Signaling through CD38 induces NK cell activation

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Abstract

Human CD38 is a signal transduction molecule, and, concurrently, an ectoenzyme catalyzing the synthesis and degradation of cyclic ADP-ribose (cADPR), a potent Ca²⁺ mobilizer. One facet of CD38 that has not yet been addressed is its role in NK cells. To this end, the events triggered by CD38 ligation with agonistic mAb were analyzed on freshly purified human NK cells. Ligation was followed by (i) a significant rise in the intracellular level of Ca²⁺, (ii) increased expression of HLA class II and CD25, and (iii) tyrosine phosphorylation of discrete cytoplasmic substrates. The phosphorylation cascade involved CD3-ζ and FcεRIγ chains, ζ-associated protein (ZAP)-70 and the proto-oncogene product c-Cbl. NK effector functions were then analyzed: CD38 signaling was able (iv) to induce release of IFN-γ and, more prominently, of granulocyte macrophage colony stimulating factor, as assessed by measuring both mRNA and protein products; and, lastly, (v) to induce cytolytic effector functions on target cells after IL-2 activation, as shown both by cytotoxicity assays and ultrastructural changes. The tyrosine-phosphorylated substrates and all the effects mediated by CD38 were similar to those observed following triggering via CD16 (FcγRIIIA); moreover, Ca²⁺ mobilization via CD38 no longer operated in NK-derived cell lines lacking CD16. These results suggest that the activation signals transduced by CD38 in NK cells elicit relevant cellular events. The effects are similar to those elicited via CD16 and possibly rely on common signaling pathways.

Introduction

CD38, initially described as an activation antigen, attracted widespread interest when it was attributed the role of ADP-ribosyl cyclase and cyclic ADP-ribose (cADPR) hydrolase, two enzymatic activities involved in the conversion of nicotinamide adenine dinucleotide (NAD⁺) first to cADPR and then to ADPR (1,2). Consequently, CD38 has been counted among the members of the ectoenzyme family, a growing number of molecules which includes >3% of all surface receptors (3).

Some ectoenzymes are paradoxical in that they also mediate other apparently unrelated functions, mostly dealing with the regulation of cell–cell contacts and transmembrane signaling. CD38 has thus moved from consideration as an orphan receptor to consideration as a pleiotropic molecule involved in adhesion (4,5) and signaling, in spite of its apparently unsuitable cytoplasmic domain. The nature of CD38 as a transducing channel was confirmed by the identification of a counter-receptor (5), identified as CD31 (6), that, following interaction with CD38, triggers the same events seen in the surface, cytoplasmic and nuclear districts when specific mimotopic mAb are used (6).

Several laboratories have undertaken the analysis of the signals implemented after CD38 ligation. The results so far...
obtained indicate that CD38 engagement in T cells leads to activation of the Raf-1/MAP kinase and the CD3–ζ-associ-
ated protein (ZAP)-70/phospholipase C (PLC)-γ1 pathways (7), Ca2+ mobilization and induction of apoptosis (8). These
events are dependent on the presence of a functional TCR–
CD3 complex (8,9). CD38 ligation in B lymphocytes inhibits
proliferation and induces apoptosis of B precursors (10); signal transduction in these cells involves tyrosine phospho-
rylation of Syk, PLC-γ (11), Tec (12), c-Cbl (13) and of the
p85 subunit of phosphatidylinositol 3-kinase (PI 3-K) (11). Mature B cells yield contrasting results: CD38 signaling is
followed by proliferation and prevention of apoptosis (14,15); several events are dependent, at least in murine models, on
the association of CD38 with BCR (16). Ligation of CD38 in
myeloid cells enhances superoxide generation induced by
chemotactic peptide (17), and induces tyrosine phosphoryla-
tion of c-Cbl (18) and its association with the p85 subunit of
PI 3-K (19). Thus, CD38 relies upon different complexes for
its signaling purposes, acting as a molecular parasite of TCR
in T lymphocytes (8), BCR in B lymphocytes (16) and HLA
class II in monocyteid cells (20).

This paper originates from the need to complete our under-
standing of the signaling properties of CD38 in human NK
cells, a population which has only relatively recently begun
to be explored (21–23). Interest in NK cells also stems from
the fact that they lack the TCR and BCR complexes, and are
thus likely to offer insights into the signaling mechanism(s)
driven by CD38. The results obtained indicate that CD38
delivers activation signals in NK cells through a complex
molecular machinery largely shared by CD16.

Methods

Antibodies

CD38 stimulation was performed with the agonistic anti-CD38
mAb IB4 (IgG2a) (24), used in the form of an F(ab′)2 fragment
to avoid binding through FcRfIIIA (CD16), unless otherwise
specified. Other mAb used were IB6 (non-agonistic anti-
CD38, IgG2b) (25), CB16 (anti-CD16, IgG2a), Moon-1 (anti-
CD31, IgG1) (5), anti-CD28 (IgG2a) and JAS (anti-gp120,
IgG2a irrelevant isotype-matched control), all produced in
our laboratory. Anti-CD3, anti-CD20 and anti-HLA class II
mAb used for NK cell purification were all locally produced.
Affinity-purified goat antibody to mouse IgG (whole molecule)
(GuMlgG; Cappel, Organon Teknika, Durham, NC) was used
as a cross-linker in the form of an F(ab′)2 preparation. 1G2
mAb coupled to agarose beads from Oncogene (Calbiochem,
Cambridge, MA) was used for anti-phosphotyrosine (pTyr)
immunoprecipitation. Other antibodies used for Western blot-
ing and immunoprecipitation were: anti-pTyr mAb PY20
(Transduction Laboratories, Lexington, KY); 1D4.1 (anti-CD3–
ζ mAb) (26) and 448 (anti-CD3–ζ serum), the kind gift from
Dr B. Alarcoñ (Centro de Investigaciones Biologicas, Madrid,
Spain); anti-ZAP-70 rabbit antiserum, generously made available by
Dr J. M. Rojo (Centro de Investigaciones Biologicas, Madrid,
Spain); and anti-c-Cbl (Santa Cruz Biotechnology, Santa Cruz,
CA). The anti-FceRI mAb C36 rabbit polyclonal antiserum was
obtained by immunizing New Zealand White rabbits with the
synthetic peptide CGVpYTGLSTRNQETpYETLKJHEKRR-
ASV conjugated to soluble keyhole limpet hemocyanin (Sigma,
St Louis, MO). The immunogen was a dually phosphorylated
peptide corresponding to the immunoreceptor tyrosine-activa-
tion motif (ITAM) of the FceRIγ. The peptide was chemically
synthesized with an additional N-terminal Cys for coupling to
affinity matrices, and with an additional C-terminal sequence,
Arg–Arg–Ala–Ser–Val (RRASV), for quantitative measurement
of the coupling efficiency of the peptide. Horseradish peroxid-
ase (HRP)-conjugated goat anti-rabbit IgG was from Promega
(Madison, WI).

NK cell isolation and NK cell lines

NK cells were purified from peripheral blood mononuclear
cells (PBMC) by negative selection with Dynabeads magnetic
particles (Dynal, Oslo, Norway) conjugated with sheep anti-
mouse IgG. Briefly, PBMC obtained from healthy donors by
centrifugation on density gradient were deprived of monocytes
by a plastic adherence step and successively incubated for
1 h at 4°C in the presence of anti-CD3, anti-CD20 and anti-
HLA class II mAb (5 μg/ml/5×106 cells). After three washings,
cells were incubated for 1 h at 4°C with immunobeads accord-
ing to the manufacturer’s instructions. The preparations
obtained were ≥97% CD16+, CD3+, HLA class II+ and CD14+,
as assessed by cytofluorimetric analysis. The continuous
human NK lines YT (27) and NKL (28), both CD38+CD16−,
were used for comparative analyses. Lack of CD16 expression
was further confirmed by PCR analysis of the specific
mRNA product.

Measurement of intracellular Ca2+

Changes in intracellular Ca2+ concentrations ([Ca2+]i) were
monitored by flow cytometry after loading cells with the
Ca2+-sensitive fluorescent dye Fluo 3-AM (Molecular Probes,
Eugene, OR), as previously described (29,30). Briefly, either
freshly isolated peripheral blood NK cells or YT and NKL
cells were washed twice in HBSS (pH 7.0) with 5% FCS,
resuspended in the same medium at a concentration of 2×106
cells/ml and incubated for 30 min at 37°C with 5 mM Fluo 3-
AM in the presence of 0.01% Pluronnic F127 (Sigma). Cells
were then washed twice, incubated for 10 min at room
temperature with the primary mAb at different concentrations
(1–5 μg/ml, depending on the mAb), washed again and
analyzed continuously at 37°C on a FACSsort flow cytometer
(Becton Dickinson, Milan, Italy) with Lysys II software. Stimula-
tion was induced by the addition of 20 μg/ml of GuMlgG
mAb. Cross-linking was performed, as the application of
soluble antibodies alone has little effect on [Ca2+]i, according
to observations reported by other authors (30,31). Standard
controls included incubations with an irrelevant isotype-
matched mAb, with GuMlgG mAb alone, with the non-agonis-
tic anti-CD38 mAb IB6 and with the ionophore A23187
(Molecular Probes). Cells were gated by size and side scatter
to eliminate both debris and dead cells from analysis. Dynamic
changes in [Ca2+]i were monitored by continuously plotting
the shift in the Fluo 3-AM fluorescence over a 540 s time-
course.

Modulation of surface molecules

Purified NK cells (2×106/ml) were cultured at 37°C for the
indicated time intervals (see figures) in RPMI 1640 medium
with 5% FCS in the presence of soluble IB4 F(ab’)2 mAb, anti-CD16 mAb, an irrelevant IgG2a mAb or a combination of IB4 plus anti-CD16 mAb. After incubations, cells were washed and resuspended in PBS containing 0.2% BSA and 1% NaN₃, and incubated with FITC- or phycoerythrin (PE)-conjugated anti-HLA class II, anti-CD25 or anti-CD69 mAb (Becton Dickinson) for 1 h at 4°C. The analysis was performed on a FACSort (Becton Dickinson). Excitation was from an argon laser at 488 nm. Background antibody binding was estimated by isotype-matched negative control mAbs. Acquired data were analyzed with Lysys II software (Becton Dickinson).

**Phosphorylation experiments**

Cells were incubated for 10 min on ice with an F(ab’)2 fraction of the IB4 mAb, anti-CD16 mAb or an F(ab’)2 fraction of an isotype-matched, unreactive mAb (anti-gp120) for the not stimulated (NS) condition. Each mAb was used at a concentration of 10 µg/10⁶ cells. The unbound mAb was eliminated by washing with cold IMDM and then the cells were incubated (10 min on ice) with an F(ab’)2 preparation of a GoMlgG. The cells were subsequently reacted with the relevant mAb at 37°C for 4 min, after which lysis was performed for 20 min on ice with 1% NP-40 lysis buffer (20 mM HEPES, pH 7.6, 150 mM NaCl, 50 mM NaF, 1 mM Na₂VO₄, 1mM EGTA, 50 mM phenylarsine oxide, 10 mM iodoacetamide, 1 mM PMSF, and 2 µg/ml of antipain, chymostatin, leupeptin and peptatin), as previously reported (7). After removal of nuclei by centrifugation, an aliquot of the lysates was diluted in Laemmli sample buffer, boiled for 5 min and stored at -80°C prior to running on SDS–PAGE. The remainder was used for immunoprecipitation incubating with the antibody of interest and recovering the immune complexes by means of recombinant Protein A–Sepharose beads (Repligen, Cambridge, MA). After washing, the beads were boiled in either reducing or non-reducing sample buffer (as specified in the figure legends) for 5 min and the elutes run on SDS–PAGE. The gel was then transferred onto a PVDF membrane with a semi-dry transfer apparatus. The membrane was washed and developed again with ECL reagents containing 20% methanol and 0.035% SDS at 0.8 mA/cm². The analysis was performed on a FACSsort (Becton Dickinson). Excitation was from an argon laser at 488 nm. Background antibody binding was estimated by isotype-matched negative control mAbs. Acquired data were analyzed with Lysys II software (Becton Dickinson).

**Cytokine release**

NK cells were resuspended at 2×10⁶/ml in RPMI 1640 medium supplemented with 5% FCS and antibiotics, and cultured in the presence of anti-CD16 and IB4 mAb at predetermined optimal concentrations (20 µg/ml) for 24, 36 and 48 h respectively. Total cellular RNA was extracted according to the guanidium isothiocyanate method (32). RNA (1 µg in a 20 µl reaction volume) was transcribed using Moloney murine leukemia virus reverse transcriptase and PCR amplification was conducted starting from as low as 1 ng of the original RNA. Cytokine-specific primer pairs were synthesized according to published sequences (DNA synthesizer; Applied Biosystems, Foster City, CA). PCR was performed in a 9600 Perkin-Elmer (Foster City, CA) thermal cycler, as previously described (33). The reaction product was visualized by electrophoresis using 10 µl of the reaction mixture. Culture supernatants were collected and used to measure IFN-γ and GM-CSF cytokine secretion by ELISA tests (Quantikine; R & D System, Minneapolis, MN), following the manufacturer’s instructions.

**Cytotoxicity assays**

The ability of the agonistic IB4 mAb to trigger the cytolytic activity of NK cells was evaluated in a conventional ⁵¹Cr-release assay. The murine mastocytoma cell line P815 was used as target, and labeled for 1 h with ⁵¹Cr (100 µCi/10⁶ cells), washed twice with medium and plated at 5×10³ cells/well in 96-well U-bottom plates. In the redirected killing assay, effector cells were activated with IL-2 (100 U/ml for 5 days), plated in triplicate at various E:T in the presence of anti-CD16, anti-CD38 and a control reactive isotype-matched mAb. Labeled target cells were added to each well and 100 µl of supernatant was collected after 4-h incubation at 37°C and analyzed in a γ-counter. The percent specific lysis was calculated as: ([experimental release – spontaneous release]/(maximum release – spontaneous release))×100.

**Electron microscopy**

Activated NK cells were treated with anti-CD38, anti-CD16 or an irrelevant reactive isotype-matched mAb for 20 min at 4°C, and subsequently added to P815 target cells (E:T = 10:1) and incubated at 37°C. The ultrastructural analysis was performed after 10 min, 20 min and 2 h. Cells were fixed with 2.5% glutaraldehyde (Polysciences, Warrington, PA) in Tris–glycine buffer containing 20% methanol and 0.035% SDS at 0.8 µm/cm². To ensure proper recovery of all migrated proteins, transfer efficiency was checked by Ponceau red stain. The membrane was blocked in 1% BSA, washed in TBS-T (10 mM Tris, pH 7.4, 100 mM NaCl, 0.1% Tween 20) and reacted with HRP-conjugated PY20 anti-pTyr mAb for 2 h. The filter was then washed again and developed using ECL reagents (Amersham, Little Chalfont, UK). The membrane was subsequently stripped by thorough washing in a buffer containing 150 mM NaCl and 10 mM Tris–HCl (pH 2.2), blocked again in 2.5–7% non-fat dry milk and incubated with the primary antibody of interest diluted in 1% milk for 1 h. After washing, HRP-conjugated goat anti-rabbit IgG was added and the membrane was washed and developed again with ECL reagents.

**Results**

**Ca²⁺ mobilization**

Previous reports showed that the interaction of CD16 with ligands (either immune complexes or anti-CD16 antibodies) induces a rapid rise in [Ca²⁺], (31). We investigated whether CD38 binding by agonistic mAb could mediate similar effects in purified NK cells (Fig. 1A, panel 1). The rise in [Ca²⁺], after CD38 ligation with the agonistic mAb IB4 became apparent only after cross-linking with GoMlgG. The profile is characterized by an ascending slope for the first 2 min, followed by a plateau which is maintained for the rest of the reading time (9 min). CD16 signaling also needed cross-linking of the specific mAb in order to yield results recordable with the
system adopted. The Ca\textsuperscript{2+} profile (Fig. 1A, panel 2) was slightly different from that observed with CD38: a faster rise with an earlier (~1.5 min) and higher peak was followed by a rapid decline, reaching the basal level at the end of the reading time. Thus, the Ca\textsuperscript{2+} fluxes recorded followed different kinetics, i.e. marked by lower but constant levels in the CD38 pathway and higher, although transient, spikes obtained after CD16 ligation. Triggering of the NK cells via CD31 (Fig. 1A, panel 3) also induced some Ca\textsuperscript{2+} movements, although of lower intensity, as previously reported in other cellular models.
Fig. 2. Modulation of HLA class II, CD25 and CD69 surface expression following CD38 and CD16 ligation. One-parameter flow cytometric analysis of HLA class II, CD25 and CD69 expression in basal conditions (grey profiles) and following treatment for either 36 h (HLA class II and CD25) or 4 h (CD69) with anti-CD38, anti-CD16 or the combination of the two mAb (white profiles). x-axis = fluorescence intensity/cells; y-axis = number of cells registered/channel. Number of cells tested = 5000. Results refer to a representative experiment performed in triplicate.

(5). None of the mAb induced Ca^{2+} mobilization when not cross-linked with GoMlgG, suggesting that engagement of more than two receptor molecules is required. Similar experiments performed using IB6 (a non-agonistic anti-CD38 mAb) (Fig. 1A, panel 4), or with an isotype-matched irrelevant IgG2a mAb (Fig. 1A, panel 5) or with GoMlgG alone (not shown), did not yield any recordable effect. As a further positive control, NK cells were treated with the ionophore A23187 (Fig. 1A, panel 6).

To investigate the potential interconnection between the CD38 and CD16 transduction pathways involved in Ca^{2+} release, the same experiments were performed on YT (27) (Fig. 1B, panel 1) and NKL (28) (Fig. 1B, panel 2) cell lines, which are reported to display an NK phenotype, but lack CD16 expression, although they are CD38\+. As shown in Fig. 1(B), ligation of CD38 on these cells did not give rise to any Ca^{2+} movement. Stimulation with an irrelevant IgG2a mAb and via CD28 were included as negative and positive controls respectively (not shown).

Modulation of HLA class II, CD25 and CD69 surface expression

To further study the activation signals driven by CD38, we examined a selected panel of surface receptors. As shown in Fig. 2, signaling via CD38 as well as via CD16 is followed by an increased epitope density of HLA class II. The combination of these two mAb did not show additive effects. A similar up-modulation was observed for CD25, an effect previously reported to be induced via CD16 (34); in this instance, simultaneous ligation of CD16 and CD38 was followed by enhanced expression. CD69, an early activation molecule, was not significantly influenced by CD38 nor by CD16 signaling alone, while combination of the two mAb induced a modest shift in the CD69 fluorescence. No modulation of any of these receptors was observed after incubation of the NK cells with an irrelevant isotype-matched mAb (not shown).

Phosphorylation of CD3-ζ, FceRγ/γ, ZAP-70 and c-Cbl

After analyzing early events triggered either via CD38 or CD16 (i.e. Ca^{2+} mobilization, expression of CD69), the transduction...
pathways activated by these molecules were then defined by Western blot analysis of the proteins tyrosine phosphorylated after short-term stimulation at 37°C. Anti-pTyr Western blot on NK immunoprecipitates enriched with tyrosine-phosphorylated proteins clearly demonstrated a differential phosphorylation of discrete cytoplasmic substrates in cells incubated with IB4 or control anti-CD16 mAb (Fig. 3A). Similar results were obtained using the F(ab')2 preparation of the IB4 mAb. The most relevant bands featuring increased intensity compared to the basal condition were at 120, 70–75 and 23 kDa (as indicated by arrows); their mol. wt are compatible with the proto-oncogene product c-Cbl, ZAP-70 and the phosphorylated form of monomeric CD3-ζ respectively. All these molecules are reported to be recruited following CD16 triggering (35–39). Figure 3B reports the results obtained by increasing exposure time of the lower part of the membrane shown in Fig. 3(A); signaling via CD38 (as well as via CD16) is followed by a marked phosphorylation of a protein migrating as a doublet at 21–23 kDa, as indicated by arrows. The identity of these bands as phosphorylated forms of monomeric ζ chains was confirmed by stripping the same membrane and reacting it with an anti-ζ serum (Fig. 3E, left). This specific antibody recognized the same bands highlighted by the anti-pTyr mAb in Fig. 3(B); furthermore, analysis on whole lysates with the same antiserum displayed a relative abundance of unphosphorylated ζ species of lower mol. wt (Fig. 3E, right). The finding of unphosphorylated ζ bands detected in almost identical fractions in all the conditions considered (Fig. 3E, right) confirmed that the same amount of total proteins was present at each experimental point. The upper part of the membrane shown in Fig. 3(A) was then reprobed with an anti-c-Cbl mAb and the results demonstrated that the 120 kDa
Fig. 4. Western blot analysis of CD3-ζ phosphorylation upon CD38 ligation on purified NK cells. (A) Whole lysates probed with anti-pTyr; the bracket indicates phosphorylated substrates whose mol. wt is compatible with ζ-ζ species. (B) Anti-CD3-ζ immunoprecipitates probed first with anti-pTyr (left half) and, following membrane stripping, with anti CD3-ζ serum (right half). Gels were run under non-reducing conditions. Results refer to a representative experiment performed in duplicate. NS, not stimulated.

To further document CD3-ζ tyrosine phosphorylation, a second set of experiments was designed where ζ chain immunoprecipitation was performed in order to obtain a part of the blot enriched with this substrate. Additionally, samples were run under non-reducing conditions to visualize the γ chain as part of ζ-ζ homodimers or, possibly, ζ-γ heterodimers, as previously described in both T and NK cells (38). Whole lysates from NK cells stimulated via CD38 or CD16 and probed with anti-pTyr mAb highlighted the phosphorylation of multiple proteins whose migration is compatible with ζ-ζ homodimers, as indicated by the bracket in Fig. 4(A); this is in line with the characteristic migratory pattern of the tyrosine-phosphorylated species of CD3-ζ, reported as multiple bands resulting from the combination of hyperphosphorylation and mult ubiquitination (36,40). On the contrary, the phosphorylated ζ-ζ forms appeared as a single band of ~46 kDa on the CD3-ζ immunoprecipitate (Fig. 4B, left). This finding is probably due to preferential binding by the 1D4.1 mAb used for immunoprecipitation to selected pζ-pζ forms. Another doublet of lower mol. wt was visible on the whole-cell lysate in Fig. 4(A): its ~36 kDa electrophoretic mobility is compatible with phosphorylated ζ-γ heterodimers. Reprobing the anti-CD3-ζ immunoprecipitates with an anti-ζ serum (Fig. 4B, right) confirmed the identity of the phosphorylated bands observed in the left half of Fig. 4(B) as pζ-pζ forms. Excess unphosphorylated ζ-ζ homodimers at ~32–34 kDa and other ζ-containing species at ~25 kDa (likely corresponding to ζ-γ heterodimers) were also observed (Fig. 4B, right).

To verify the possible involvement of FcεRI in CD38 signaling, the same lysates deprived of CD3-ζ in the experiments described in Fig. 4 underwent a second immunoprecipitation with an anti-γ chain serum. The results, shown in Fig. 5(A), indicate the presence of a tyrosine-phosphorylated protein of ~25 kDa with a migratory pattern compatible with γ-γ homodimers (36), visualized by running the samples under non-reducing conditions. Subsequent recognition of the band by an anti-γchain serum confirmed that the substrate engaged by CD38 ligation is indeed FcεRI (Fig. 5B). Similar results were obtained after CD16 signaling. Longer exposure of the bottom part of the filter (Fig. 5C) led to the identification of unphosphorylated γ-γ migrating at ~14 kDa. FcεRI phosphorylation was less prominent than that of CD3-ζ, a finding which is probably due to preferential physical associations of the homodimeric ζ-ζ species with CD16, as already reported (41), or with CD38. Indeed, CD38 and CD16 molecules do display marked physical lateral associations on the membrane of NK cells (42).

Cytokine release: mRNA expression and production of IFN-γ, GM-CSF and tumor necrosis factor (TNF)-α

CD16 is reported to mediate transcription and release of IFN-γ and GM-CSF (34,43). We performed comparative tests after
CD38 ligation. Freshly purified NK cells cultured for 24, 36 or 48 h in the presence of F(ab')2 preparations of the IB4 mAb were characterized by a consistent accumulation of mRNA for IFN-γ and GM-CSF, whereas expression of mRNA for TNF-α was only slightly increased (Fig. 6). The initial increments of IFN-γ mRNA levels over the basal levels observed after 24 and 36 h became significantly marked at 48 h (Fig. 6, top panel). Such effects were faster and higher in amplitude upon ligation of CD16. Further, the simultaneous addition of mAb specific for CD38 and CD16 was not apparently followed by significant additive or synergistic effects. GM-CSF mRNA was apparent also in basal conditions (Fig. 6, second panel). However, CD38 ligation was paralleled by increased levels of the specific transcripts, quantitatively similar to those elicited by anti-CD16 mAb. A possible additive effect was observed for GM-CSF, although the qualitative nature of the PCR assay and the high levels of transcript obtained with the two mAb separately make comparison undependable. The accumulation of TNF-α mRNA after exposure to the IB4 anti-CD38 mAb was less significant; the same was true for anti-CD16 mAb, with no synergies between CD38 and CD16 (Fig. 6, third panel). The expression of β-actin mRNA used as control was similar in all samples (Fig. 6, bottom panel).

Cytokine induction was also quantitatively evaluated by assaying the protein product release (Fig. 7). Time-course experiments in NK cells cultured in the presence of either anti-CD38 or anti-CD16 mAb indicate that the levels of IFN-γ secreted become significant after 36 h (not shown). Thus,
CD38 signaling in NK cells

Fig. 6. Kinetics and profile of cytokine mRNA expression in purified NK cells cultured in the presence of anti-CD38, anti-CD16 or both. After 24, 36 and 48 h, mRNA was extracted and reverse transcribed. The cDNA obtained was used to assay for the presence of specific cytokine mRNA by PCR. Results refer to a representative experiment performed in triplicate.

Fig. 7. ELISA analysis of cytokine secretion in purified NK cells cultured in the presence of anti-CD38, anti-CD16 or both. After 36 h, culture supernatants were collected and assayed for IFN-γ (A) and GM-CSF (B) contents. Results are expressed as mean ± SD of three experiments performed.

this time-point was selected for these experiments. CD38 engagement induced a modest increase in IFN-γ secretion as compared to basal levels (P < 0.01); on the contrary, CD16 signaling was followed by a marked response (Fig. 7A). Moreover, IFN-γ release was not significantly increased when NK cells were simultaneously incubated with anti-CD38 and anti-CD16 mAb (Fig. 7A). Additional effects were seen in the regulation of GM-CSF secretion (Fig. 7B). Both CD38 and CD16, when independently engaged, give rise to increased levels of the cytokine (P < 0.01 for both stimulations as compared with control). The simultaneous signaling was paralleled by levels of GM-CSF secreted in the supernatant higher than those obtained when signals were given independently.

Cytotoxicity

The involvement of CD38 in the cytolytic function of NK cells was evaluated in a redirected cytotoxicity assay against target P815 cells (Fig. 8). The assay was performed using NK cells treated with IL-2, since the lysis observed after ligating CD38 on resting NK was negligible. The results obtained indicate that CD38 signaling in IL-2-treated NK cells is followed by a triggering of cytolytic programs and the amount of specific lysis parallels E:T. CD16 signaling included as positive control also yielded a relevant lysis of target cells; in contrast, exposure of the same cells to an irrelevant isotype-matched binding mAb did not give rise to significant effects. The simultaneous addition of anti-CD16 and anti-CD38 mAb in the assay did not display additive or synergistic effects (not shown).

Ultrastructural features of NK cells activated via CD38

The redirected cytotoxicity assay against target P815 cells was also used to study the ultrastructural changes occurring after CD38 triggering (Fig. 9). Figure 9(A) shows the ultrastructure of the target P815 cells. The mast cell lineage of the P815 cells is indicated by the presence of granules containing characteristic ‘scrolls’. Figure 9(B) shows the structure of the IL-2-activated NK cells: granules containing abundant electron-dense matrix and tubular structures are present, as shown to better advantage in Fig. 9(C). Morphological equivalent of activation are induced when NK cells were treated with IB4 (anti-CD38) mAb. Figure 9(D) shows an effector to target contact: the activated NK cells lack electron-dense granules, which are replaced by vacuoles that are empty or contain scanty tubular structures. Simultaneously, apoptotic changes of target cells became apparent, indicating the ongoing cytotoxicity. Similar effects were observed following incubation with anti-CD16 mAb (not shown), as previously reported (44). P815 cells incubated with NK cells pre-treated...
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Fig. 9. (A) Ultrastructure of the P815 murine mastocytoma cells used as targets (T) in the redirected killing assay (bar = 0.3 µm). (B) Ultrastructure of NK cells used as effectors (E) in the redirected killing assay (bar = 0.71 µm); cells were kept under the same experimental conditions as those of the assay. (C) Granules containing electron-dense matrix and tubular structures at higher magnification (bar = 0.26 µm). (D) An effector (E) to target (T) contact is shown in the redirected killing assay after treating NK cells with anti-CD38 mAb. Large, empty vacuoles are predominant in the effector cell (E) (arrows), some of which contain small tubular structures. No electron-dense granules are detected. The target cell (T) shows evidence of advanced apoptosis, as indicated by dispersed masses of strongly electron-dense chromatin (bar = 0.64 µm). The results shown are those obtained after incubating for 20 min the effector cells with the target P815 cells.

with an irrelevant reactive isotype-matched mAb as negative control did not show any significant ultrastructural change on either side (not shown).

Discussion

The characteristic ability of human CD38 to operate as an ectoenzyme and also as an adhesion molecule involved in immune regulation was primarily described in T cell models, and was later observed also in the B and myeloid lineages. The role played by CD38 in NK cells has only been marginally studied and exclusively in terms of surface expression (42,45). More recently, CD38 has been shown to trigger lytic and secretory responses in IL-2-activated human NK cells, thus entering the list of receptors able to mediate natural cytotoxicity (23).

This paper reports on the results of concerted efforts to determine the CD38 functions as a receptor and a signaling molecule in NK cells. The capability of CD38 to deliver regulatory signals for key cellular functions in NK cells was our first working hypothesis. Such behavior is quite unexpected for two reasons: (i) because of its role as an ectoenzyme involved in the demolition of NAD$^+$ and (ii) because of the apparent unsuitability of its cytoplasmic domain for transduction purposes. To investigate this issue, we monitored early (e.g. Ca$^{2+}$ currents, phosphorylation of cytoplasmic substrates, expression of surface CD69 marker), intermediate (cytokine messages) and late events (expression of surface HLA class II and CD25, cytokine release, cytotoxicity).

Inferences derived from studies of T and B cells clearly indicate that CD38 exploits the signaling machinery of the TCR and, at least in murine models, the BCR respectively (8,9,16). Consequently, our second hypothesis postulated the existence of a surface signaling molecule in NK cells which co-operates with CD38. Our attention was focused on CD16, the only surface IgG-binding molecule expressed by NK cells (46). CD16 is involved in antibody-dependent cell cytotoxicity and is a signaling molecule which shares several structural and functional homologies with TCR (47). Further evidence for CD16 as a candidate for this task was the report on the existence of lateral associations with CD38 (42).

Results obtained in the present work confirm our first working hypothesis. Indeed, the CD38 molecule engaged by agonistic mAb operates as a receptor involved in the regulation of Ca$^{2+}$ currents. Analysis of the Ca$^{2+}$ profiles in resting NK cells indicates that CD38 ligation is followed by a longer-lasting signal than that elicited by CD16, in contrast with observations reported in IL-2-activated NK cells (23). Up-modulation of late surface activation antigens (i.e. HLA class II and CD25) was also efficiently induced through both CD38 and CD16, with additive effects in the case of CD25.

Analysis of the phosphorylation of selected cytoplasmic substrates showed that CD38 ligation was followed by phosphorylation of the CD3-ζ and FcεRγ chains, ZAP-70
and of the proto-oncogene product c-Cbl. The transduction pathways followed were apparently similar to those described for CD16. CD16 signaling abilities are reported to rely upon non-covalent associations with disulfide-linked ζ and γ chain homo- and heterodimers (35,36,38,41,48–51), which are polypeptide subunits specialized in coupling to the intracytoplasmic transduction machinery (47). These subunits express ITAMs in their intracytoplasmic domain which are phosphorylated by Lck upon CD16 triggering (52); thus, they are capable of recruiting and activating the SH2 domains of ZAP-70/Syk (39,53–56) and shc (36). Following the multiple cascades initiated via these early phosphorylation steps, CD16 stimulation leads to proximal responses such as increases in intracellular Ca\(^{2+}\) concentration (31) and release of intracytoplasmic NK granules as well as distal responses, such as gene transcription and expression of activation molecules (i.e., CD25) and lymphokines (i.e., IFN-γ) (34,43). Further evidence of CD38 signaling was provided by the analysis of the effects on cytokines selected from among those playing a role in the NK cell economy. The messages for IFN-γ and GM-CSF were clearly influenced by the signals delivered by CD38 ligation, even if to a lesser extent than that triggered via CD16. Further, CD38 signaling was followed by the release of appreciable amounts of IFN-γ, while it was more efficient in enhancing GM-CSF release; in the latter case, the combined effects of CD38 and CD16 ligations were additive.

The last issue considered was the influence of CD38 signaling on cytokytic functions, the most relevant biological effects driven by NK cells. The signals elicited by CD38 ligation were followed by significant lysis of the target cells. Such effects were visible on IL-2-activated NK cells and paralleled the effects induced via CD16, although to a lesser extent. These events were also documented at ultrastructural levels by electron microscopy. The CD38-driven cytokytic functions required IL-2 activation of the effector cells, a feature shared by other receptors (57). The IL-2 requirement is likely due to the CD16 itself or downstream of CD16. A more precise definition of this issue will be the immediate follow-up of the present investigation: experiments are currently in progress in our laboratory to assess whether CD16 transfection in these CD16- NK cell lines is able to reconstitute the signaling properties.

A likely scenario is where CD38 has the ability to take part in the reorganization of the membrane structure, leading to an enrichment in microdomains which are rich in kinases and adaptor molecules on the inner side, and in the molecules involved in signaling on the outer side (59). A structure similar to immunological synapses could be envisaged for NK cells (60,61); this might include active participation of the cytoskeleton (62), as already demonstrated in the case of CD38 (63). The attribution of a precise co-localization of CD38 in such structures, which are gaining relevance in co-stimulatory signals, will constitute further follow-up of this research.

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Abbreviations

cADPR cyclic ADP ribose
\([\text{Ca}^{2+}]_i\) intracellular Ca\(^{2+}\) concentration
GexMlG goat anti-mouse IgG
GM-CSF granulocyte macrophage colony stimulating factor
HRP horseradish peroxidase
ITAM immunoreceptor tyrosine-activation motif
NAD\(^+\) nicotinamide adenine dinucleotide
PBMC peripheral blood mononuclear cells
PI 3-K phosphatidylinositol 3-kinase
PLC phospholipase C
pTyr phosphotyrosine
TNF tumor necrosis factor
ZAP-70 ζ-associated protein 70

References


