

Fig. 1. Effect of HIV-1 Tat protein on tetanus-induced lymphocyte proliferation. Several concentrations of Tat were incubated with 1×10^5 PBMCs for 1 hour. Tetanus toxoid was then added to the cultures, and cells were grown as described in Table 1. Incorporation of [³H]thymidine was measured on day 6. Results are means \pm 1 SD of six replicates.

Tat could occur at any of these steps. Since Tat does not inhibit PHA-induced proliferative responses, it seems reasonable that Tat may interfere with signal transduction via the T cell antigen receptor or with antigen processing or presentation. Alternatively, Tat might induce production of cellular factors or cytokines that mediate the inhibition.

One of the hallmarks of acquired immunodeficiency syndrome (AIDS) is depletion of T4 cells, with the subsequent development of immunodeficiency. However, it is

recognized that destruction of CD4⁺ cells does not adequately explain all of the immunopathogenic effects of HIV infection (10). For example, even early in infection, patients' lymphocytes have a defect in their ability to recognize and respond to soluble antigen in vitro, although there are still normal numbers of CD4⁺ T lymphocytes (11). In contrast, lymphocytes from HIV-1-infected patients retain the ability to proliferate in response to mitogens. The effect of Tat on in vitro lymphocyte proliferation mimicked this difference between antigen- and mitogen-induced proliferation. Under our in vitro conditions, 50 nM Tat was sufficient for 50% inhibition, suggesting that Tat may be a potent immunosuppressive agent. We do not yet know whether Tat must be provided extracellularly or whether Tat produced internally can elicit these effects. If inhibition of lymphocyte function occurs in vivo, Tat produced inside HIV-infected cells may act directly on those cells or Tat may be released extracellularly, perhaps by cell lysis. More studies are needed to determine the biological significance of an immunosuppressive activity of Tat.

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14. We thank B. Felber and G. Pavlakis for the H938, U38, and HL3T1 cell lines, B. Liu for help with rabbit immunizations, and R. Young and A. Aldo-vini for helpful comments. This work was supported in part by the Lucille P. Markey Charitable Trust and NIH National Institute of Allergy and Infectious Diseases grant AI29135 (A.D.F.).

7 September 1989; accepted 10 November 1989

Mechanisms for Regulating Expression of Membrane Isoforms of FcγRIII (CD16)

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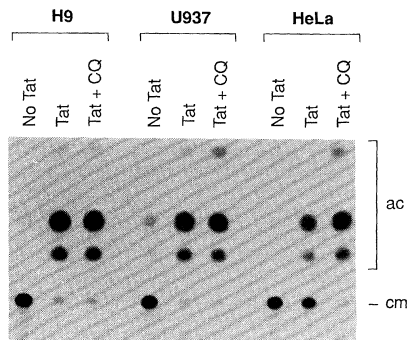


Fig. 2. Transactivation of the HIV-1 promoter in lymphocytes, promonocytes, and HeLa cells by purified Tat protein. H9 lymphocytes and U937 promonocytes (10^6 cells) that have an integrated HIV-1 LTR-CAT plasmid [H938 and U38 cells, respectively (12)] were incubated in RPMI 1640 medium containing 10% fetal bovine serum (1 ml in 25-mm wells) at 37°C (no Tat), treated with 5 µg of purified Tat protein (Tat), or treated with 5 µg of Tat protein and 100 µM chloroquine (Tat + CQ). Cells were harvested 24 hours after Tat treatment and assayed for CAT activity (13). HeLa cells (10^6 cells) that have an integrated HIV-1 LTR-CAT plasmid (HL3T1) (12) were incubated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (1 ml in 25-mm wells) and similarly treated with Tat protein, or with Tat and chloroquine, and assayed for CAT activity. Unacetylated (cm) and acetylated (ac) forms of [¹⁴C]chloramphenicol were separated by thin-layer chromatography.

Granulocyte and natural killer (NK) cell Fc receptors for immunoglobulin G (CD16) differ in only a few amino acids, yet have phosphatidylinositol glycan (PIG) or polypeptide membrane anchors, respectively. Mutagenesis shows that anchoring is regulated by a serine residue near the PIG anchor attachment site in the extracellular domain. The NK cell isoform was not expressed on the surface of COS cells unless cotransfected with a subunit that was expressed in NK cells and that was identical to the γ subunit of the high affinity IgE Fc receptor (FcεRI). However, the CD16 sequence and not expression of the γ subunit is dominant in regulating PIG reanchoring.

FC RECEPTORS (FCRs) SPECIFIC FOR the Fc domain of immunoglobulin (Ig) play a vital role in the function of the immune system. These receptors belong to the Ig gene superfamily (1). The IgG Fc receptor type III (FcγRIII or CD16) is predominantly found on NK cells, granulocytes, and tissue macrophages and mediates

low affinity binding to human IgG1 and IgG3. On granulocytes two alleles, NA1 and NA2, have been described for this receptor. CD16 is anchored to the membrane of granulocytes through a PIG moiety, but on NK cells and tissue macrophages it has a peptide transmembrane anchor (2-10). The type of membrane anchor appears to determine the functional capacity of this receptor, because NK cell CD16 is able to trigger killing and other functions like lymphokine secretion and IL2 receptor expression, whereas granulocyte CD16 is unable to do so (6, 11). There are two similar genes for

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CD16 that differ only by six amino acid substitutions and the presence of a cytoplasmic domain (7, 8). Five of these differences are scattered throughout the two extracellular Ig-like domains, whereas one of these substitutions changes a Phe residue, which is located just proximal to the transmembrane domain in the polypeptide-anchored NK cell CD16 isoform, to a Ser residue. In addition, the predicted membrane-spanning domain has a charged Asp residue. Polypeptides with charged residues in their transmembrane domains are either expressed in association with other transmembrane polypeptides, as in the case of the α chain of Fc ϵ RI (12), the $\alpha\beta$ - and $\gamma\delta$ -T cell receptors and CD3 polypeptides (13), or are destined to become PIG reanchored like Qa-2 (14).

Granulocyte and NK cell CD16 differed in their ability to be expressed in COS cells. Granulocyte CD16 was expressed well in COS cells (Fig. 1A, panel 1) and was attached by a PIG anchor as demonstrated by its release with phosphatidylinositol-specific phospholipase C [PIPLC; (Fig. 1A, panel 2) and in (3, 6)]. However, the NK cell form of CD16 was expressed poorly, if at all (Fig. 1A, panel 5). The transmembrane domain is replaced with a PIG anchor in granulocyte CD16 but is predicted to be retained in the NK cell CD16 isoform. There is a similarity between the transmembrane domains of CD16 and the α subunit of the high affinity Fc ϵ RI; an Asp in the middle of the transmembrane segment and eight contiguous residues are identical (1).

The IgE-binding α subunit of Fc ϵ RI is associated with a β subunit with four transmembrane segments and a dimer of disulfide-linked γ subunits with one transmembrane segment each (12). This similarity provoked us to examine whether coexpression with β or γ subunits of Fc ϵ RI would allow expression of the NK cell CD16 isoform. Indeed, efficient expression of NK cell CD16 was obtained by cotransfection with the γ subunit of the rat Fc ϵ RI (Fig. 1A, 7). In contrast to granulocyte CD16, NK CD16 was expressed with a polypeptide membrane anchor as indicated by its resistance to PIPLC (Fig. 1A, panel 8). Expression of NK cell CD16 was also achieved with the γ subunit of the human Fc ϵ RI. Cotransfection with the β subunit cDNA did not allow CD16 NK cell isoform expression and in combination with the γ subunit did not improve CD16 expression. Northern blot analysis indicated that the γ subunit transcript is found in the human NK cell line NK3.3 (Fig. 2). Cloning by polymerase chain reaction and sequencing of the entire coding region revealed that the NK cell γ subunit protein sequence is identical to the human mast cell γ sequence (15).

COS cells cotransfected with NK cell CD16 and γ subunit cDNAs, but not COS cells transfected with NK cell CD16 cDNA alone, could bind immune complexes in a standard IgG-opsonized erythrocyte rosetting assay. Approximately 20% of the transfectants were able to bind the opsonized erythrocytes, and this binding was completely abrogated by CD16 antibody.

Immunoprecipitation of 125 I-labeled material confirmed differences between the NK cell and granulocyte CD16 isoforms expressed in COS cells (Fig. 3). N-glycanase-treated CD16 that had been isolated from NK cells and from COS cells expressing both the NK form and the rat γ subunit was larger than CD16 isolated from both NA1/NA1 and NA1/NA2 neutrophils and from COS cells that expressed the NA1 form of granulocyte CD16. The larger size of the NK cell isoform of CD16 that was coexpressed in COS cells with the γ subunit indicates the presence of transmembrane and cytoplasmic domains. No CD16 was precipitated from COS cells transfected with the NK cell CD16 cDNA in the absence of the rat γ subunit cDNA.

We then examined which sequence differences between the CD16 isoforms regulated PIG reanchoring and whether sequence differences or γ chain expression were dominant. The stop codon in the granulocyte isoform of CD16 was converted to the Arg encoded in the NK cell isoform at residue 234 (mutation *234R). This added to the granulocyte isoform a cytoplasmic tail of 21

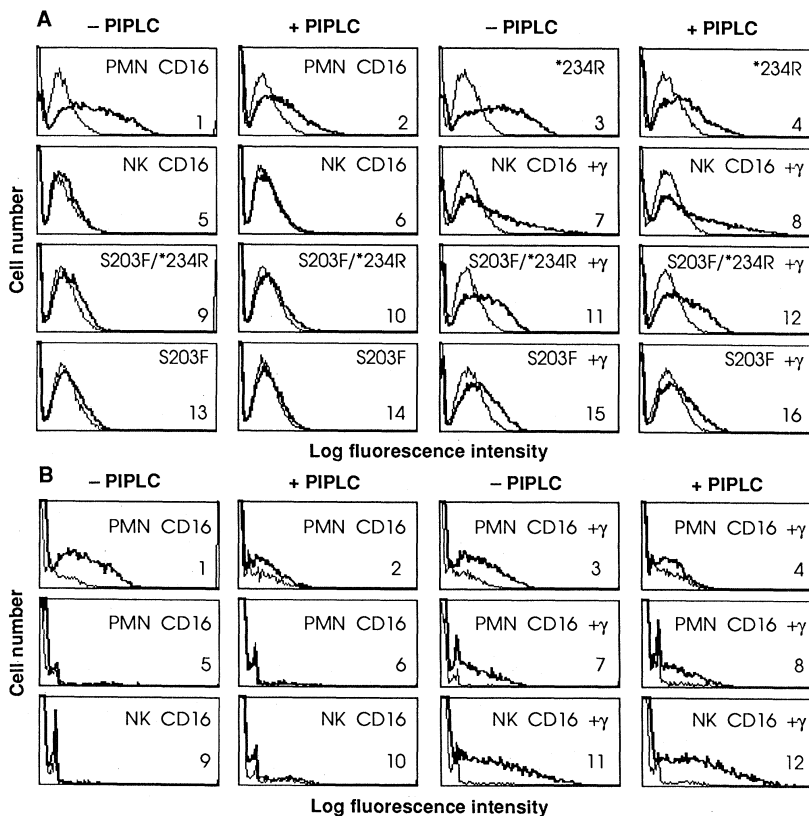


Fig. 1. Immunofluorescence flow cytometric analysis of COS and L cells transfected with PIG-anchored, transmembrane-anchored, and mutant forms of CD16 in the presence and absence of the γ subunit of the rat high affinity Fc ϵ RI. **(A)** COS cells transfected with plasmids encoding granulocyte CD16 (PMN CD16), mutants of PMN CD16, or NK cell CD16 with or without the γ subunit as indicated, and subjected to PIPLC treatment (3) as indicated, were labeled with either anti-CD16 [monoclonal antibody (MAB)3G8] (thick line) or with a non-binding control MAB X63 (thin line). **(B)** COS cells (panels 1 to 4) or L cells (panels 5 to 12) were transfected with plasmids encoding the indicated subunits, treated with PIPLC as indicated, and were labeled as in (A). The cDNA encoding the NA1 polymorphic form of granulocyte CD16 (7) was contained in the transient expression vector CDM8. Mutants of the granulocyte form of CD16 were prepared by oligonucleotide-directed mutagenesis in CDM8; the second strand was synthesized on uracil-containing templates made in a *dut⁺ ung⁻* mutant host with mutants then selected against the wild-type, uracil-containing template in a *dut⁺ ung⁺* host (32, 33). All mutations were verified by nucleotide sequencing. The cDNAs encoding the NK cell form of CD16 and the rat γ subunit of the high affinity Fc ϵ RI were subcloned separately into the expression vector pSVL and transcribed from the SV-40 promoter. COS and L cells were transfected with DEAE-Dextran and transferred to fresh plates 1 day before analysis (34).

amino acids that differed only by a Leu instead of a Phe from the NK cell cytoplasmic domain. Independently, or in combination with the above mutation, the Ser residue six amino acids external to the membrane in the granulocyte isoform was converted to a Phe as in the NK cell isoform (S203F and S203F/*234R, respectively). Unlike results with other proteins such as LFA-3 (16–19), the CD16 mutant *234R was expressed in COS cells as a PIPLC-sensitive and hence PIG-anchored protein, its long cytoplasmic tail did not prevent attachment of the PIG anchor (Fig. 1A, panels 3 and 4). Neither mutant S203F nor the mutant encompassing both changes, S203F/*234R, were expressed when transfected alone (Fig. 1A, panels 9 and 10 and 13 and 14). In contrast, when cotransfected with the γ subunit, these two mutants were expressed as transmembrane proteins resistant to PIPLC (Fig. 1A, panels 11 and 12 and 15 and 16). Thus, the substitution S203F regulated cleavage of the CD16 polypeptide chain and transfer of the extracellu-

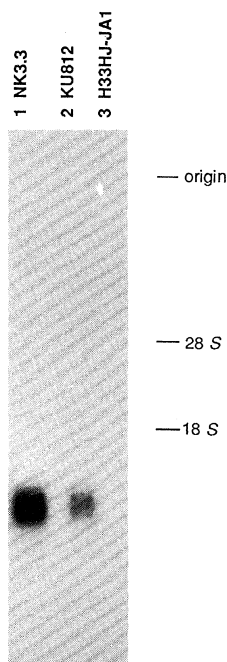
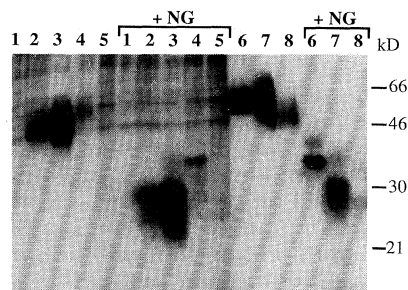


Fig. 2. Northern blot analysis of γ subunit mRNA. Total RNA (7.5 μ g) isolated from the human NK cell line NK3.3 (lane 1), the human basophil cell line Ku812 (lane 2), and the H33HJ-JA1 human T cell line (lane 3) was probed with the nick translated 472-bp Nde I fragment of the human γ subunit cDNA. 28S and 18S ribosomal RNA markers are indicated. The NK3.3 cell line and the Ku812 cell line have been described (35, 36). H33HJ-JA1 is a human IL-2-producing T cell line obtained from the American Type Culture Collection (ATCC# CRL 8163). RNA extraction, Northern blotting, and hybridization were performed as in (37).

Fig. 3. N-glycanase treatment and SDS-PAGE analysis of the different isoforms of CD16. COS cells transfected with either no DNA (lane 1), the granulocyte CD16 isoform and the γ subunit (lane 2), the granulocyte CD16 isoform alone (lane 3), the NK cell CD16 isoform and the γ subunit (lane 4), or the NK cell CD16 isoform alone (lane 5). CD16 was also obtained from freshly prepared NK cells (lane 6), granulocytes from NA1/NA2 heterozygotes (lane 7) and granulocytes from NA1 homozygotes (lane 8). Lanes marked “+NG” contains N-glycanase treated CD16. Molecular weight markers are shown. Granulocytes and NK cells were isolated from peripheral blood leukocytes (3, 6). CD16 was immunoprecipitated from 125 I surface-labeled COS cells, NK cells, or granulocytes and digested with N-glycanase (6) except that 100 mM octyl-glucoside was used to solubilize the COS cells.



lar domain to the PIG anchor. Attachment of the PIG anchor is predicted, based on chemical studies on other proteins, to occur at or near Ser²⁰³ (20). CD16 molecules with residue Phe²⁰³ of the NK cell isoform required γ for surface expression. The cytoplasmic domain was not essential for association with γ but improved efficiency of surface expression (Fig. 1A, compare panels 11 and 12 to 15 and 16).

In addition to regulation by the amino acid sequence, the γ subunit could perhaps regulate PIG anchoring by associating with the CD16 transmembrane segment and thereby preventing reanchoring. Cotransfection of the granulocyte form of CD16 with the γ subunit slightly decreased the expression of granulocyte CD16, in contrast to an increase for NK cell CD16 (Fig. 1B, panels 1 and 3). The granulocyte isoform of CD16 that was coexpressed with the γ subunit was sensitive to PIPLC treatment (65% removed; Fig. 1B). This was comparable to the amount of CD16 released from COS cells transfected with granulocyte CD16 alone (70%; Fig. 1B) and from neutrophils (3). PIPLC release of PIG-anchored proteins is never complete, due to the acylation of the inositol in a subpopulation of the anchors (21). To confirm PIG anchoring, material immunoprecipitated from surface-labeled COS cells cotransfected with the granulocyte isoform of CD16 and the γ subunit was treated with or without N-glycanase and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 3). If any of the granulocyte CD16 were anchored to the membrane through a polypeptide chain, this material should be approximately 3 kD larger than the PIG-anchored material. However, the material immunoprecipitated from COS cells transfected with both granulocyte CD16 and the rat γ subunit was identical in size to that immunoprecipitated from COS cells transfected with granulocyte CD16 alone (Fig. 3, lanes 2 and 3). Thus, COS cells transfected with granulocyte CD16 and the γ subunit only

expressed PIG-anchored CD16.

The relative dominance of the amino acid sequence of CD16 over the expression of the γ subunit in the regulation of PIG anchoring was further examined in L cells that lacked the appropriate machinery for attachment of a PIG anchor (22). The granulocyte isoform of CD16 was not expressed on the surface in L cells (Fig. 1B, panel 5), but when cotransfected with the γ subunit some surface expression of CD16 was apparent (Fig. 1B, panel 7) although less than when the NK cell CD16 isoform was coexpressed with the γ subunit in L cells (Fig. 1B, panel 11). In agreement with requirement of γ for expression, the granulocyte isoform of CD16 expressed in L cells was not PIG-anchored; PIPLC had no effect (Fig. 1B, panels 7 and 8). Thus, in normal cells the amino acid sequence of CD16 at residue 203 determined PIG reanchoring and was dominant over γ subunit expression in regulating anchoring. Expression of the γ subunit did not alter processing of CD16 with Ser²⁰³ but was required for expression of CD16 with Phe²⁰³. CD16 molecules with Ser²⁰³ could be expressed at the surface in the presence of the γ subunit in a non-PIG-anchored form, but only with no functional PIG-anchoring machinery. This suggests that normal PIG reanchoring is efficient, and dominates this alternative pathway for surface expression. Our findings demonstrate that the transmembrane region of CD16 is critical for association with the γ subunit, and the cytoplasmic domain is not required, but increases the efficiency of association or surface expression. Similar results to those reported here have been obtained independently (23).

CD16 illustrates a mechanism for regulating PIG reanchoring in which a single amino acid substitution near or at the predicted site of PIG anchor attachment plays the most critical role. Previous physiologic mechanisms for regulating PIG reanchoring involve mRNA splicing rather than duplicated, similar genes and have implicated a

cytoplasmic domain in LFA-3 (16–19), or completely different cytoplasmic, transmembrane, and extracellular amino acid sequences in the neural cell adhesion molecule (24, 25). Serine and other small hydrophilic side chain amino acid residues are commonly found at and near the site of PIG anchor attachment (20). However, their role in regulating anchor attachment had not been appreciated, although it is known that a segment including the 20 amino acids in the extracellular domain, together with the transmembrane domain, are required to signal reanchoring of decay accelerating factor (26). The studies here further illuminate the relative dominance of protein sequence and post-translational mechanisms, including subunit association and availability of the appropriate enzymes for PIG assembly and attachment in regulating PIG reanchoring.

We have not demonstrated association in NK cells of CD16 and the γ subunit, although the requirement for coexpression in COS or L cells and the presence of a γ subunit of identical sequence in NK cells is suggestive. A mouse IgG Fc receptor, Fc γ RIIa, also requires γ chain for cell surface expression (27) as does the Fc ϵ RI (28). For these receptors, demonstration of γ association requires special conditions. This may explain previous (5–7, 29), and our own (unpublished), lack of identification of this subunit in association with CD16 in NK cells. The γ subunit has little exposure on the outside of the cell (12). Lack of significant homology in cytoplasmic domains of CD16, Fc ϵ RI, and mouse Fc γ RIIa, together with lack of requirement of the CD16 cytoplasmic domain for association, suggests association is governed primarily by the transmembrane region. The γ subunit is related to the T cell receptor ζ subunit (30) and may participate in related signaling pathways. Recently, ζ has also been shown to be expressed in NK cells (31) and is also a candidate for a subunit that may associate with CD16.

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- The authors wish to thank B. Seed for the granulocyte CD16 cDNA clone contained in CDM8, J. Ravetch for the NK cell CD16 cDNA clone and for communicating the NK cell CD16 sequence prior to publication, M. Low for PIPLC, J. Unkeless for the 3G8 hybridoma cell line, P. A. T. Tetteroo for CLBFcGran-1 cell line, and E. Luther for flow cytometric analysis for transfectants. M.L.H. was supported by a grant from the Jane Coffin Childs Memorial Fund for Medical Research and O.C. was supported by grants from Emil Aaltonen Foundation and the Finnish Cultural Foundation. This work was supported by NIH grants.

19 September 1989; accepted 9 November 1989

Membrane Anchoring of a Human IgG Fc Receptor (CD16) Determined by a Single Amino Acid

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CD16 is a low-affinity immunoglobulin G (IgG) Fc receptor that is expressed on natural killer (NK) cells, granulocytes, activated macrophages, and some T lymphocytes. Two similar genes, CD16-I and CD16-II, encode membrane glycoproteins that are anchored by phosphatidylinositol (PI)-glycan and transmembrane polypeptides, respectively. The primary structural requirements for PI-linkage were examined by constructing a series of hybrid cDNA molecules. Although both cDNA's have an identical COOH-terminal hydrophobic segment, CD16-I has Ser²⁰³ whereas CD16-II has Phe²⁰³. Conversion of Phe to Ser in CD16-II permits expression of a PI-glycan-anchored glycoprotein, whereas conversion of Ser to Phe in CD16-I prevents PI-glycan linkage.

THE CD16 IgG Fc RECEPTOR (Fc γ RIII) is identified by monoclonal antibodies (MAb) 3G8, B73.1, Leu 11, and others (1). The binding of immune complexes or anti-CD16 MAb to NK cells causes a rapid increase in intracellular Ca²⁺ and inositol triphosphate generation, with subsequent transcription of lymphokines and triggering of cell-mediated cytotoxicity (2, 3). In contrast, on granulocytes CD16 may function simply as a binding structure for IgG complexes (3, 4).

Biochemical differences between CD16 on NK cells and granulocytes may account

for these functional distinctions (3). The CD16 polypeptide on granulocytes is ~30 kD, but is ~36 and ~38 kD on NK cells. CD16 on granulocytes is susceptible to cleavage with phosphatidylinositol phospholipase C (PI-PLC), indicating phosphatidylinositol (PI)-glycan linkage, whereas CD16 on NK cells is resistant to PI-PLC (5). CD16 is diminished on granulocytes but is unaffected on macrophages in patients with paroxysmal nocturnal hemoglobinuria, a disease selectively affecting PI-anchored proteins (6). Two genes exist for CD16 (7). Granulocytes and NK cells preferentially transcribe CD16-I and CD16-II, respectively (7, 8). We examined the structural requirements for PI linkage of CD16-I.

A λ gt11 cDNA library from human NK

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