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CD38 Triggers Cytotoxic Responses in Activated Human Natural Killer Cells

By Giuseppe Sconocchia, J ulie A. Titus, Alessandra Mazzoni, Alberto Visintin, Federica Pericle, Stuart W. Hicks, Fabio Malavasi, and David M. Segal

Receptors used by natural killer (NK) cells to mediate natural cytotoxicity are poorly defined, although it is now clear that a number of adhesion molecules can serve this function. CD38 transduces signals on T- and B-cell lines, and we asked whether it could trigger lytic and secretory responses in human NK cells. By using an anti-CD38 monoclonal antibody in reverse antibody-dependent cellular cytotoxicity experiments, it is shown that CD38 engagement triggers cytotoxic responses by activated NK cells, but not by cytotoxic T lymphocytes or fresh NK cells. Cross-linking with anti-CD38 F(ab′)2 caused activated NK cells to release granymes and cytokines, but did not trigger an increase in intracellular Ca2+. Fresh NK cells acquired CD38-dependent lytic function during activation with interleukin-2 (IL-2), and inhibitor studies suggested that IL-2 stimulated the de novo expression of proteins that act between CD38 and the lytic machinery in NK cells. The induction of proteins that link commonly expressed adhesion molecules to effector mechanisms could provide a paradigm for pathogen recognition by the innate immune system.

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NATURAL KILLER (NK) CELLS, the primary lymphoid mediators of natural cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC), are controlled by positive and negative cytolytic signals.1,2 Negative signals are transduced by cellular cytotoxicity (ADCC), are controlled by positive and negative signals.1,2 The best-characterized positive triggering molecule on NK cells is CD16 (FcγRIIA), the receptor responsible for mediating ADC.1,4 CD16 associates noncovalently with γ and ζ homodimers and heterodimers that contain tyrosine-based activation motifs (ITAMs) in their cytoplasmic domains. Cross-linking of CD16 leads to phosphorylation of these ITAMs and of ηγ, ZAP-70, and phospholipase C-γ (PLC-γ), followed by a number of downstream events, including the release of intracellular Ca2+, that ultimately result in the exocytosis of cytolytic granules at the effector-target interface. It has been recently shown that activating forms of KIRs and CD155, ZAP-70, and phospholipase C-γ (PLC-γ), followed by a number of downstream events, including the release of intracellular Ca2+, that ultimately result in the exocytosis of cytolytic granules at the effector-target interface. It has been recently shown that activating forms of KIRs and Ly-49 follow a similar positive signaling pathway but use DAP-12, a homologue of ηγ, as a signal transducer.3,7,8

Receptors mediating natural cytotoxicity, the innate ability of NK cells to lyse tumor or virally infected target cells in the absence of antibody, have been more difficult to characterize because multiple different receptors can trigger cytolysis. Thus, a monoclonal antibody (MoAb) to any 1 triggering molecule would only marginally inhibit lysis. However, it has been possible to bypass the normal receptor-ligand interaction and to study the capacity of individual molecules to transduce cytolytic signals by using receptor-specific IgG MoAbs to redirect lysis of FcγR+ target cells.3,9,10 By using this approach, a number of cytotoxic triggering molecules have been identified, including CD2,13-15 CD44,14-16 CD69,17 NKRP-1,18 CD40,19 B7.2,19 and NK-TR,20 suggesting that these molecules might be involved in natural cytotoxicity. Another molecule expressed by NK cells is CD38, a type II integral membrane receptor that binds to CD31 (PECAM-1) and has adenosine diphosphate (ADP)-ribose cyclase ectoenzymatic activity.1-21 CD38 is expressed early in the differentiation of CD34+ stem cells to lymphocytes and remains in mature, CD56+, CD16+ NK cells,22 but not in resting B or T cells, although it is re-expressed in activated T cells. Ligation of CD38 on Jurkat T cells,26,27 immature B cells,28 and differentiated HL-60 cells29 by anti-CD38 MoAbs induces protein tyrosine phosphorylation. In Jurkat, but not in immature B cells or MHC-nonrestricted T cells,30 CD38 cross-linking also increases in intracellular Ca2+. A number of important functional consequences of CD38 engagement include upregulation of receptors such as B7.2,31 and CD73,32 apoptosis,27 proliferation,31 and cytokine release.30

The potent signaling properties of CD38 in T-cell, B-cell, and granulocytic cell lines prompted us to ask whether CD38 might also serve as a cytotoxic triggering molecule on NK cells. We show here that an agonistic anti-CD38 MoAb redirectss cell-mediated cytosis and induces cytokine and granzyme release in activated NK cells. We conclude that the CD38 adhesion molecule is indeed a cytotox trigger on NK cells that could contribute significantly to the positive triggering events leading to natural cytotoxicity.

MATERIALS AND METHODS

Antibodies and reagents. Phycoerythrinth (PE)-anti-CD38 (H1T2), PE-anti-CD16 (3G8), and unconjugated anti-CD11a (G43-25B, IgG2b and HI111, IgG1) and anti-CD56 (B159, IgG1) were from Pharmingen (San Diego, CA).3G8 (IgG1 anti-CD38) and M300 (IgG1 myeloma protein, MOPC 300) were from the American Type Culture...
CD38 IS A CYTOTOXIC TRIGGER IN HUMAN NK CELLS

CD38 is a cytotoxic triggering molecule in activated NK cells. Although CD38 transduces a variety of signals in B-cell, T-cell, and granulocytic cell lines and induces a cytotoxic response in an MHC-unrestricted T-cell line, it is not known whether it has the capacity to trigger cytolysis in isolated subsets of PBLs. Therefore, PBLs from normal donors were incubated in reverse ADCC experiments with Fe3+, P815 target cells in the presence of either anti-CD38 MoAb or MoAbs against CD3 and CD16, known cytotoxic triggers in CTLs and NK cells, respectively. In these experiments, IgG MoAbs redirect lysis by simultaneously binding to Fe3+ on the target cells and to triggering molecules on the effector cells. P815 cells are of mouse origin and are not specifically recognized by anti-human CD38 or other mouse antihuman MoAbs used in this study. Figure 1A shows that IL-2–activated PBLs in culture medium (RPMI 1640 plus 10% fetal calf serum [FCS], incubated for 3 hours at 37°C in 5% CO2) after incubation, 50 µL aliquots of supernatants were transferred to a flat-bottom microtiter plate and 50 µL of 1 mmol/L 5,5'-Dithio-bis-(2-nitrobenzoic acid) (DTNB; Sigma) in Hanks’ balanced salt solution (HBSS) was added to each well. Immediately after the addition of DTNB, plates were read at 414 nm using a Titertech (Huntsville, AL) Multiskan enzyme-linked immunosorbent assay (ELISA) reader to obtain blank values. One hundred microliters of 1 mmol/L BLT (Calbiochem, San Diego, CA) in HBSS was added to each well, and the plates were incubated at room temperature until a visible yellow color developed. The plates were again read in the ELISA reader, and blank values were subtracted.

**RESULTS**

CD38 is a cytotoxic triggering molecule in activated NK cells. Although CD38 transduces a variety of signals in B-cell, T-cell, and granulocytic cell lines and induces a cytotoxic response in an MHC-unrestricted T-cell line, it is not known whether it has the capacity to trigger cytolysis in isolated subsets of PBLs. Therefore, PBLs from normal donors were incubated in reverse ADCC experiments with Fe3+, P815 target cells in the presence of either anti-CD38 MoAb or MoAbs against CD3 and CD16, known cytotoxic triggers in CTLs and NK cells, respectively. In these experiments, IgG MoAbs redirect lysis by simultaneously binding to Fe3+ on the target cells and to triggering molecules on the effector cells. P815 cells are of mouse origin and are not specifically recognized by anti-human CD38 or other mouse antihuman MoAbs used in this study. Figure 1A shows that IL-2–activated PBLs do in fact contain cells that mediate CD38-directed cytolysis. Purified NK cells and CTLs were then used to determine which types of cells mediate this activity. The purified NK cell preparation (Fig 1B) mediated moderate amounts of lysis of P815 cells, a relatively NK-resistant target, in the absence of MoAb. Lysis was not affected by anti-CD3

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MoAb, indicating that levels of CTL contamination were negligible, but was markedly enhanced by the anti-CD38 and anti-CD16 MoAbs. By contrast, conventional CTL, depleted of NK cells and a small subset of T cells by CD56 negative selection, exhibited potent CTL activity, but failed to mediate CD38- or CD16-directed lysis. Removal of the Fc portion of IB4, the anti-CD38 MoAb used above, abrogated its capacity to mediated reverse ADCC, as expected, and OKT10, a less agonistic anti-CD38 MoAb than IB4,43 consistently mediated lysis at a lower level than IB4 (Fig 1D). FACS analysis (Fig 2) showed that all NK (CD16+) cells and the great majority of CTL expressed CD38. Thus, the inability of the anti-CD38 MoAb to trigger lysis by CTLs was not due to the lack of expression of CD38 on these cells. We conclude that CD38 is a cytotoxic trigger on activated NK cells but not on conventional CTLs.

Comparison of triggering functions of NK surface molecules. Not all MoAbs against NK surface molecules consistently induced lytic responses. Table 1 shows that activated PBLs from most donors mediate CD38- and CD16-dependent lysis of P815 target cells at levels significantly above the antibody-independent background. By contrast, anti-MHC I and anti-CD11a MoAbs induced positive responses in only 18% and 0% of donors, respectively, although these same MoAbs bound to essentially all NK cells by FACS analysis (data not shown). Moreover, both triggering and nontriggering MoAbs were fully capable of inducing NK cell-target cell conjugate formation (Fig 3), indicating that conjugate formation, per se, is not a lytic signal in NK cells.
Induction of CD38-dependent lysis. Unstimulated NK cells mediated little if any CD38-dependent lysis, but culture with IL-2 induced both CD38-dependent lysis and Ab-independent cytotoxicity (Fig 4). By contrast, unstimulated NK cells did mediate CD16-directed lysis, indicating that they were competent killer cells before activation (Fig 4). CD38 expression was similar on both activated and unstimulated cells (Fig 2B and A), indicating that the acquisition of CD38 triggering function was not due to enhanced CD38 surface expression. To determine whether de novo gene expression was required for the induction of CD38-directed lysis, PBLs were activated in the presence of varying concentrations of the transcription inhibitor, actinomycin D. As shown in Fig 5, actinomycin D, even at 10 ng/mL, had little effect on the acquisition or maintenance of lysis mediated by CD16, indicating that it did not impair the cytolytic function of NK cells in general. By contrast, actinomycin D strongly inhibited the induction of CD38-dependent lysis and Ab-independent cytotoxicity (Fig 5), but had a negligible effect on CD38 expression (Fig 2C). These data suggest that IL-2 induced the expression of at least 1 protein, distinct from CD38 itself, that was required for CD38 lytic function.

CD38 IS A CYTOTOXIC TRIGGER IN HUMAN NK CELLS

Table 1. Percentage of Donors Mediating Directed Lysis of P815

<table>
<thead>
<tr>
<th>MoAb Specificity</th>
<th>% Active Donors*</th>
<th>N†</th>
</tr>
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<tbody>
<tr>
<td>CD38</td>
<td>64</td>
<td>34</td>
</tr>
<tr>
<td>CD16</td>
<td>85</td>
<td>27</td>
</tr>
<tr>
<td>MHC I</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>CD11a</td>
<td>0</td>
<td>5</td>
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*Percentages of donors in which the indicated MoAb increased lysis more than 1.5-fold over natural cytotoxicity at at least 2 different E:T ratios.
†Number of donors tested. IL-2-activated PBL were used as effectors.

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CD38 cross-linking triggers degranulation and cytokine release. Cell-mediated cytolysis requires the formation of multicellular conjugates between effector and target cells and could involve ligation of several different receptors on the killer cells. To determine whether the cross-linking of CD38 alone could trigger functional responses, activated, purified NK cells were incubated with immobilized MoAbs, and the supernatants were tested for esterase or cytokine release (Fig 6). Immobilized anti-CD38 F(ab’)2 promoted esterase, IFN-γ, and TNF-α release at levels that were clearly above the medium control, but
that were usually lower than those induced by anti-CD16 MoAb. By contrast, MoAbs against CD56 and MHC I, 2 molecules expressed on NK cell surfaces, failed to trigger cytokine or esterase release. Esterase release results from the exocytosis of stored cytolytic substances such as perforin and granzymes, whereas cytokine release requires de novo protein synthesis. Thus, the cross-linking of CD38 with immobilized F(ab')2 triggered 2 fundamentally different processes used in NK effector responses.

Lack of CD38-induced Ca2+ mobilization in NK cells. Cross-linking of CD38 induces an increase in intracellular Ca2+ in Jurkat, but not in immature B cells or MHC-nonrestricted CTLs. We therefore asked whether CD38 cross-linking would trigger Ca2+ mobilization in NK cells. Fluo-3–loaded NK cells, isolated from PBLs that mediated high levels of CD38-dependent lysis (Fig 1A) were treated with either anti-CD38 F(ab')2, anti-CD16 MoAb (positive control), or anti-MHC I MoAb (negative control), followed by further cross-linking (right arrow) with antimouse Ig. As seen in Fig 7, only the anti-CD16 MoAb induced an increase in intracellular Ca2+. Similar results were obtained using biotin-MoAbs and streptavidin as a cross-linker (data not shown). We conclude that CD38 cross-linking in NK cells does not trigger Ca2+ mobilization and that the CD38 and CD16 signaling pathways differ in this regard.

**DISCUSSION**

It is well known that NK cells lyse tumor and virally infected cells in the absence of Ab, but the receptors used in target cell recognition are poorly defined. In this report, we show that CD38 is a receptor that could potentially contribute to natural cytotoxicity mediated by IL-2–activated NK cells. The signaling capacity of CD38 was demonstrated by using an anti-CD38 MoAb to mimic its interaction with CD31, a ligand for CD38 that is present in especially high amounts on endothelial cells. The choice of target cell is particularly important in these studies. P815 was chosen not only because it expresses FcR and is of mouse origin (and therefore does not interact with the mouse antihuman receptor antibodies used here), but also because it is relatively resistant to natural cytotoxicity. Natural cytotoxicity is the result of several positive signaling interac-
CD38 is a Cytotoxic Trigger in Human NK Cells

Fig 7. Cross-linking of CD16 but not CD38 induces an increase in intracellular Ca\(^{2+}\). Purified, IL-2–activated NK cells were loaded with Fluo-3, warmed to 37°C, and, at the time indicated by the left arrow, treated with anti-CD38 (IB4) F(ab\(^8\))\(_2\) anti-MHC-1 (W6/32) MoAb (\(\square\)), or anti-CD16 (3G8) MoAb (\(\blacktriangle\)). At the time indicated by the right arrow, antibodies were further cross-linked by the addition of rabbit F(ab\(^8\))\(_2\) antimouse IgG. Cytolysis mediated by PBLs from this donor is shown in Fig 1A. This experiment has been repeated 4 times, with similar results.

The killing capacity of CD38 appeared to be mediated primarily by activated NK cells. Conventional CTLs and unactivated NK cells expressed CD38 on their surfaces and mediated lysis through CD3 and CD16, respectively, but failed to kill through CD38. Because of our separation procedures, we could not establish whether the CD56\(^+\) T cells were also triggered by CD38 ligation. This NK-like subset of T cells comprises 2% to 3% of total PBLs and is the primary mediator of CD3 directed lysis in IL-2–activated PBLs. By contrast, the more abundant conventional CTL are CD3\(^+\), CD56\(^-\) and require T-cell receptor (TCR) cross-linking in addition to cytokine for activation. Recent studies by Cesano et al\(^{10}\) provide evidence on the role of CD38 in the activation of CD56\(^+\), CD3\(^+\) T cells. In their experiments, the IB4 anti-CD38 MoAb triggered low levels of cytolysis by the CD56\(^+\), CD3\(^+\) TALL line and no lysis by unfractionated LAK cells that were 90% T cells. In addition, anti-CD38 failed to induce an increase in intracellular Ca\(^{2+}\) levels in both cell types. However, IB4 did induce low, but significant levels of esterase release by both cell types, and gave robust cytokine responses. Thus, to the extent that the TALL cells and unfractionated LAK represent CD56\(^+\) T cells, it appears that this subset of cells behaves similar to NK cells in response to anti-CD38, except that they are less cytotoxic.

The acquisition of CD38 triggering capacity by NK cells during activation with IL-2 was blocked by actinomycin D, an inhibitor of transcription, suggesting that CD38 lytic function depends on the de novo synthesis of 1 or more proteins that are essential components of the lytic pathway. Because actinomycin D had no significant effect on CD38 expression, the newly synthesized proteins must have acted downstream of CD38. There are 2 known pathways by which lymphocytes lyse target cells in 4 hours (the time used in the current study): granule exocytosis and cross-linking of death receptors such as Fas on target cells. We demonstrate here that both anti-CD38 and anti-CD16 MoAbs induce degranulation (BLT-esterase release) of NK cells; in addition, the target cells used in this study did not express Fas (CD95) either by functional or FACS analysis (data not shown). Thus, CD16 and CD38 most likely killed through the granule exocytosis pathway. Because actinomycin D had little effect on CD16-mediated lysis on either activated or fresh NK cells, it is unlikely that the putative, IL-2–induced proteins were involved in the degranulation process itself. Thus, it is likely that IL-2 induced the synthesis of proteins that act between CD38 and degranulation. Previously, we demonstrated that CD44, like CD38, acquired cytotoxic triggering capacity during NK cell activation and that this gain in function was actinomycin inhibitable but not dependent on receptor expression. Thus, the de novo synthesis of proteins that link adhesion molecules to the killing machinery may be an important component of NK cell-mediated lysis in general.

In the Jurkat T-cell line, CD38 ligation induces the tyrosine phosphorylation of several proteins used in TCR signaling, including \(\zeta\), ZAP-70, and PLC-\(\gamma\), as well as proteins in the Raf-1/ MAP kinase pathway. Cross-linking of CD38 on Jurkat cells also induces an increase in intracellular Ca\(^{2+}\) and apoptosis, both of which require an intact TCR. CD16 ligation on NK cells initiates a signaling pathway that mirrors that of the TCR in that it induces phosphorylation of \(\zeta\) and homologous \(\gamma\) subunits, phosphorylation of ZAP-70 and syk, and an increase in intracellular Ca\(^{2+}\). In addition, it has been reported that CD16 associates with CD38 on NK cell surfaces. It seemed possible that the CD38 and CD16 triggering pathways on NK cells might merge either at the level of CD16 itself or downstream of CD16. However, the fact that CD38 cross-linking does not induce an increase in intracellular Ca\(^{2+}\), whereas CD16 ligation does, suggests that the 2 triggering molecules use different signaling pathways.

The number of adhesion molecules that can serve as cytotoxic triggers on NK cells, which now include CD2, CD38, CD44, and CD69, continues to increase, suggesting that the process of natural cytotoxicity may not be triggered by specialized NK receptors, but may instead reflect the level of expression of downstream proteins that confer triggering capacity on commonly expressed receptors. This mechanism of triggering might be fundamental to the cellular arm of innate immunity, but would be avoided by cellular mediators of acquired immunity, in which it would override a highly specific interaction with one that was less specific. Thus, it is not surprising that CD38 and CD44 trigger lysis on NK cells, but fail to do so when expressed on bulk CTLs generated from PBLs (although some
CTL clones mediate CD44-directed lysis\(^{17,48}\). It has been shown that CD44 is a cytotoxic trigger in neutrophils,\(^{49}\) but it remains to be established how extensively cellular mediators of innate immunity use adhesion molecules to initiate cytolytic responses.

REFERENCES


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