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Lineage-independent activation of immune system effector function by myeloid Fc receptors

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An emerging theme in immunology finds receptors which initiate cellular effector programs forming multichain complexes in which the ligand recognition elements associate with one or more 'trigger molecules' whose aggregation initiates a signal transduction cascade. The sequence motifs constituting the active sites of these trigger molecules are found in the T cell and B cell antigen receptors, and some Fc receptors, and appear to be central to effector function activation. For example, of the many molecules that mimic or potentiate the action of the T cell antigen receptor (TCR), none have yet been found to initiate effector programs autonomously in cells lacking TCR. We have devised two strategies to study activation mediated by myeloid Fc receptors, which appear not to associate with trigger molecules: the use of primary human cytolytic T cells as surrogate effector cells for genetically delivered receptors, and the use of vaccinia virus vectors to introduce genetically modified receptors into primary human monocytes. Using these approaches, we have found that the cytoplasmic domains of two Fc receptors show comparable function to equivalent domains of the trigger molecule family, but are not homologous to members of that family.

Key words: cytolysis/FcγRII/Fc receptor/macrophages

Introduction

Receptors for the constant portion of immunoglobulin G (Fcγ receptors) form a complex class of cell surface proteins mediating phagocytosis of immune complexes, transcytosis and various forms of antibody dependent cellular cytotoxicity (Mellman, 1988; Unkeless *et al.*, 1988; Fanger *et al.*, 1989; Ravetch and Kinet, 1991). Human and murine Fcγ receptors have been divided into three classes, corresponding to a high affinity type (FcγRI), a family of low affinity receptors (the FcγRII receptors) and a third low affinity type (FcγRIII) which has two forms in humans, and one in mice. Some aspects of receptor function are fairly well delineated. FcγRIIA has recently been shown to mediate phagocytosis (Engelhardt *et al.*, 1991; Indik *et al.*, 1991; Odin *et al.*, 1991), while FcγRIIB2 mediates internalization of Ig-coated targets into clathrin coated pits, (Hunziker and Mellman, 1989; Miettinen *et al.*, 1989). The closely related FcγRIIB1 bears all of the sequences of FcγRIIB2, but is prevented from association with the endocytotic apparatus by a 47 amino acid insertion in the cytoplasmic domain (Amigorena *et al.*, 1992; Miettinen *et al.*, 1992) which, in B cells, favors association of the receptor with surface IgM by co-capping (Amigorena *et al.*, 1992). Both B forms of FcRII are capable of inhibiting B-cell activation mediated by the IgM antigen receptor (Amigorena *et al.*, 1992).

A low affinity receptor of the third class, FcγRIIIA, mediates immune cell activation through its association with one or more members of a small family of 'trigger molecules' (Hibbs *et al.*, 1989; Kurosaki and Ravetch, 1989; Lanier *et al.*, 1989; Ra *et al.*, 1989; Anderson *et al.*, 1990; Bonnerot *et al.*, 1992).

These trigger molecules, T cell receptor (TCR) ζ chain (Samelson *et al.*, 1985; Weissman *et al.*, 1988) TCR η chain (Jin *et al.*, 1990) and Fc receptor γ chain (Blank *et al.*, 1989; Ra *et al.*, 1989; Bonnerot *et al.*, 1992) interact with ligand recognition domains of different immune system receptors and can autonomously initiate cellular effector programs, including tyrosine kinase activation, cytokine secretion and cytolysis, following their aggregation (Irving and Weiss, 1991; Letourneur and Klausner, 1991; Romeo and Seed, 1991; Bonnerot *et al.*, 1992). Within the ζ, η and FcR γ intracellular domains are sequence motifs also represented in CD3 γ, δ and ε chains, in the B cell antigen receptor mb1 and B29 proteins, and in the FcεRI β chain (Kinet, 1989; Reth, 1989; Wegener *et al.*, 1992). Recent studies have shown that CD3ε (Letourneur and Klausner, 1992) and a complex comprising CD3γ, δ and ε (Wegener *et al.*, 1992) have the capacity to activate T cells, suggesting that the function of the sequence motif common to these chains may be to initiate a common or related activation pathway.

Early events in the activation of receptors associating with trigger molecules include tyrosine phosphorylation, formation of inositol phosphates and accumulation of free intracellular Ca^{2+} (e.g. Gardner, 1989; Sefton and Campbell, 1991; Keegan and Paul, 1992). In many cases the later stages of activation are accompanied by a change in transcriptional potential which results in cytokine synthesis or a developmentally activated cell state. The causative chain of events which translates cell surface receptor aggregation into cellular activation remains poorly understood even in the best studied examples.

The triggering of cell type-specific effector function can be distinguished from the diverse signals which either influence responsiveness to central activating signals or mediate activation pathways which do not engage effector function, e.g. as found in adhesion or chemotaxis. As yet, no molecules apart from members of the trigger family have been shown to autonomously activate T cells, the most extensively characterized of immune system effector cells (Yokoyama and Shevach, 1989). Aggregation of phospholipid-linked proteins (Kroczek *et al.*, 1986; Yeh *et al.*, 1987; Balk and Terhorst, 1989) or treatment of CD2 with pairs of monoclonal antibodies (Meuer *et al.*, 1984) can activate effector function in T cells, but this activation depends on the presence of one or more members of the trigger family (Schmitt-Verhulst *et al.*, 1987; Moingeon

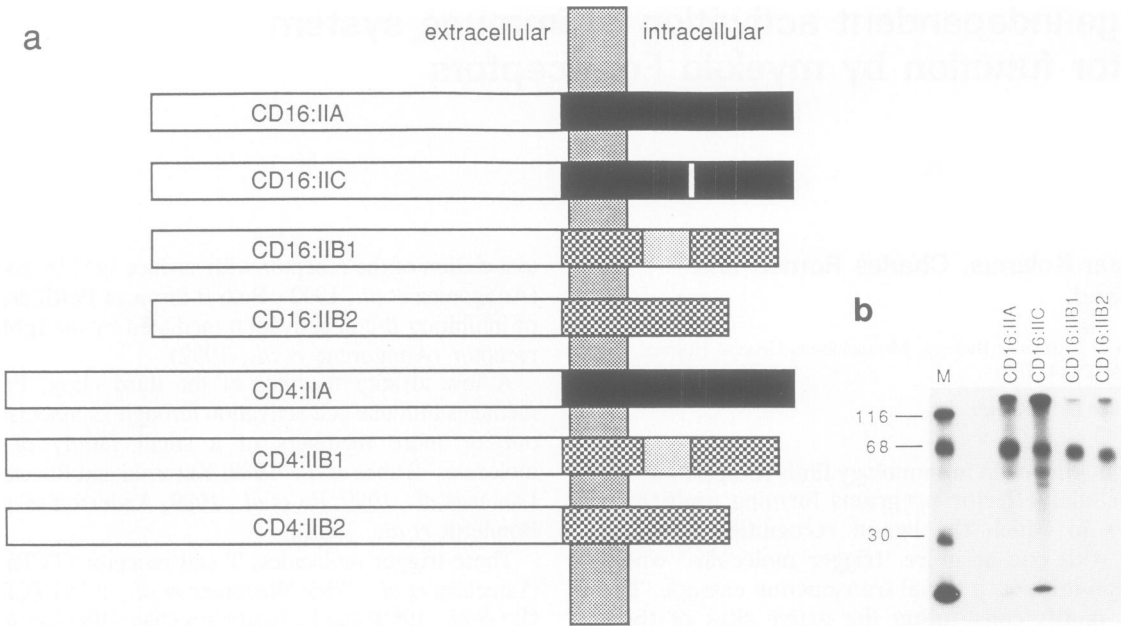

Fig. 1. Creation and analysis of FcγRII chimeras. (**a**) Schematic diagram of CD16: FcγRII and CD4:FcγRII chimeras. The intracellular domains of FcγRIIA and C differ by a single residue (268 from the amino-terminus of the precursor), while the intracellular domains of FcγRIIB1 and B2 differ by the presence or absence of an additional exon (light stippling). (**b**) Immunoprecipitation of CD16:FcγRII chimeras. An autoradiogram of a reducing gel of immunoprecipitated FcγRIIA, C, B1 and B2 chimeras is shown with molecular mass standards on the left. At present we believe that the lower molecular mass species in the FcγRIIA and C tracks represent partially glycosylated precursors.

et al., 1992). In less extensively characterized cell types there is relatively little information as to the indispensability of the trigger molecules. In no small part this scarcity of information can be attributed to the absence of suitable cell lines which both possess effector function and are amenable to genetic and biochemical analyses.

The initiation of cytolysis by Fc receptors in macrophages (or monocytes, their blood-borne precursors) is an example of an activation process whose study has been stymied for want of a suitable *in vitro* system. Although several myeloid cell lines have been developed, none display the cytolytic activity of freshly isolated monocytes or macrophages. Conversely, monocytes in primary culture exhibit little proliferative potential and are sufficiently refractory to transfection to make transient expression approaches infeasible. In this work we have pursued two alternative strategies to explore Fc receptor mediated activation: the use of primary human cytolytic T cells as surrogate effector cells for genetically delivered Fc receptors, and the use of vaccinia virus vectors to introduce Fc receptors into primary human monocytes. Using these approaches we have found that protein chimeras based on FcγRIIA as well as similar chimeras based on the closely related FcγRIIC trigger cytolytic T cell effector programs in a manner whose efficacy and lineage independence are reminiscent of the action of the TCR/Fc receptor-associated ζ and γ chains.

Results

Construction of Fc receptor chimeras

To evaluate the actions of the different receptor subtypes, we created chimeric molecules in which the extracellular domain of the human CD4 or CD16 antigens were joined to the transmembrane and intracellular domains of the FcγRIIA, B1, B2 and C subtypes (nomenclature of Ravetch and Kinet, 1991). The chimeras were created by genetically fusing DNA segments encoding the different extracellular domains to fragments encoding Fc receptor sequences obtained from previously described clones (Stengelin *et al.*, 1988) or from polymerase chain reactions based on other reported sequences (Stuart *et al.*, 1989; Qiu *et al.*, 1990; Figure 1). The gene fusions were constructed in vaccinia virus expression vectors and subsequently inserted into wild type vaccinia by recombination at the thymidine kinase locus, using selection for cointegration of *Escherichia coli gpt* (Falkner and Moss, 1988; Boyle and Coupar, 1988) to facilitate identification of the desired recombinants. Immunoprecipitation of Jurkat (T cell leukemia) cells infected with the recombinant viruses revealed chimeric molecules of the expected molecular masses (Figure 1 and data not shown).

Calcium mobilization in T cells is independent of T cell receptor

Introduction of the chimeras into a TCR-negative mutant of the Jurkat line (Weiss and Stobo, 1984) showed that the intracellular domains of FcγRIIA and FcγRIIC were capable of mediating an increase in cytoplasmic free calcium ion after the extracellular domains were crosslinked by antibody, whereas the intracellular domains of FcγRIIB1 and B2 were inactive under comparable conditions (Figure 2). The CD4 and CD16 hybrids of FcγRIIA shared essentially equal capacity to promote the calcium response (Figure 2 and data not shown). These data are consistent with studies documenting calcium mobilization by intact FcγRIIA in a murine macrophage cell line (Odin *et al.*, 1991), although in the latter case the possible association with zeta-family trigger molecules has not been excluded.

Cytolytic activation in primary human T cells

The ability to promote accumulation of free Ca^{2+} following crosslinking suggested that the active receptors might be

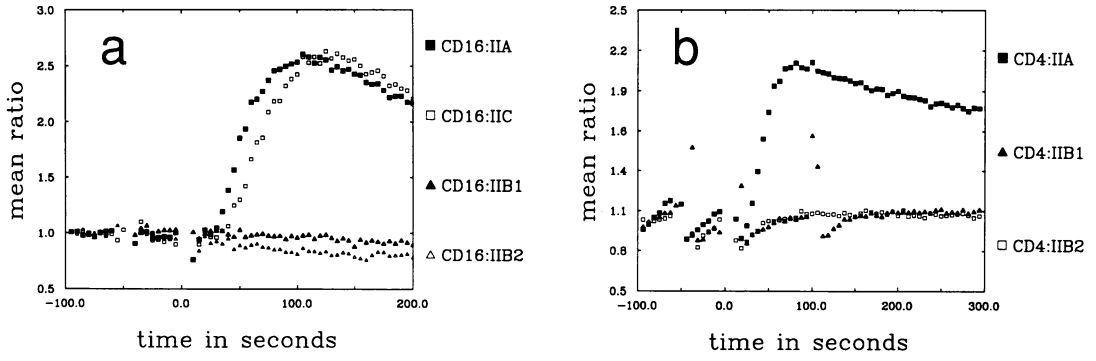

Fig. 2. Calcium mobilization following crosslinking of CD4:FcγRII and CD16:FcγRII chimeras. (**a**) The ratio of violet to blue fluorescence emitted by TCR^- mutant Jurkat cells loaded with the calcium sensitive fluorophore Indo-1 is shown as a function of time following crosslinking of the CD16 extracellular domain with antibodies. (**b**) Similar analysis of the increase in ratio of violet to blue fluorescence of cells bearing CD4:FcγRII chimeras, following crosslinking with antibodies.

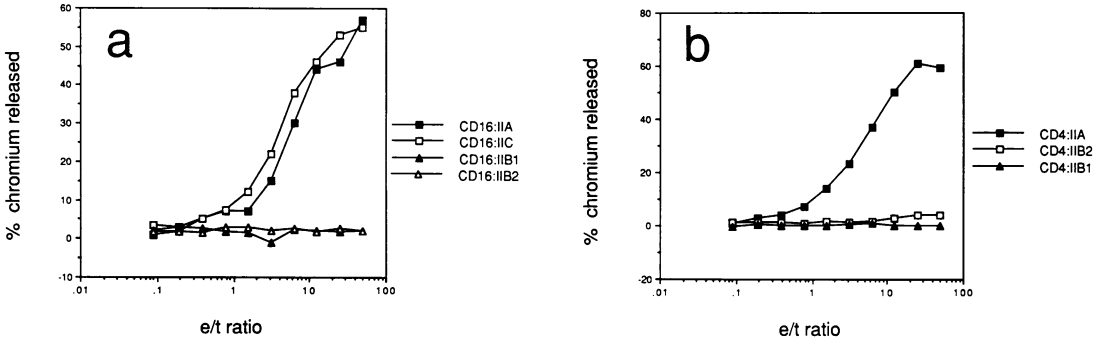

Fig. 3. Cytolysis assay of CD16:FcγRII chimeras (a) and CD4:FcγRII chimeras (b). (**a**) The percent of ^{51}Cr released from anti-CD16 hybridoma (target) cells when the cells were exposed to increasing numbers of cytotoxic T lymphocytes expressing CD16:FcγRII chimeras (effector cells) is plotted as a function of the ratio of the number of effector cells to target cells. (**b**) Cytolysis experiments were conducted as in (a) except that the effector cells expressed CD4 chimeras, and the targets were HeLa cells expressing HIV gp120/41. The expression of fusion protein by the vaccinia infected cells was measured by indirect immunofluorescence and flow cytometry. Mean fluorescence intensities for the experiment in panel (a) were: CD16:FcγRIIA, 752; CD16:FcγRIIC, 777; CD16:FcγRIIB1, 1194; and CD16:7:FcγRIIB2, 1204. Mean fluorescence intensities for the experiment in panel (b) were: CD4:FcγRIIA, 467; CD4:FcγRIIB1, 120; and CD4:FcγRIIB2, 299.

capable of initiating a cytolytic response. Because there were no satisfactory systems for evaluating the cytolytic potential of gene constructs introduced into myeloid cells, we initially opted to evaluate their potential in a human cytolytic T cell line previously shown to respond to the FcγRIIIA-associated ζ and γ chains (Romeo and Seed, 1991). Human cytotolytic T lymphocytes (CTL) were infected with vaccinia recombinants expressing CD16:FcγRIIA, IIB1, IIB2 and IIC chimeras, and the infected cells were cocultured with ^{51}Cr-loaded hybridoma cells (3G8 clone 10-2; Fleit *et al.*, 1982; Shen *et al.*, 1989) which expressed cell surface antibody to CD16. In this assay (Graziano and Fanger, 1987a,b; Romeo *et al.*, 1992) CTL bearing the CD16 chimera kill hybridoma target cells (allowing release of free ^{51}Cr) if the CD16 extracellular domain of the chimera has been joined to an intracellular segment capable of activating the lymphocyte effector programs. Figure 3 shows that CTL armed with CD16:FcRγIIA and C but not FcγRIIB1 or B2 are capable of lysing target cells expressing cell surface anti-CD16 antibody.

To eliminate the possibility that the specific cytolysis was in some way attributable to interaction with the CD16 moiety, we also conducted cytolysis experiments in which the FcRII intracellular domains were attached to CD4 extracellular domain. In this case the target cells were HeLa cells expressing HIV envelope gp120/41 proteins (Romeo and Seed, 1991). As in the CD16 system, target cells expressing HIV envelope were susceptible to lysis by T cells expressing CD4:FcγRIIA chimera, but not FcγRIIB1 or B2 (Figure 3).

Analysis of Fc receptor chimeras in monocytes

Primary monocytes represent ~15% of the mononuclear fraction of blood leucocytes, and exhibit little or no proliferative capacity in culture. However, they can be relatively easily purified, based on their tendency to adhere to solid surfaces and each other (Connor *et al.*, 1990). Using purified preparations of monocytes, we were able to document expression of Fc receptor chimeras delivered by vaccinia virus expression vectors. Figure 4 shows that primary monocytes represent good targets for vaccinia virus infection, and that high levels of expressed protein can be achieved in the infected cells. Free calcium ion accumulates in the monocyte cytoplasm following crosslinking of the Fc receptor chimeras in a fashion similar to that observed in the Jurkat T cell line (Figure 5).

Purified monocytes in culture also show cytolytic activity, albeit at lower levels than cytolytic T lymphocytes. The confounding effects of nonspecific cytotoxicity are higher, which we attribute to the fact that monocytes adhere non-specifically to target cells more than cytotoxic T cells do. However, despite these limitations, it is possible to demonstrate mobilization of the cytotoxic potential of primary monocytes by Fc receptor chimeras bearing CD4

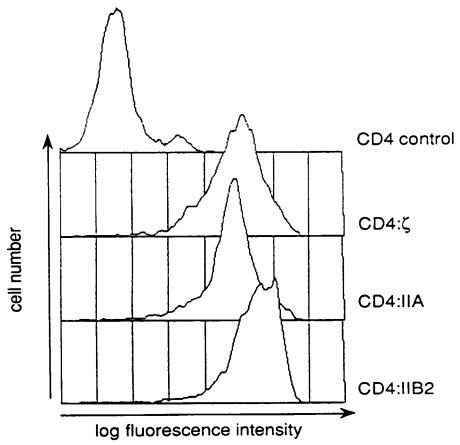

Fig. 4. Primary human monocytes are good targets for vaccinia virus expression vectors. Human monocytes were freshly isolated from whole blood and infected with vaccinia viruses bearing the indicated fusion proteins under the control of the p7.5 promoter. The infected cells were stained with phycoerythrin-conjugated antibody to CD4 and analyzed by flow cytometry. The panels depict the increase in specific fluorescence over that shown by uninfected monocytes, which express some CD4.

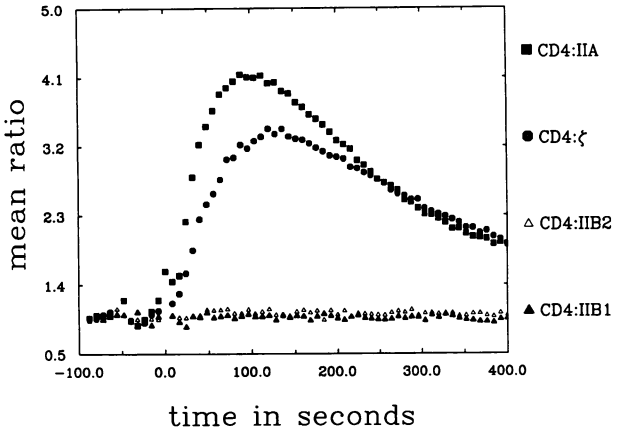

Fig. 5. Calcium mobilization in primary human monocytes expressing CD4 chimeras. Primary human monocytes were isolated from whole blood and infected with recombinant vaccinia viruses expressing various CD4 fusion proteins. Gated calcium flux analyses were conducted by flow cytometry after loading the infected monocytes with the dye Indo-1. The ratio of violet to blue fluorescence (a surrogate marker for calcium ion) for the positive cell population (gated for both CD4 expression and scatter, using CD14 expression to establish scatter windows) is shown as a function of time.

extracellular domains joined to Fc receptor transmembrane and intracellular domains (Figure 6). As is the case for T cells, Fc receptor chimeras are essentially as effective as TCR ζ chain chimeras in the redirected cytolysis assay (Figure 6).

Analysis of functional subdomains of FcγRII intracellular sequences

The intracellular portions of FcγRIIA and C share no appreciable sequence homology with other proteins, including the members of the extended TCR ζ family. To define the sequence elements responsible for induction of cytolysis, we prepared 5′ and 3′ deletions of the intracellular domain coding sequences, and evaluated the efficacy of the resulting deleted fusion proteins in the calcium mobilization

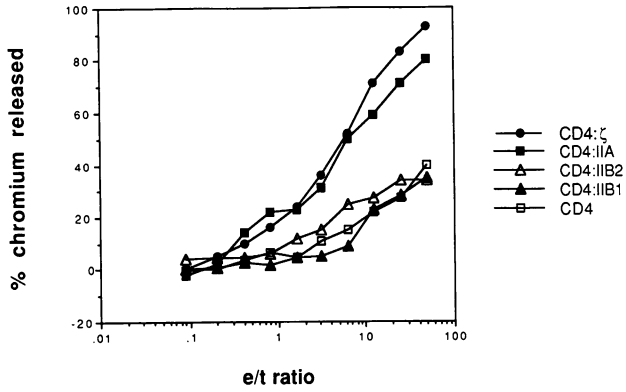

Fig. 6. Cytolysis in primary human monocytes expressing CD4 chimeras. Primary human monocytes were isolated from whole blood and infected with recombinant vaccinia viruses. The infected monocytes were then cultured with ^{51}Cr-loaded CV1 cells which had previously been infected with recombinant vaccinia viruses expressing HIV envelope glycoproteins. The figure displays the % of chromium release as a function of the effector to target ratio. Mean fluorescence intensities for cells treated with phycoerythrin-conjugated anti-CD4 antibody in this experiment were: for CD4:ζ, 706; for CD4:FcγRIIA, 1000; for CD4:FcγRIIB1, 75.6; for CD4:FcγRIIB2, 912; and for CD4 alone, 28.6.

and cytolysis assays. In the experiments in which the amino-terminal portion of the intracellular domain was removed, the transmembrane domain of FcγRII was replaced with the transmembrane domain of the unrelated CD7 antigen, to eliminate the possible contribution of interactions mediated by the membrane-spanning domain (Figure 7a).

Removal of the 14 carboxyl-terminal residues, including Tyr298, resulted in a complete loss of cytolytic capacity and a substantial but incomplete reduction in ability to mobilize calcium in mutant cells lacking T cell receptor (Figure 7b). Such cells are often hypersensitive to calcium-eliciting stimuli, however, and a nearly complete loss of activity was recorded in cells retaining T cell antigen receptor (Figure 7c). The latter data give results comparable to those obtained with intact receptor, in which the removal of 17 residues was found to eliminate calcium mobilization in P388D$_1$ cells (Odin *et al.*, 1991). Further truncation from the carboxyl-terminus to just before Tyr282 gave an identical phenotype.

Deletion from the amino-terminus of the intracellular domain to residue 268 had no substantial effect on either the calcium profile or the cytolytic potency, whereas deletion to residue 275 markedly impaired free calcium release but had little effect on cytolysis. At present we suspect that the inability of the FcγRIIA(276−311) fragment to mobilize calcium in the flow cytometric assay does not reflect its ability to support calcium ion accumulation over the longer time span of the cytolysis assay, inasmuch as the cytotoxicity in this assay is inhibited by the inclusion of EGTA in the medium (data not shown). However, it is possible that the effects of EGTA are limited to inhibition of exocytosis, rather than triggering. An EGTA-insensitive cytolytic activity has been reported for some (but not all) cytolytic T cell lines (Ostergaard *et al.*, 1987; Trenn *et al.*, 1987, 1989; Young *et al.*, 1987). Further deletion from the amino-terminus, to residue 282, produced FcγRII tails that lacked the ability either to mobilize calcium or to trigger cytolysis.

The 'active element' defined in this manner is relatively large (36 amino acids) and contains two tyrosines separated

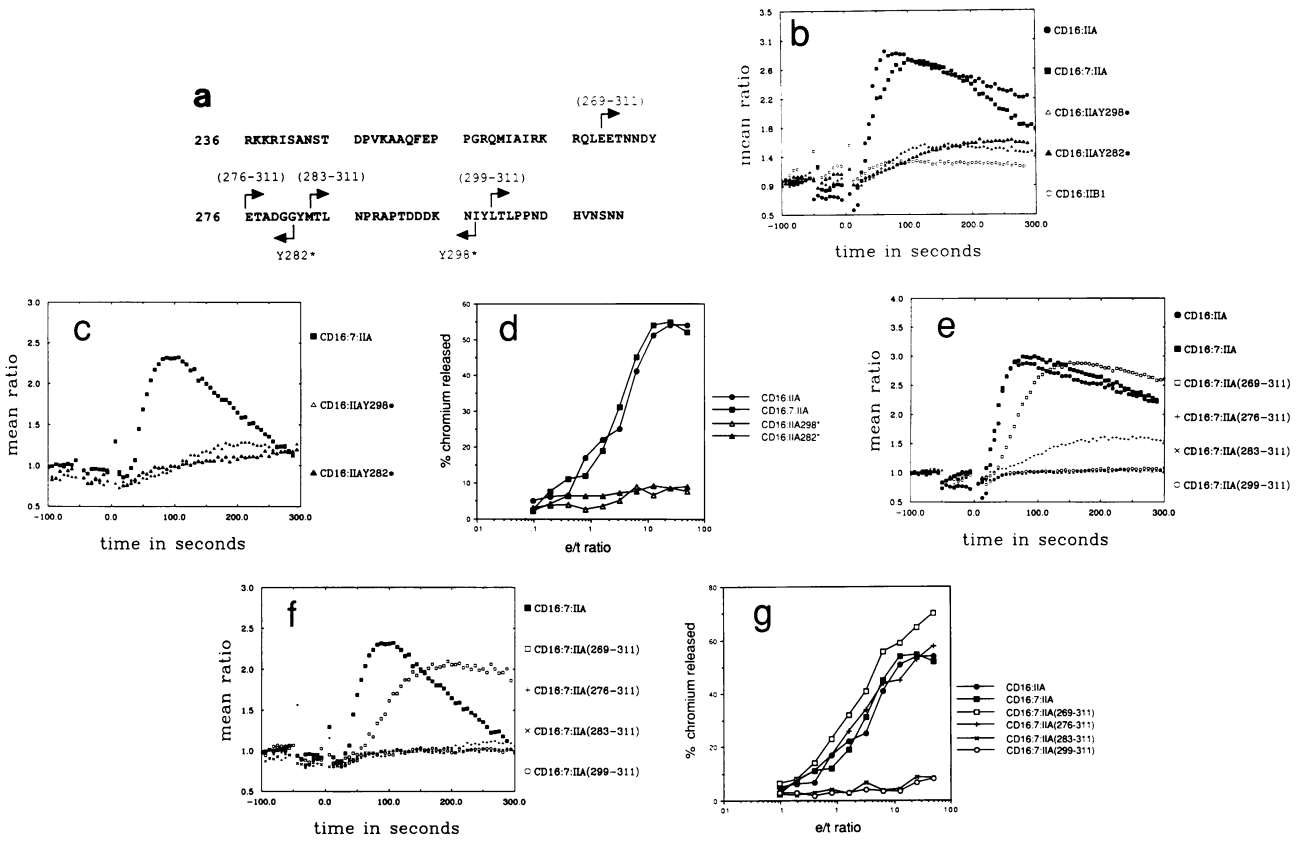

Fig. 7. Deletion mapping of residues in the FcγRIIA tail which are important for cytolysis. (**a**) Schematic diagram of the deletion constructs. Deletions of the amino-terminal portion of the extracellular domain (arrows above sequence) were constructed by addition of the FcγRII fragment to the transmembrane domain of CD7, which in turn was joined to the extracellular domain of CD16. (**b**, **c** and **d**) Calcium mobilization in TCR^- (b) and TCR^+ (c) variants of the Jurkat cell line and cytolysis (d) by carboxyl-terminal deletion variants of CD16:FcγRIIA. (**e**, **f** and **g**) Calcium mobilization in TCR^- (e) and TCR^+ (f) variants of the Jurkat cell line and cytolysis (g) by tripartite chimeras bearing progressively less of the amino terminus of the intracellular tail of CD16:FcγRIIA. The mean fluorescence intensities for CD16 expression by the cells in (d) and (g) were: CD16:FcγRIIA, 850; CD16:FcγRIIA Y298*, 695; CD16:FcγRIIA Y282*, 319; CD16:7:FcγRIIA, 419; CD16:7:FcγRIIA(269−311), 908; CD16:7:FcγRIIA(276−311), 799; CD16:7:FcγRIIA(283- 311), 490; and CD16:7:FcγRIIA(299−311), 333.

by 15 residues. To evaluate the potential role of the tyrosine residues, point mutations converting each tyrosine codon to a serine codon were introduced into the cytoplasmic domain of full length tripartite chimeras bearing the CD4 extracellular domain, CD7 transmembrane domain and FcγRIIA cytoplasmic tail. In each case, mutation of the tyrosine abolished the ability of the resulting chimeras to mobilize calcium in TCR^- T cells and monocytes, and also blocked their capacity to mediate HIV envelope-directed killing by either CTL or monocytes (Figure 8).

Discussion

In this report we establish that the FcγRIIA and IIC subtypes possess a cellular activation potential previously thought to be limited to members of the ζ family of trigger molecules. Although the sequence motifs characteristic of the ζ family are not found in the cytoplasmic domains of FcγRIIA and C, there are structural similarities between the two classes of activation domain, notably the requirement for two tyrosine residues for both calcium mobilization and cytolysis (Romeo *et al.*, 1992). The spacing between the tyrosines is substantially greater for FcγRIIA and C than for members of the ζ family (15 as opposed to 10 residues), and the characteristic acidic residues flanking the distal tyrosines in the ζ family are not found in FcγRIIA or C. In the ζ motif studied earlier (Romeo *et al.*, 1992), the presence of a leucine residue immediately preceding the amino-terminal tyrosine was found to be important for signalling, but a similar residue is not found in the same position for FcγRIIA or C. However, one similarity between the FcγRII family and the ζ family is the appearance of a leucine (or occasionally another aliphatic residue, among ζ family members) three residues carboxyl-terminal, resulting in a consensus of the form Y-X-X-L. There is not a great deal of specificity inherent in this pattern, however, and similar Y-X-X-M or Y-X-X-Aliphatic sequences are found among the tyrosine phosphorylation sites recognized by src homology 2 and 3 (SH2 and SH3) domains of tyrosine kinases and other proteins.

Although there is no evidence pointing to an evolutionary relationship between the ζ family proteins and the FcγRII subtypes, recent data support the idea that both classes of activation molecule function through, or engage, tyrosine kinases in the process of activation. In platelets (Huang *et al.*, 1992) and the monoblastoid cell line U937 (Liao *et al.*, 1992), crosslinking of FcγRII leads to *de novo* accumulation of tyrosine phosphate. Although the case cannot be made unambiguously, the present results make it likely that it is the FcγRIIA subtype which is responsible for the increased kinase activity in both examples. Similarly, Odin *et al.* (1991) have shown that in a myeloid cell line, crosslinking

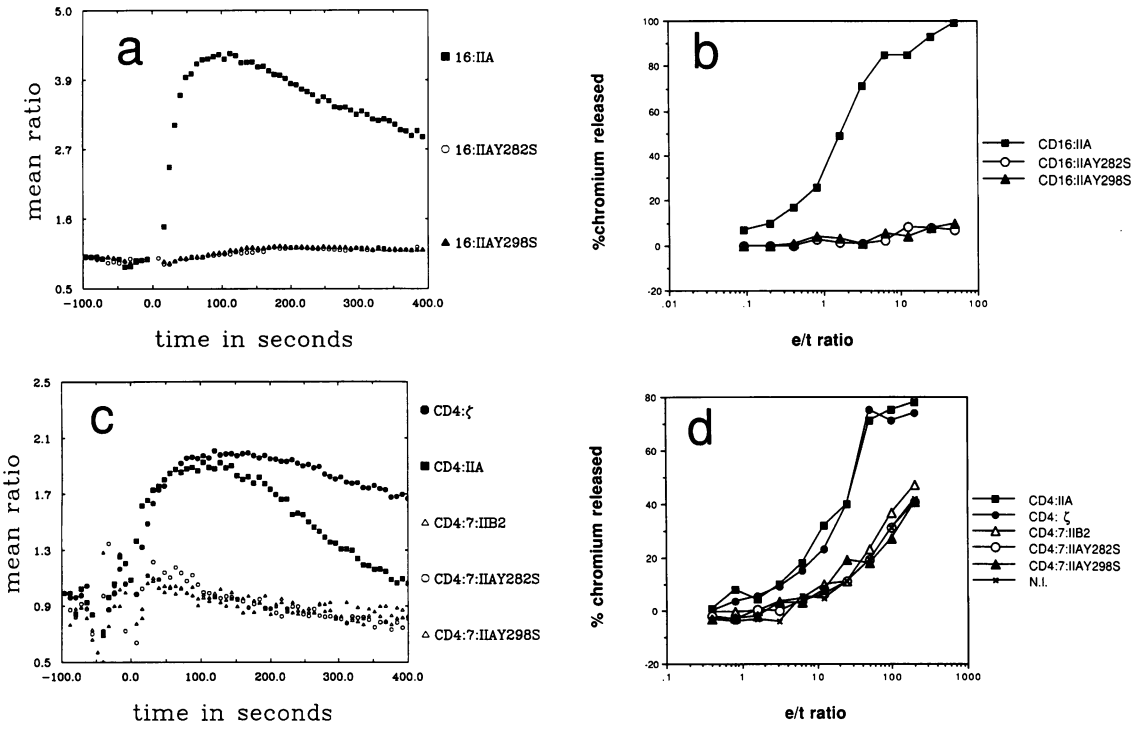

Fig. 8. Analysis of the importance of tyrosine residues within the FcγRIIA cytoplasmic domain. (**a**) Calcium mobilization analysis in the TCR$^-$ cell line JRT3T3.5. The response of cells bearing wild-type chimera, chimera bearing Tyr282 substituted with Ser, and chimera bearing Tyr298 substituted with Ser is shown. (**b**) Cytolysis in CTL directed by FcγRIIA, and the mutants Tyr282Ser and Tyr298Ser. (**c**) Calcium mobilization in monocytes by CD4 chimeras CD4:ζ, CD4:FcγRIIA, or tripartite chimeras bearing CD7 transmembrane sequences and either intact FcγRIIB2 intracellular domain, FcγRIIA mutant Tyr282Ser or FcγRIIA mutant Tyr298Ser. Calcium mobilization analysis was performed on cells gated for CD4 and scatter as described in the legend to Figure 5. (**d**) Cytolysis in monocytes expressing the chimeric molecules described in (c). N.I., not infected. Mean fluorescence intensities for CD16 expression by the cells in (b) were: CD16:FcγRIIA, 246; CD16:FcγRIIA Y298S, 314; and CD16:FcγRIIA Y282S, 304. Mean fluorescence intensities for CD4 expression by the cells in (d) were: CD4:FcγRIIA, 109; CD4:ζ, 162; CD4:7:FcγRIIA Y298S, 75; CD4:7:FcγRIIA Y282S, 120; CD4:7:FcγRIIB2, 84; and not infected, 26.

of the FcγRIIA subtype can mediate free calcium ion accumulation, an idea supported and extended by our work with chimeras. Thus both the ζ family trigger molecules and the FcγRIIA or C subtypes produce calcium transients and appear to (or are known to) increase tyrosine phosphorylation following a crosslinking stimulus. The finding that phospholipase $C\gamma_1$ is specifically phosphorylated following FcγRII clustering in U937 cells (Liao *et al.*, 1992) provides a reasonable candidate explanation for the similarities, suggesting that tyrosine phosphorylation precedes and results in inositol phosphate production and calcium mobilization through activation of PLC-γ_1. A similar phosphorylation of PLC-γ_1 has been observed following crosslinking of the high affinity IgE receptor (Park *et al.*, 1991). In U937 cells, tyrosine phosphorylation of PLC-γ1 can be induced by crosslinking either $Fc\gamma R_I$ or FcγRII (Liao *et al.*, 1992); the mechanism may not be similar, however, since the cytoplasmic domain of FcγRI shows no relatedness to that of FcγRII, and in particular contains no tyrosine residues.

Although activation of cytolysis is a central feature of cellular immunity, historically it has been more difficult to study than activation of cellular helper function. In large part this stems from the relative scarcity of cell lines which are amenable to transfection and which are also capable of mediating a functional cytolytic response. It may be that this is inevitable, and that loss of function is an unavoidable consequence of attempting to propagate cells with a high cytotoxic potential.

Whatever the explanation, the absence of appropriate cell lines is a substantial technological obstacle. In this work we have introduced two related strategies to study cytolytic pathways initiated by myeloid Fc receptors. One is the use of primary human T cells as surrogate effector cells, and the other is the use of vaccinia virus vectors to introduce receptors into primary human monocytes. Primary human cytolytic T lymphocytes represent a renewable population of effector cells with high target specificity and high cytolytic potential. Although not especially susceptible to transfection, they are easily infected with recombinant vaccinia viruses, and their cytolytic capacity does not appear to be impaired by infection. Moreover, as this study shows, they have the ability to respond to exogenous receptors whose natural distribution does not fall in their lineage.

More problematic is the analysis of cytolytic effector function in cell types other than CTL. For example, primary natural killer cells, although potently cytotoxic, are also substantially less specific than CTL; and primary monocytes offer the worst of both worlds, being both less cytolytic and less specific than CTL. However, it is possible by suitable choice of target cell and monocyte isolation conditions to make the study of monocyte cytolysis amenable to genetic methods of analysis. Like CTL, primary monocytes are easily infected with vaccinia viruses, and give good expression of cell surface proteins under the control of the vaccinia p7.5 mixed early/late promoter. Although the tendency of vaccinia to shut off host protein and RNA

synthesis must be counted a liability, there is reason to be optimistic that the scope and variety of subjects which are susceptible to study in monocytes will continue to expand.

Materials and methods

Preparation of chimeras

To prepare the chimeras, cDNA sequences corresponding to the transmembrane and cytoplasmic domains of FcγRI and the FcγRIIA, IIB1 and IIB2 isoforms were amplified using synthetic oligonucleotides primers either from existing clones (Stengelin *et al.*, 1988; Allen and Seed, 1989) or from a human tonsil cDNA library. The cDNA fragment corresponding to the FcRIIC cytoplasmic domain, which differs from the IIA isoform at one amino acid residue (L for P at residue 268; Stuart *et al.*, 1989) was generated by site directed mutagenesis by overlap PCR. The forward and reverse primers contained cleavage sites for the enzymes *Bam*HI and *Not*I respectively, indented six residues from the 5′ end. The *Not*I site was immediately followed by a stop anticodon, either CTA or TTA. All primers contained 18 or more residues complementary to the 5′ and 3′ ends of the desired fragments.

The PCR fragments were inserted into expression vectors which contained CD16 or CD4 extracellular domains terminating in a *Bam*HI site just proximal to the membrane spanning domain (Romeo and Seed, 1991; Romeo *et al.*, 1992). The identity of all isoforms was confirmed by dideoxy sequencing.

Immunoprecipitation

Approximately 10^7 JRT3.T3.5 (Weiss and Stobo, 1984) cells were infected for 1 h in serum-free IMDM medium with recombinant vaccinia at a multiplicity of infection (m.o.i.) of at least 10. Twelve hours after infection, the cells were harvested and surface labeled with 0.5 mCi ^{125}I per 10^7 cells using the lactoperoxidase/glucose oxidase method. The labeled cells were collected by centrifugation and lysed in 1% NP-40, 0.1% SDS, 0.15 M NaCl, 0.05 M Tris pH 8, 5 mM $MgCl_2$, 5 mM KCl, 0.2 M iodoacetamide and 1 mM PMSF. Nuclei were removed by centrifugation and CD16 fusion proteins were immunoprecipitated with antibody 3G8 and anti-mouse IgG agarose. Samples were electrophoresed through 10% polyacrylamide−SDS gels under reducing conditions.

Isolation of peripheral blood monocytes

Monocytes were isolated either from buffy coats or from fresh blood as described by Connor *et al.* (1990). Cells were centrifuged over Ficoll-Hypaque and the interface layer was collected. After three washes in IMDM, the cells were suspended in IMDM containing 20% fetal bovine serum at 10^7 cells per ml in 15 ml polypropylene tubes and were rotated at 20 r.p.m. (Lab-Quake, Tektator Inc.) about an axis parallel to the long axis of the tubes for 15−25 min at 4°C to induce monocyte aggregation. The aggregated cells were sedimented on ice at 1 *g* for 15 min, resuspended in 2 ml of medium and layered onto an equal volume of ice-cold fetal bovine serum. After sedimentation through the fetal bovine serum for 20 min at 4°C, the lower phase contained between 80 and 97% monocytes, the remainder being lymphocytes. Alternatively, cells from the Ficoll-Hypaque interface were washed three times in IMDM and plated at 5×10^6 cells per ml into 100 mm tissue culture dishes. Monocytes were allowed to adhere to the plastic for at least 2 h and were then detached by scraping for further analysis. The purity of monocytes was usually >90% as determined by staining with Leu-M3 antibody (Becton-Dickinson).

Calcium flux analysis

Cells of the Jurkat mutant subline JRT3.T3.5 (Weiss and Stobo, 1984) or peripheral blood monocytes prepared as described above were infected with recombinant vaccinia viruses for 1 h in serum-free IMDM at an m.o.i. of 10 and incubated for 3−9 h in IMDM, 10% FBS. Cells were collected by centrifugation and resuspended at 3×10^6/ml in complete medium containing 1 mM Indo-1 acetomethoxyester (Grynkiewicz *et al.*, 1985) (Molecular Probes) and incubated at 37°C for 45 min. The Indo-1 loaded cells were pelleted and resuspended at 1×10^6/ml in serum-free IMDM and stored at room temperature in the dark. Cells were analyzed for free Ca^{2+} by simultaneous measurement of the violet and blue fluorescence emission by flow cytometry (Rabinovitch *et al.*, 1986). To initiate calcium flux, either unconjugated 3G8 (anti-CD16) monoclonal antibody (at 1 μg/ml) was added to the cell suspension followed by 10 μg/ml of phycoerythrin (PE)-conjugated Fab'_2 goat anti-mouse IgG at time 0, or a PE-conjugated anti-CD4 antibody (Leu-3a, Becton Dickinson) was added, followed by unconjugated secondary antibody. Histograms of the violet/blue emission ratio were collected from the PE positive (infected) cell population, which typically represented 40−80% of the cells. The violet/blue emission ratio prior to the addition of antibody was used to establish the normalized initial ratio, set equal to unity.

Lymphocyte cytolysis assay

A $CD8^+$ $CD4^-$ HLA B44 restricted cytolytic line (WH3), kindly provided by J.Kurnick and F.Harris, was maintained in IMDM, 10% human serum with 100 U/ml of IL-2 and was periodically stimulated with irradiated (3000 rad) mononuclear cells having the HLA B44 haplotype. Cells were grown for at least 10 days following stimulation before use in cytotoxicity assays. The cells were infected with recombinant vaccinia at an m.o.i. of at least 10 for 1 h in serum-free medium, followed by incubation in complete medium for 3 h.

Cells were harvested by centrifugation and resuspened at a density of 1×10^7/ml. 100 μl were added to each well of a U-bottom microtiter plate containing 100 μl/well of complete medium. Cells were diluted in 2-fold serial steps. Two wells for each sample did not contain lymphocytes, to allow spontaneous chromium release and total chromium uptake to be measured. An aliquot of 10^6 target cells, either 3G8 10-2 (Shen *et al.*, 1989) or HeLa cells infected with vaccinia recombinant vPE16 (Earl *et al.*, 1990) was centrifuged and resuspended in 50 μl of sterile [^{51}Cr]sodium chromate (1 mCi/ml, DuPont) for 1 h at 37°C with intermittent mixing, then washed three times with PBS. 100 μl of labeled cells resuspended in medium at 10^5 cells/ml were added to each well. The microtiter plate was spun at 750 *g* for 1 min and incubated for 4 h at 37°C. At the end of the incubation period the cells in each well were resuspended by gentle pipetting, a sample was removed to determine the total counts incorporated and the microtiter plate was spun at 750 *g* for 1 min. 100 μl aliquots of supernatant were removed and counted in a gamma ray scintillation counter. The effector to target ratio was corrected for the percent of effector cells infected (usually >70%).

Monocyte cytolysis assay

Cytolysis assays using human peripheral blood monocytes as effector cells were carried out in the same fashion as for the CTL assays, with the following modifications: monocytes, cultured overnight in IMDM, 20% fetal bovine serum, were detached by scraping and infected with recombinant vaccinia at a multiplicity of infection of at least 10 for 1 h in serum free medium, followed by incubation for 4 h in Teflon beakers in IMDM, 20% fetal bovine serum. CV1 cells infected with vaccinia recombinant vPE16 (Earl *et al.*, 1990) were used as targets. The microtiter plate containing effector/target mixtures at 10 ratios from 50:1 to 0.09:1 plus control wells for determination of spontaneous and maximal ^{51}Cr release was incubated at 37°C for 16h.

In vitro mutagenesis

Carboxyl-terminal FcRIIA deletion mutants were constructed by PCR in the same fashion as for the full length constructs, converting the sequences encoding tyrosine at positions 282 and 298 into stop codons (TAA). The amino-terminal deletions were generated by amplifying fragments encoding successively less of the intracellular domain by PCR, using oligonucleotides which allowed the resulting fragments to be inserted between *Mlu*I and *Not*I restriction sites into a previously constructed expression plasmid encoding the CD16 extracellular domain fused to the CD7 transmembrane domain, the latter terminating in a *Mlu*I site at the junction between the transmembrane and the intracellular domain.

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References

Amigorena,A. *et al.* (1992) *Science*, **256**, 1808−1812.

Anderson,P., Caligiuri,M., O'Brien,C., Manley,T., Ritz,J. and Schlossman,S.F. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 2274−2278.

Balk,S. and Terhorst,C. (1989) *Immunol. Ser.*, **45**, 411−416.

Blank,U., Ra,C., Miller,L., White,K., Metzger,H. and Kinet,J.-P. (1989) *Nature*, **337**, 187−189.

Bonnerot,C., Amigorena,S., Choquet,D., Pavlovich,R., Choukroun,V. and Fridman,W.H. (1992) *EMBO J.*, **11**, 2747−2757.

Boyle,D.B. and Coupar,B.E.H. (1988) *Gene*, **65**, 123–128.
Connor,R.I., Shen,L. and Fanger,M.W. (1990) *J. Immunol.*, **145**, 1483–1489.
Engelhardt,W., Gorczytza,H., Butterweck,A., Monkemann,H. and Frey,J. (1991) *Eur. J. Immunol.*, **21**, 2227–2238.
Falkner,F.G. and Moss,B. (1988) *J. Virol.*, **62**, 1849–1854.
Fanger,M.W., Shen,L., Graziano,R.F. and Guyre,P.M. (1989) *Immunol. Today*, **10**, 92–99.
Fleit,H.B., Wright,S.D. and Unkeless,J.C. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 3275–3279.
Gardner,P. (1989) *Cell*, **59**, 15–20.
Graziano,R.F. and Fanger,M.W. (1987a) *J. Immunol.*, **138**, 945–950.
Graziano,R.F. and Fanger,M.W. (1987b) *J. Immunol.*, **139**, 3536–3541.
Grynkiewicz,G., Poenie,M. and Tsien,R.Y. (1985) *J. Biol. Chem.*, **260**, 3440–3450.
Hibbs,M.L., Selvaraj,P., Carpén,O., Springer,T.A., Kuster,H., Jouvin,M.-H.E. and Kinet,J.-P. (1989) *Science*, **246**, 1608–1611.
Huang,M.M., Indik,Z., Brass,L.F., Hoxie,J.A., Schreiber,A.D. and Brugge,J.S. (1992) *J. Biol. Chem.*, **267**, 5467–5473.
Hunziker,W. and Mellman,I. (1989) *J. Cell Biol.*, **109**, 3291–3302.
Indik,Z., Kelly,C., Chien,P., Levinson,A.I. and Schreiber,A.D. (1991) *J. Clin. Invest.*, **88**, 1766–1771.
Irving,B.A. and Weiss,A. (1991) *Cell*, **64**, 891–901.
Jin,Y.J., Clayton,L.K., Howard,F.D., Koyasu,S., Sieh,M., Steinbrich,R., Tarr,G.E. and Reinherz,E.L. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 3319–3323.
Keegan,A.D. and Paul,W.E. (1992) *Immunol. Today*, **13**, 63–68.
Kinet,J.-P. (1989) *Cell*, **57**, 351–354.
Kroczek,R.A., Gunter,K.C., Germain,R.N. and Shevach,E.M. (1986) *Nature*, **322**, 181–184.
Kurosaki,T. and Ravetch,J.V. (1989) *Nature*, **342**, 805–807.
Lanier,L.L., Yu,G. and Phillips,J.H. (1989) *Nature*, **342**, 803–805.
Letourneur,F. and Klausner,R.D. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 8905–8909.
Letourneur,F. and Klausner,R.D. (1992) *Science*, **255**, 79–82.
Liao,F., Shin,H.S. and Rhee,S.G. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 3659–3663.
Mellman,I. (1988) *Curr. Opin. Immunol.*, **1**, 16–25.
Meuer,S.C., Hussey,R.E., Fabbi,M., Fox,D., Acuto,O., Fitzgerald,K.A., Hodgdon,J.C., Protentis,J.P., Schlossman,S.F. and Reinherz,E.L. (1984) *Cell*, **36**, 897–906.
Miettinen,H.M., Rose,J.K. and Mellman,I. (1989) *Cell*, **58**, 317–327.
Miettinen,H.M., Matter,K., Hunziker,W., Rose,J.K. and Mellman,I. (1992) *J. Cell Biol.*, **116**, 875–888.
Moingeon,P., Lucich,J.L., McConkey,D.J., Letourneur,F., Malissen,B., Kochan,J., Chang,H.C., Rodewald,H.R. and Reinherz,E.L. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 1492–1496.
Odin,J.A., Edberg,J.C., Painter,C.J., Kimberly,R.P. and Unkeless,J.C. (1991) *Science*, **254**, 1785–1788.
Ostergaard,H.L., Kane,K.P., Mescher,M.F. and Clark,W.R. (1987) *Nature*, **330**, 71–72.
Park,D.J., Min,H.K. and Rhee,S.G. (1991) *J. Biol. Chem.*, **266**, 24237–24240.
Qiu,W.Q., de-Bruin,D., Brownstein,B.H., Pearse,R. and Ravetch,J.V. (1990) *Science*, **248**, 732–735.
Ra,C., Jouvin,M.-H.E. and Kinet,J.-P. (1989) *J. Biol. Chem.*, **264**, 15323–15327.
Rabinovitch,P.S., June,C.H., Grossmann,A. and Ledbetter,J.A. (1986) *J. Immunol.*, **137**, 952–961.
Ravetch,J.V. and Kinet,J.P. (1991) *Annu. Rev. Immunol.*, **9**, 457–492.
Reth,M. (1989) *Nature*, **338**, 383–384.
Romeo,C. and Seed,B. (1991) *Cell*, **64**, 1037–1046.
Romeo,C., Amiot,M. and Seed,B. (1992) *Cell*, **68**, 889–897.
Samelson,L.E., Harford,J.B. and Klausner,R.D. (1985) *Cell*, **43**, 223–231.
Schmitt-Verhulst,A.M., Guimezanes,A., Boyer,C., Poenie,M., Tsien,R., Buferne,M., Hua,C. and Leserman,L. (1987) *Nature*, **325**, 628–631.
Sefton,B.M. and Campbell,M.A. (1991) *Annu. Rev. Cell Biol.*, **7**, 257–274.
Shen,L., Graziano,R.F. and Fanger,M.W. (1989) *Mol. Immunol.*, **26**, 959–969.
Stengelin,S., Stamenkovic,I. and Seed,B. (1988) *EMBO J.*, **7**, 1053–1059.
Stuart,S.G., Simister,N.E., Clarkson,S.B., Kacinski,B.M., Shapiro,M. and Mellman,I. (1989) *EMBO J.*, **8**, 3657–3666.
Trenn,G., Takayama,H. and Sitkovsky,M.V. (1987) *Nature*, **330**, 72–74.
Trenn,G., Taffs,R., Hohman,R., Kincaid,R., Shevach,E.M. and Sitkovsky,M. (1989) *J. Immunol.*, **142**, 3796–3802.
Unkeless,J.C., Scigliano,E. and Freedman,V.H. (1988) *Annu. Rev. Immunol.*, **6**, 251–281.
Wegener,A.-M.K., Letourneur,F., Hoeveler,A., Brocker,T., Luton,F. and Malissen,B. (1992) *Cell*, **68**, 83–95.
Weiss,A. and Stobo,J.D. (1984) *J. Exp. Med.*, **160**, 1284–1299.
Weissman,A.M., Baniyash,M., Hou,D., Samelson,L.E., Burgess,W.H. and Klausner,R.D. (1988) *Science*, **239**, 1018–1020.
Yeh,E.T., Reiser,H., Daley,J. and Rock,K.L. (1987) *J. Immunol.*, **138**, 91–97.
Yokoyama,W.M. and Shevach,E.M. (1989) *Year Immunol.*, **4**, 110–146.
Young,J.D., Clark,W.R., Liu,C.C. and Cohn,Z.A. (1987) *J. Exp. Med.*, **166**, 1894–1899.

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