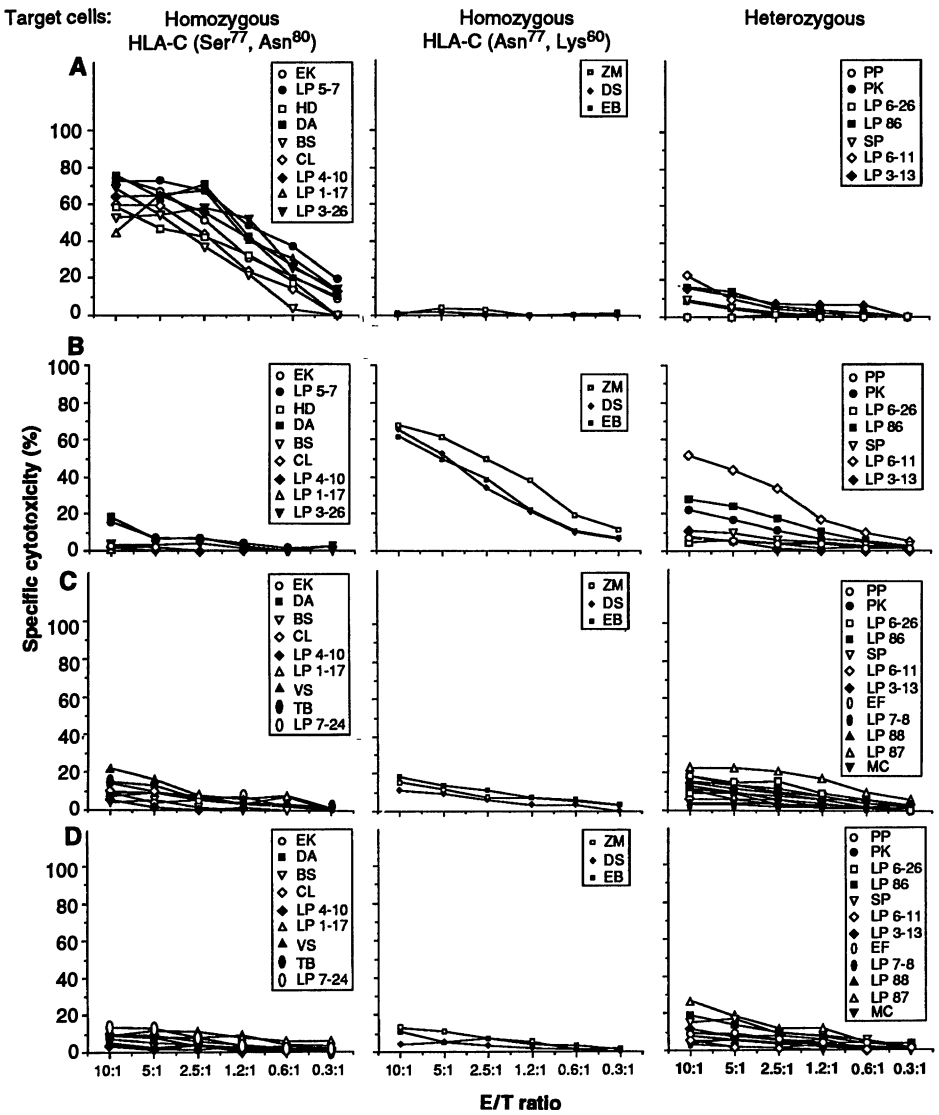

Fig. 3. Lysis pattern of EB1 (**A**), HD1 (**B**), MC1 (**C**), and MC2 (**D**) NK cell lines. The data represent the percentage of cytotoxic responses at different effector-to-target ratios (E/T ratio). HLA-C(Ser^{77}, Asn^{80}) homozygous target cells are shown on the left, HLA-C(Asn^{77}, Lys^{80}) homozygous target cells in the middle, and heterozygous cells on the right. The target cells are those in Table 1. Cytotoxic activity of NK cells was determined against PHA-activated T cell blasts in a standard 4-hour ^{51}Cr-release assay.

an effector-to-target ratio of 100:1) (*24*). Again, previous sensitization of F_1 animals with lymphocytes from one of the two parental strains reduced the NK-mediated lysis of T cell blasts.

Second, in some instances heterozygotes might be reactive to homozygotes. For example, heterozygotes that bear HLA-Cw14 may behave as homozygotes because of low or no expression of the HLA-Cw14 allele. Thus, they might develop NK cells that lyse NK-1^+ (but not NK-2^+) homozygous cells, paralleling at least in part the situation observed in murine hybrid resistance. Both of these interpretations would make the present observations consistent with the view that human NK alloreactivity and murine hybrid resistance are analogous. In any event, the phenomena described here are important in understanding the biology of NK recognition and may have a bearing on the matching of bone marrow donors and recipients in allogeneic bone marrow transplantation in humans.

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10. A 338-bp fragment spanning the first and second exons of HLA-C was selectively amplified from genomic DNAs by the polymerase chain reaction (PCR). The primers used for amplification were CCCGAACCCTC(C/A)TCCTGCT (5′, nucleotides 17 to 35) and ACGGCGCCCGCGGCTCCC (3′, nucleotides 206 to 223). Reaction conditions for the amplification were as described (*6*). Amplified fragments were gel-purified, subcloned into pCR1000 (Invitrogen), and sequenced by dideoxynucleotide chain termination. Out of four independent clones analyzed from each PCR product, at least one showed a sequence identical to the reported sequence for HLA-Cw14 (*7, 12*); the other clones represented the second HLA-C allele of the heterozygote.
11. For oligonucleotide typing of HLA-Cw14, HLA-C was amplified from genomic DNAs (*10*), denatured, applied to Hybond-membranes (Amersham), and hybridized with the following ^{32}P oligonucleotides: GTATTTCTCCACATCCGTGTC (nucleotides 90 to 110, specific for HLA-Cw4, HLA-C BeWo C.1, and HLA-Cw14), TTTCTCCACATCCGTGTCCCGGCCC (nucleotides 93 to 117, specific for Cw14), and CCGTGTCCTGGCCCGGCC (nucleotides 104 to 121, specific for Cw4 and HLA-C BeWo C1). Hybridization conditions were as described in Fig. 1. Membranes were washed in 3 N tetramethylammonium chloride, 50 mM tris-HCl (pH 8.0), and 2 mM EDTA for 30 min at 59°C, 65°C, and 59°C, respectively.
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Mechanotransduction Across the Cell Surface and Through the Cytoskeleton

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Mechanical stresses were applied directly to cell surface receptors with a magnetic twisting device. The extracellular matrix receptor, integrin β_1, induced focal adhesion formation and supported a force-dependent stiffening response, whereas nonadhesion receptors did not. The cytoskeletal stiffness (ratio of stress to strain) increased in direct proportion to the applied stress and required intact microtubules and intermediate filaments as well as microfilaments. Tensegrity models that incorporate mechanically interdependent struts and strings that reorient globally in response to a localized stress mimicked this response. These results suggest that integrins act as mechanoreceptors and transmit mechanical signals to the cytoskeleton. Mechanotransduction, in turn, may be mediated simultaneously at multiple locations inside the cell through force-induced rearrangements within a tensionally integrated cytoskeleton.

The process of recognizing and responding to mechanical stimuli is critical for the growth and function of living cells. Many sensory functions including touch, hearing, baroreception, proprioception, and gravity sensation involve specialized mechanotransduction mechanisms. Development of tissue pattern is also exquisitely sensitive to changes in mechanical stress (*1*). Nevertheless, the molecular mechanism by which individual cells recognize and respond to external forces is not well understood. Stretch-sensitive ion channels, adenylate cyclase, and protein kinase C change their activity in response to applied stress (*2–4*). However, these signaling pathways are likely to lie downstream from the initial mechanoreception event at the cell surface. For example, activation of these signaling molecules appears to be mediated though changes in the cytoskeleton (CSK) (*2, 4, 5*). Although changes in CSK organization are a ubiquitous response to mechanical perturbation (*4, 6, 7*), the mechanism by which forces are transmitted across the cell surface and transduced into a CSK response remains unknown.

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Analysis of mechanotransduction in specialized force-sensing cells, in both plants and animals, suggests that the cell's extracellular matrix (ECM) attachments are the sites at which forces are transmitted to cells (*6, 8*). As in any architectural structure, mechanical loads are transmitted across the cell surface and into the cell by means of structural elements that are physically interconnected. Transmembrane ECM receptors, such as members of the integrin family, are excellent candidates for mechanoreceptors because they bind actin-associated proteins within focal adhesions and thereby physically link ECM with CSK microfilaments (*9*). The possibility that ECM receptors mediate mechanotransduction is supported by the finding that stretching flexible ECM culture substrata alters CSK organization and induces biochemical changes in adherent cells (*10*). However, in these stretching studies, it is not possible to separate effects due to transmembrane force transfer from those due to global shape changes and generalized deformation of the plasma membrane and CSK.

To determine whether ECM receptors provide a specific molecular path for mechanical signal transfer to the CSK, we devised a method in which controlled mechanical loads could be applied directly to specific cell surface molecules without producing large-scale changes in cell shape (Fig. 1). We modified a cell magnetometry