staining with SA-HRP or SA-AP (Southern Biotechnology) to detect the GCs (*19*). Bound HRP and AP activities were visualized with 3-aminoethyl carbazole (3-AEC) (red stain) and fast blue BB (blue strain), respectively. Commercially obtained rabbit IgG specific for terminal deoxynucleotidyl transferase or rat Ig failed to label splenic and PP GCs.

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14. Two microliters of cDNA from each 20-μl sample reverse transcribed from extracted RNA was amplified in RT-PCR assays which used the following primer pairs: *Rag1*, 5′-CCAAGCTGCAGACATTCTAGCACTC-3′, 5′-CTGGATCCGGAAAATCCTGGCAATG-3′; *Rag2*, 5′-CACATCCACAAGCAGGAAGTACAC-3′, 5′-GGTTCAGGGACATCTCCTACTAAG-3′; and HPRT, 5′-GCTGGTGAAAAGGACCTCT-3′, 5′-CACAGGACTAGAACACCTGC-3′. Taq polymerase (Gibco, Gaithersburg, MD) was used in all amplifications. PCR was carried out as follows: for *Rag1*—at 94°C for 2 min followed by eight cycles at 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min, and 22 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min; for *Rag2*—at 94°C for 2 min followed by 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; for HPRT—at 94°C for 2 min followed by 35 cycles at 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min. The amplified products (6 μl) were loaded onto a 1% agarose gel and visualized with ethidium bromide. To ensure that RT-PCR products were not derived from contaminating genomic DNA, the primer pairs for *Rag1* and *Rag2* each span an intron and primers for *HPRT* span two introns. The molecular sizes of the PCR products were determined by comparison to standards of 50, 100, 200, 300, 400, 500, 700, and 1000 base pairs (bp) (Marker XI, Boehringer-Mannheim).
15. Splenic cells obtained from C57BL/6 mice immunized with NP-CGG were stained with fluorescein isothiocyanate–conjugated antibody to mouse B220 (PharMingen, San Diego, CA) and GL-7–biotin followed by staining with SA coupled to phycoerythrin (PE). GL7$^+$B220$^+$ and GL-7$^-$B220$^+$ cell populations were sorted into TRIzol reagent (Gibco). Some cells were sorted into cold medium for re-analysis. Routinely, GL-7$^+$B220$^+$ cells were 33 to 55% pure, whereas GL-7$^-$B220$^+$ populations were ≥96% pure. Similarly, CD4$^+$CD8$^+$ thymocytes were also sorted to serve as positive controls; these double-positive thymocytes were ~99% pure. Total RNA recovered from TRIzol digests of the different sorted populations was reverse-transcribed with SuperScript (Gibco) with the following specific primers according to the manufacturer's instructions: *Rag1*, 5′-CTTGGGAAGTAGACCTGAC-3′; *Rag2*, 5′-CCCATGCTTTTCCCTCGACT-3′; and HPRT, 5′-GACGCAGCAACTGACATTTC-3′.
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28. We thank J. Cerny and M. Schlissel for their review of this manuscript; S. V. Desiderio for RAG2-specific antibody; R. Hodes, K. Holmes, and A. Lanzavecchia for describing unpublished observations; and F. W. Alt for the communication of unpublished results confirming Ig class switching in the absence of RAG proteins. Supported in part by USPHS grant AI32524 to D.G.S. and grants AI24335, AG10207, and AG13789 to G.K. E.S. is the recipient of a grant from the Leukemia Research Foundation. D.G.S. is an assistant investigator of the Howard Hughes Medical Institute.

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Enhancement of Class II–Restricted T cell Responses by Costimulatory NK Receptors for Class I MHC Proteins

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An important feature of the human immune system is the ability of T cells to respond to small quantities of antigen. Class II major histocompatibility complex (MHC)–restricted T cells that expressed a costimulatory natural killer (NK) cell receptor for class I MHC proteins were cloned. In the presence of low doses of superantigen, the proliferative response of these T cell clones was three- to ninefold greater when the T cells were costimulated by way of the NK receptor. Thus, the action of costimulatory NK receptors on T cells may play a significant role in initiating and sustaining immune responses.

T cells recognize antigens through contacts made between the T cell receptor (TCR) and peptides presented in association with specific MHC proteins on an antigen-presenting cell (APC). However, the T lymphocyte response is also shaped by many other interactions between cell-surface molecules on T cells and APCs, as well as by the action of cytokines (*1*). Although no distinct "antigen receptor" analogous to the TCR has been found on NK cells, NK cell–mediated lysis can be inhibited by NK receptors that also bind to class I MHC proteins (*2*, *3*). In particular, lysis by NK1 and NK2 cells is inhibited by target cells expressing human leukocyte antigen (HLA)–Cw2, -Cw4, -Cw5, or -Cw6 and HLA-Cw1, -Cw3, -Cw7, or -Cw8, respectively (*4*, *5*). Also, lysis by NKB1$^+$ NK3 cells is inhibited by target cells expressing an HLA-B allele containing the Bw4 epitope at residues 77 to 83 (*6*). Such inhibition is initiated by the recruitment of protein tyrosine phosphatases on the cytoplasmic tail of the NK receptor (*7*). T cells share with NK cells a common lineage and many phenotypic markers (*8*) including NK inhibitory receptors. Indeed, ~28% of cytotoxic T cells express p58 NK inhibitory receptors (*9*), and the p70 NK inhibitory receptor, NKB1, is expressed on 0.2 to 15% of T cells (*10*). The action of these NK inhibitory receptors can affect T cell function because NKB1$^+$ T cell clones that can kill superantigen-coated target cells cannot kill the same target cells transfected with class I MHC alleles expressing the Bw4 epitope (*10*).

Recently, an isoform of NK inhibitory receptors was described. These p50 NK receptors share similar extracellular sequences with the p58 receptors but have altered transmembrane regions, including the addition of a charged lysine residue, and truncated cytoplasmic tails (*3*, *11*), so that they lack the YXXL sequences (L, Leu; Y, Tyr; X, any amino acid) to which protein tyrosine phosphatases can bind. Such short-tailed NK receptors do not mediate inhibition of NK cell–mediated lysis but instead activate or coactivate NK clones (*11*). To investigate the possible effect of NK activating receptors on T cell function, we studied T cell clones isolated as by-products in NK cell cloning (*12*).

Two of these clones, TANK-1 and TANK-9, were prepared from a donor whose HLA type is HLA-A1, -A2, -B7,

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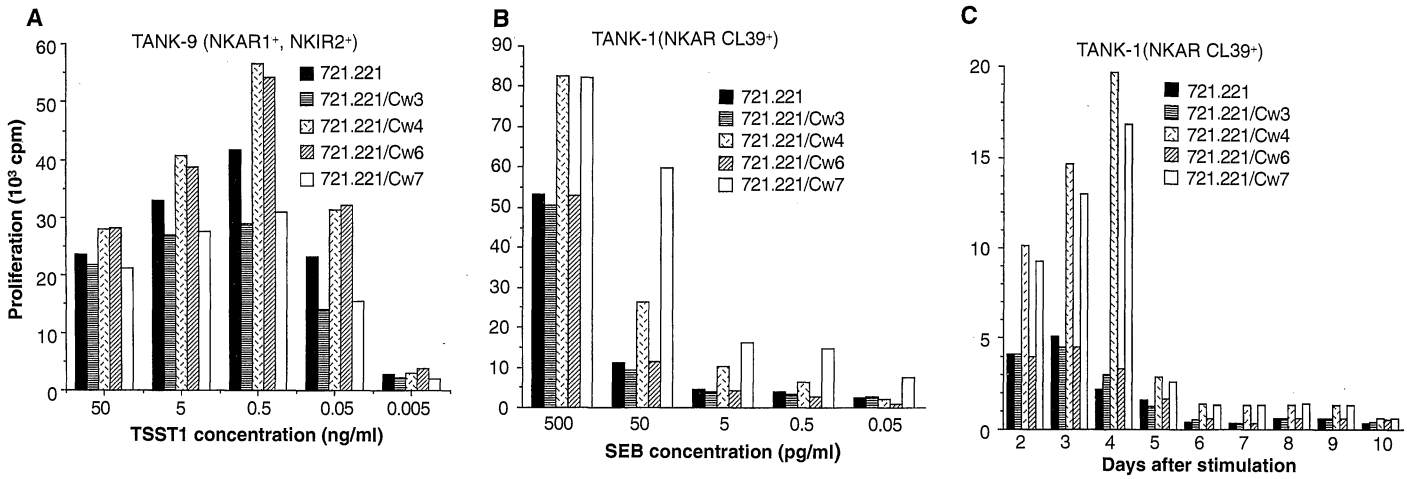

Fig. 1. Proliferation of TANK cell clones in the presence of superantigen is dependent on the class I MHC allotype of the APC. Proliferation assays were done as described (*18*). (**A**) TANK-9 and (**B**) TANK-1 were incubated with various concentrations of TSST1 and SEB, respectively, in the presence of human B cells deficient in cell-surface expression of class I MHC proteins (721.221) or 721.221 cells transfected with HLA-Cw3, HLA-Cw4, HLA-Cw6, or HLA-Cw7. (**C**) The proliferation of TANK-1 in response to SEB-coated 721.221 cells (0.5 ng/ml) and the 721.221 transfectants, measured on subsequent days after the initial mixing of the target and effector cells. In all experiments, each data point was measured in triplicate. The range in each measurement was less than 5% of the mean of the triplicates. The 721.221 transfectants were generated as in (*5*). The experiment shown here is representative of six data sets that were obtained. In the absence of superantigen, less than 400 cpm were measured regardless of the type of B cell or T cell present.

-B8, -Cw7, -DR1, -DR17, -DQw2, and -DQw5. Clone TANK-1 was positive for TCRBV17 (V_β17) and expressed neither NK1, NK2, nor NK3 receptors by flow cytometry (*13*). Cloning by polymerase chain reaction (PCR) and sequencing of cDNA derived from TANK-1 (*14*) revealed the expression of the short-tailed activating NK receptor, clone 39 (*3*). Clone TANK-9 was positive for TCRBV2 (V_β2) and expressed both NK1 and NK2 receptors by flow cytometry (*13*). PCR typing of cDNA derived from TANK-9 (*15*) revealed that TANK-9 expresses the activating, short-tailed form of the NK1 receptor (NKAR1) and the inhibitory, long-tailed form of the NK2 receptor (NKIR2), as well as TCRVA24 (V_α24). These T cells with activating NK receptors (TANK cells) were positive for TCR$\alpha\beta$, CD4, and CD3 and negative for TCR$\gamma\delta$, CD8, CD16, CD56, and CD94 by flow cytometry (*13, 16*).

To determine the effect of NK activating receptors on T cell responses, we examined the proliferative response of TANK-1 and TANK-9 in the presence of superantigen-coated 721.221 cells, human HLA-DR1$^+$ B cells deficient in cell-surface expression of class I MHC proteins (*17*), and 721.221 transfectants (*18*). As expected from the TCRVB gene segment expressed by each clone, TANK-9 responded to toxic shock syndrome toxin 1 (TSST1) (Fig. 1A) and not to staphylococcal enterotoxin A (SEA) or SEB (*19*), whereas TANK-1 responded to SEB (Fig. 1B) and not to SEA (*19*). At all the tested concentrations of TSST1, the proliferation of TANK-9 was greater when the target cell was transfected with either HLA-Cw4 or -Cw6 and reduced when the target cell was transfected with either HLA-Cw3 or -Cw7 (Fig. 1A).

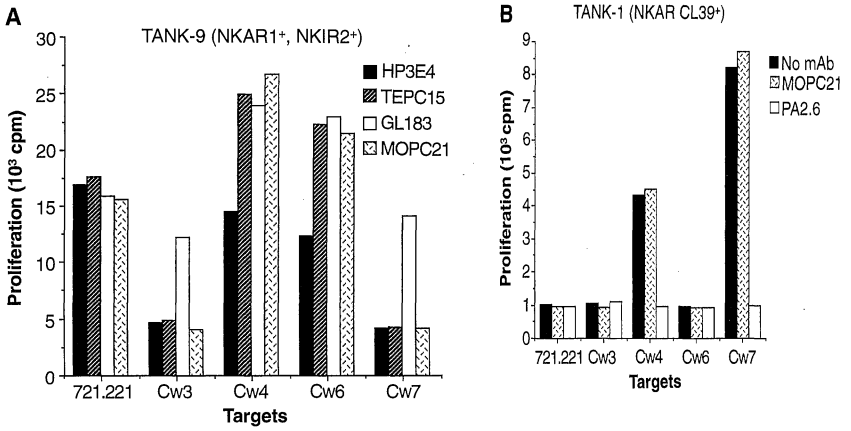

Fig. 2. Antibodies to NK receptors on the effector cells or to class I MHC proteins on the target cells block the alteration in TANK cell proliferation. (**A**) Proliferation of TANK-9 stimulated by TSST1 (0.05 ng/ml) was measured in the presence of 721.221 cells and various 721.221 transfectants, with antibodies to the NK1 or NK2 receptors (HO3E4 and GL183, respectively), a control immunoglobulin M (IgM) myeloma protein (TEPC15, Sigma), or a control IgG1 myeloma protein (MOPC21, Sigma). (**B**) Proliferation of TANK-1 stimulated by SEB (0.5 ng/ml) was measured in the presence of 721.221 cells or 721.221 transfectants plus the antibody to class I MHC proteins (PA2.6) or an isotype control (MOPC21, Sigma). To show the largest possible effect of blocking NK receptor ligation with PA2.6, we harvested the cells 3 days after incubation. Otherwise, proliferation assays were done as described (*18*). Each data point was measured in duplicate. The range in each measurement was less than 10% of the mean of the duplicates. The experiments shown here are representative of three data sets that were obtained.

Target cells that enhanced the proliferative response expressed class I MHC proteins that were recognized by NK1 receptors, whereas target cells that inhibited the response expressed alleles that were recognized by NK2 receptors (*9, 11*). This result implies that the short-tailed NK1 receptor present on the cell surface of TANK-9 facilitates the enhancement of the T cell proliferative response, whereas the long-tailed NK2 receptor mediates inhibition of such a response. The decrease in proliferation of TANK-9 in response to TSST1 at 50 ng/ml compared to the proliferative response at 0.5 ng/ml is likely to be due to the induction of anergy (*20*).

Proliferation of TANK-1 in response to SEB presentation was enhanced by target cells transfected with either HLA-Cw4 or

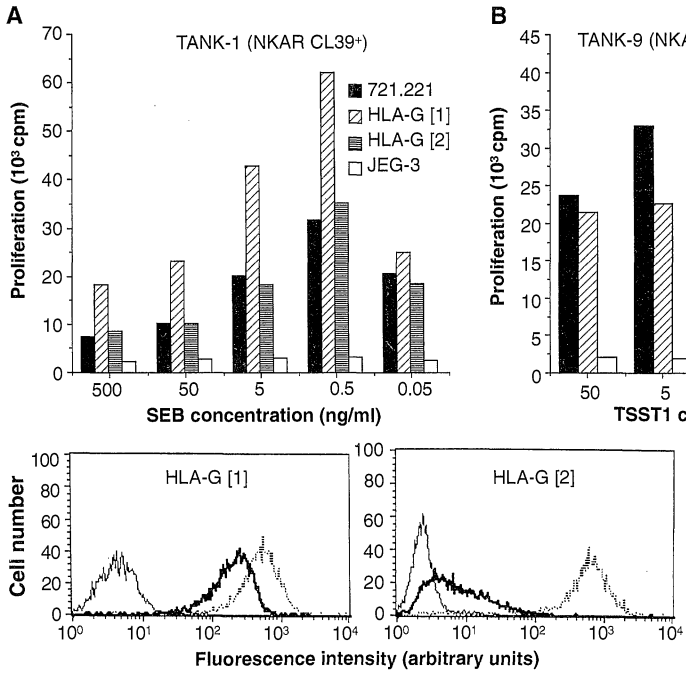

Fig. 3. Cell-surface expression of HLA-G on target cells affects the proliferation of TANK clones. (**A**) (**Top**) TANK-1 cells were incubated with various concentrations of SEB and 721.221 cells, 721.221 cells transfected with HLA-G [1], another 721.221 clone transfected with HLA-G [2], or the choriocarcinoma cell line JEG-3. (**Bottom**) Analysis of the two 721.221 lines transfected with HLA-G by flow cytometry, with fluorescein isothiocyanate (FITC)–labeled goat antibody to mouse IgG alone (plain line); W6/32, an antibody to class I MHC proteins, followed by FITC-labeled antibody to mouse IgG (bold line); or LB3.1, an antibody to class II MHC proteins, also revealed by FITC-labeled antibody to mouse IgG (dotted line). One 721.221 line transfected with HLA-G expressed large amounts of the class I protein on its cell surface [1], whereas the other HLA-G transfectant did not [2]. (**B**) Proliferation of TANK-9 stimulated by various concentrations of TSST1 was measured in the presence of 721.221 cells, 721.221 cells transfected with HLA-G [1], or the choriocarcinoma cell line JEG-3. Proliferation assays were done as described (*18*). Each data point was measured in duplicate. The range in each measurement was less than 10% of the mean of the duplicates. The experiments shown here are representative of three data sets that were obtained.

HLA-Cw7 (Fig. 1B). Two other TANK cell clones behaved similarly (*19*). Because the only NK receptor expressed on TANK-1 was the activating receptor, clone 39, it can be inferred that clone 39 facilitates an enhanced T cell response in the presence of the class I MHC alleles HLA-Cw4 and HLA-Cw7. These two class I MHC proteins belong to the two distinct groups of HLA-C proteins that are recognized by either NK1 or NK2 receptors. The molecular basis for the recognition of both HLA-Cw4 and -Cw7 by clone 39 is unknown, but the sequence of clone 39 is chimeric between NKAR1 and NKAR2.

The costimulatory effect of HLA-Cw4 or -Cw7 on the proliferative response of TANK-1 is greatest at lower concentrations of superantigen, causing a 300 to 900% increase in stimulation compared with untransfected target cells (Figs. 1, B and C, and 2A). Thus, the function of costimulatory NK receptors on T cells in vivo might be to allow a subset of T cells to be particularly responsive to small quantities of antigen. In addition, the enhanced T cell response of TANK-1 costimulated by HLA-Cw4 or -Cw7 peaks on the fourth day after initial stimulation and is maintained for a further 5 days (Fig. 1C). This suggests that the function of the costimulatory NK receptor on T cells might also be to sustain the immune response for an extended period. Possible reasons for the sharp decrease in the proliferation of TANK-1 at about 5 days after initial stimulation (Fig. 1C) include the up- or down-regulation of a particular protein on either the T cells or target cells, depletion of cytokines, or an exhaustion of nutrients within the media.

To determine directly whether the activating NK receptor on T cells facilitates the enhancement in proliferation, we used the monoclonal antibodies (mAbs) HP3E4 and GL183 to NK1 and NK2, respectively, in assays of TANK-9 proliferation in the presence of TSST1 at 0.05 ng/ml. Monoclonal antibody HP3E4 blocked the enhancement of TANK-9 proliferation mediated by 721.221 cells transfected with HLA-Cw4 or -Cw6, and GL183 blocked the inhibition of TANK-9 mediated by HLA-Cw3– or HLA-Cw7–expressing target cells (Fig. 2A). Analogous data were obtained with superantigen at 0.5 ng/ml. These data confirm that the NK1 and NK2 receptors on TANK-9 do indeed mediate the alterations in the proliferative response to TSST1.

Because none of the available mAbs binds specifically to clone 39, the role of this molecule in enhancing the proliferative response of TANK-1 could not be directly assessed by antibody blocking. However, the increase in proliferation of TANK-1 in the presence of SEB at 0.5 ng/ml, mediated by 721.221 cells transfected with HLA-Cw4 or -Cw7, was blocked by mAb PA2.6 to class I MHC proteins (Fig. 2B). Analogous data were obtained with SEB at 0.05 ng/ml. These data confirm that the increased proliferation of TANK-1 is mediated by class I MHC receptors.

The nonclassical class I MHC protein, HLA-G, has recently been shown to inhibit both NK1 and NK2 clones (*21*). Transfection of HLA-G into 721.221 cells enhanced the proliferation of TANK-1 in the presence of SEB, compared to untransfected cells (Fig. 3A). In contrast, the carcinoma cell line JEG-3, which expresses HLA-G but is deficient in class II MHC expression, could not stimulate TANK-1 in the presence of SEB (Fig. 3A). This result implies that superantigen cannot be presented by the class I MHC protein to cause significant proliferation of TANK-1, and that stimulation must be mediated primarily by superantigen associated with class II MHC molecules. In support of this conclusion, the proliferative response of TANK-1 to SEB was diminished in the presence of mAb LB3.1 to class II MHC (*19*).

SEB-coated 721.221 cells that had been transfected with HLA-G but that had lost most cell-surface expression of the protein after an extended period in culture (HLA-G[2]) caused the same extent of TANK cell proliferation as untransfected cells (Fig. 3A). Thus, it is the expression of HLA-G that facilitates the enhanced proliferation of TANK-1, rather than any artefactual alteration in the phenotype of transfected 721.221 cells. Although HLA-G can facilitate action through both NK1 and NK2 receptors, HLA-G–transfected 721.221 cells inhibited the proliferative response of TANK-9 to TSST1 (Fig. 3B) (*22*).

Thus, both NK activating receptors and NK inhibitory receptors are present on T cell clones, where they can alter the proliferative response of T cells stimulated by superantigen. NK inhibitory receptors can also inhibit the proliferative response of T cells stimulated by peptide antigen (*19*) as well as by superantigen (*10*), and it is reasonable to infer that NK activating receptors costimulate T cells activated by peptide antigens. Therefore, distinct combinations of activating and inhibitory receptors on each T cell could adjust the response of the human immune system to specific antigens.

Because the presence of an activating

NK receptor on a T cell potentially facilitates a response to small amounts of antigen, it is possible that TANK cells are important in initiating immune responses. In particular, because, by flow cytometry, NK receptors occur primarily on T cells of a memory phenotype (*19, 23*), costimulatory NK receptors may especially facilitate the rapid induction of secondary T cell–mediated immune responses. In addition, the expression and function of activating class I MHC receptors may allow activation of a T cell whose TCR may interact weakly with self peptide. Thus, expression of such receptors could also be significant in triggering the onset of autoimmune disease.

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12. TANK cell clones were prepared in the same manner as the NK clones used in (*5*), except that the $CD4^+$ T cells were not depleted.
13. Cells were typed by flow cytometry for the presence of CD3, CD4, CD8, CD16, CD56, TCRαβ, and TCRγδ (with antibodies from Becton-Dickinson), the type of TCR (with antibodies from Coulter Immunology), CD94 (with mAb HP3B1), and NK receptors [with mAbs specific for NK1 receptor (HP3E4), NK2 receptor (GL183, Coulter Immunology), and NK3 receptor (DX9)].
14. Oligonucleotides complementary to nonpolymorphic regions of the known NK receptor sequences were chosen as GATGGTACATGTCATAGGAGCTCC (at the 3′ end) and GAAAACCTTCCCTCCTGGCCC (at the 5′ end). The resultant PCR product that was amplified from cDNA derived from the T cell was cloned into pCRII (Invitrogen). The sequence of the insert was determined by automated sequencing at the Molecular Biology Core Facilities at the Dana-Farber Cancer Institute, Boston, MA. This sequence was then aligned against sequences currently held in the GenBank database with the program BLAST. The insert obtained from the cDNA of clone TANK-1 was 100% identical to that of the NK receptor, clone 39.
15. Reverse transcriptase–PCR typing of NK receptors was performed with oligonucleotides complementary to polymorphic regions of the extracellular portions of the known NK receptors and with oligonucleotides complementary to the long and short cytoplasmic tail sequences of NK receptors (H. T. Reyburn *et al.*, in preparation).
16. TANK cells are not exclusively $CD4^+$ $TCR\alpha\beta^+$ because $TCR\gamma\delta^+$ and $CD8^+$ $TCR\alpha\beta^+$ TANK cells have also been obtained (O. Mandelboim *et al.*, in preparation).
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18. Proliferation assays were performed as follows: The target cells were irradiated on a cesium source for 30 min (~30 Gy). Thereafter, 50,000 T cells, 25,000 target cells, various amounts of superantigen, and any other appropriate reagents were mixed in a total volume of 200 μl of RPMI–10% fetal calf serum in each well of a 96-well plate. After incubation at 37°C and 5% CO_2 for 2 days (or the time indicated), 1 μCi of [^{3}H]thymidine was added to each well, and the cells were further incubated at 37°C overnight. The cells were then harvested (Harvester 96 Mach III M, Tomtec) and counted on a liquid scintillation counter (1450 Microbeta Plus, Wallac). In analysis of the counts per minute (cpm) from each well, the background cpm from a well in which identical reagents and target cells were placed in the absence of any T cells was subtracted.
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22. This may be because HLA-G has a greater binding affinity for NK2 than for NK1 receptors or because HLA-G can interact with other inhibitory receptors on lymphocytes yet to be determined. Alternatively, the signals for inhibition of the proliferative response could be dominant over activating signals when both are triggered in this clone.
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Inhibition of Adipogenesis Through MAP Kinase–Mediated Phosphorylation of PPARγ

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Adipocyte differentiation is an important component of obesity and other metabolic diseases. This process is strongly inhibited by many mitogens and oncogenes. Several growth factors that inhibit fat cell differentiation caused mitogen-activated protein (MAP) kinase–mediated phosphorylation of the dominant adipogenic transcription factor peroxisome proliferator–activated receptor γ (PPARγ) and reduction of its transcriptional activity. Expression of PPARγ with a nonphosphorylatable mutation at this site (serine-112) yielded cells with increased sensitivity to ligand-induced adipogenesis and resistance to inhibition of differentiation by mitogens. These results indicate that covalent modification of PPARγ by serum and growth factors is a major regulator of the balance between cell growth and differentiation in the adipose cell lineage.

Adipose differentiation is influenced by a large number of mitogens and growth factors (*1*). In general, polypeptides that stimulate cell growth block fat cell differentiation. Platelet-derived growth factor, epidermal growth factor (EGF), fibroblast growth factor, and tumor promoters all inhibit fat cell differentiation in culture or in vivo (*2*). Various cytokines, including tumor necrosis factor–α (TNF-α), interleukin-1 (IL-1), IL-6, transforming growth factor–β, and interferon-γ also inhibit adipogenesis (*3*). Insulin has a prominent and complex role in the development of adipose cells, serving as a growth or differentiation factor depending on the specific cell type. Adipose cell precursors (preadipocytes), which express small amounts of insulin receptors, generally require insulin or insulinlike growth factor–1 for optimal differentiation (*4*). Adipose cells, which contain large numbers of insulin receptors but are postmitotic, respond to insulin with a lipogenic response as a result of the activation of lipogenic enzymes and the stimulation of Glut4-mediated glucose transport (*5*). In contrast, fibroblasts that express ectopically large amounts of insulin receptors usually respond to insulin with cell growth rather than differentiation (*6*).

Two families of factors are especially prominent in the transcriptional control of adipogenesis: the PPARs and C/EBPs. PPARγ is a member of the nuclear hormone receptor family that is expressed preferentially in adipose tissue (*7*). It is expressed in small amounts in preadipocytes, and its synthesis is increased during the process of adipogenesis (*8*). PPARγ binds specific ligands, including synthetic antidiabetic thiazolidinediones and 15-deoxy-$\Delta^{12,14}$prostaglandin J_2 (*9*), resulting in a full and powerful adipogenic response. Thus, PPARγ appears to be a key component in the determination and differentiation process in vivo (*9, 10*).

Ectopic expression of C/EBP-β and C/EBP-δ stimulates adipogenesis in fibroblasts as well (*11, 12*). This occurs through

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