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Mechanism of Induction of NK Activation by 2B4 (CD244) via Its Cognate Ligand

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We have previously shown that coincubation of purified B cells with IL-2–propagated NK cells can result in the induction of IL-13 mRNA and that the induction requires the presence of CD48 on B cells and 2B4 on NK cells. Because both of these molecules are expressed on NK cells, it is surprising that very little IL-13 mRNA can be detected in the absence of B cells. We have now found that incubation of NK cells on plates containing immobilized anti-CD48 Abs results in the clustering of CD48 and colocalization with 2B4 on the same cell. This colocalization, together with the requirement for SAP, the signal transducer for 2B4, is necessary for the induction of IL-13 mRNA expression. Activation of NK cell via CD48 on another cell may require a similar ability to alter the configuration of 2B4 to activate downstream signaling. By the use of double CD2/2B4 knockout mice, we have also shown that the induction of NK cell activation by via CD48 or by B cells is not due to the release of inhibitory effects of 2B4. The Journal of Immunology, 2010, 185: 000–000.

The signaling lymphocyte activation molecule (SLAM) family of immune cell receptors is closely related to the CD2 family of the Ig superfamily of molecules. Most molecules belonging to the SLAM family possess two to four extracellular Ig domains, a transmembrane segment, and an intracellular tyrosine-rich region (1, 2). The members are differentially expressed on a variety of immune cell types and have been shown to modulate interactions between these cells. Murine 2B4 (CD244), the first member of the SLAM family identified (3, 4), is expressed on all NK cells and on some T cells (4, 5). Whereas 2B4 functions mainly as an activating receptor on human NK cells, on murine NK cells 2B4 has been shown to exert both activating and inhibitory effects on the cytotoxic activity of NK cells (4, 6–10). Unlike most of the other SLAM family members that have self-ligands, 2B4 has a unique ligand, CD48 (6). Because CD48 is expressed on most cells of the hematopoietic lineage, 2B4 on NK cells has the potential to interact with many cell types, including other NK cells. For this reason, studies of 2B4 function in IL-2–propagated NK cells present some difficulties due to possible activating or inhibitory activities exerted by the extent of contact between the cells during culture. It may be for this reason that baseline cytotoxicity as well as levels of IFN-γ produced by NK cells can vary significantly depending on the density and length of culture. It is interesting that, unlike IFN-γ, the production of another cytokine, IL-13, by propagated NK cells is much less variable and is virtually undetectable without additional stimulation. For this reason, we have used the induction of IL-13 mRNA as a measure of NK cell activation. Using this approach, we have shown previously that some subsets of B cells are effective inducers of NK cell activation via the CD48–2B4 interaction (7). In addition, 2B4 can also function as a ligand to activate B cells via CD48, although CD2 is a more effective stimulator in this case (8). In an effort to understand how CD48 activates NK cells, we have extensively analyzed the effect of ligation of CD48 by anti-CD48 on NK cells. These studies show that activation of NK cells via CD48 may be initiated by clustering of 2B4 with 2B4 within the same cell and that the interaction with 2B4 is essential for the activation because NK cells deficient in both CD2 and 2B4 cannot be activated with immobilized anti-CD48.

Materials and Methods

Cell preparations and culture

NK cells were purified from BALB/c, BALB/c IFN-γ knockout (KO) (9), C57BL/6, or from CD2 KO (10), 2B4 KO (11), or CD2/2B4 KO mice. CD2/2B4 KO mice were generated by F1 matings between CD2 KO and 2B4 KO mice (both provided by Dr. Michael Bennett, University of Texas Southwestern Medical Center, Dallas, TX). Both 2B4 KO and CD2 KO were derived from C57BL/6 mice. B6. Sle1 and B6.Sle1/hap KO mice were provided by Dr. Edward Wakeland (University of Texas Southwestern Medical Center). Spleen cells from the mice were first passed over a nylon wool column to remove adherent cells then depleted of T cells by complement mediated lysis. NK cells were then isolated by positive selection using anti-NKp46 or anti-CD49b (DX5) Abs and magnetic beads (BD Biosciences). For further stimulation, cells were cultured at 0.5 × 10^5 to 1 × 10^5 per milliliter in the presence of 100 U/ml IL-2 as described previously (12). When necessary, residual T or NKT cells, if detected by FACS, in the propagated NK cells were depleted with biotinylated anti-CD3e and streptavidin-conjugated magnetic beads (BD Biosciences). For further stimulation, cells were cultured at 0.5 × 10^5 to 1 × 10^5 per milliliter in the presence of 100 U/ml IL-2 in 24- or 48-well Falcon tissue culture plates (BD Biosciences) and soluble anti-FcR to block nonspecific binding. For Ab precoating cultures, plates were incubated with various Abs at 20 μg/ml for at least 4 h at 23°C or overnight at 4°C prior to washing to remove nonbound Abs.

For B cell preparations, T lymphocytes were depleted from splenocytes of B6 or IFN-γ KO mice by complement-mediated lysis and further purified by negative selection by binding to CD43 and CD11b MicroBeads (Miltenyi Biotec, Auburn, CA) to deplete remaining non-B cells. B cells were found routinely to be >95% positive for the CD19 marker.

Abs and reagents

Hamster anti-CD48 (HM48-1), Hamster IgG, anti-mouse CD49b (clone DX5), and rat anti-mouse CD3e were purchased from BD Biosciences. Rabbit anti-NKp46 and goat anti-mouse 2B4 were purchased from R&D Systems (Minneapolis, MN), and rat anti-mouse CD48 was from Santa
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Cruz Biotechnology (Santa Cruz, CA), Rat anti-FcγR, [2.4G2 (13)], hamster anti-TCR-α/β, mouse anti-NK1.1 [PK136 (14)], and rat anti-2B4 were purified from hybridoma culture supernatants using γ Bind (Pharmacia, Fine Chemicals, Piscataway, NJ). Recombinant IL-12 and recombinant IL-18 were purchased from R&D Systems.

Fluorescence-activated cell sorting analysis

Stained cells were collected on the FACScan Flow Cytometer and analyzed on CellQuest (BD Biosciences).

Semiquantitative RT-PCR analysis

RNA was prepared using the TRizol reagent (Invitrogen, Carlsbad, CA), and RT-PCR was performed as previously described (15). Primers for IL-13 and Ly49 mRNA have been described earlier (11). Primers for hypoxanthine phosphoribosyltransferase mRNA were as follows: forward, 5′-GCT GGT GAA AAG GAC CTC TCT-3′; reverse, 5′-CAC ACT AGA ACA CCT GC-3′. Primers for CITAM were as follows: forward, 5′-AAACGTCTCTC-TCCAGTGGC-3′; reverse, 5′-TGAGAACATTCCAGCACAAC-3′. The forward primer for MHC class II was 5′-GAGGCTAGGACCATGGG-3′, and the reverse was 5′-AGATGCTCCAGATTTCCGG-3′. All primers were ascertained to span intronic regions. Amplified products for each set of primers were authenticated by size and restriction enzyme analysis. To quantify RT-PCR products, at least one of each primer pair was 5′-end labeled with [γ-32P]ATP and used to spike reaction mixtures. Amplified products were quantified using the ImageQuant software package (Molecular Dynamics, Sunnyvale, CA). For all primer pairs, titration curves were performed to ascertain that the cycle number used fell within the linear range as cDNA concentrations were increased.

Confocal microscopy

IL-2–propagated NK cells were stained with PE–anti-CD48 on ice for 15 min followed by two washes with complete medium. Poly-L-lysine coated coverslips (Biocoat; BD Biosciences) were coated overnight at 4°C with a 20 μg/ml solution of various Abs, followed by three washes with PBS. PE–anti-CD48 stained cells were gently placed on the coated coverslips and incubated at 37°C for different time intervals. Incubation was stopped by fixing with 2% paraformaldehyde in PBS. After washing the cells with PBS, they were blocked by 1% BSA or goat serum. Then the cells were stained with anti-2B4-biotin (BD Biosciences) or anti-NKp46-biotin followed by streptavidin–AF647. The stained cells were washed twice and mounted using Gold antifade reagent (Invitrogen). The cells were stained with anti–2B4-biotin (BD Biosciences) and rat anti-mouse 2B4 and rat anti-mouse CD48 with appropriate HRP-conjugated Abs. The presence of apparent induction of NK cell production of IL-13 mRNA expression in NK cells. IL-2–propagated NK cells from the indicated mouse strains were incubated in tissue culture wells with 2 μg/ml soluble (A) or immobilized (B) anti-CD48, other Abs, or cytokines, as indicated, for 24 h. RNA extracted from the cultured cells was analyzed by semiquantitative RT-PCR analysis as described in Materials and Methods. Relative intensities of amplified IL-13 mRNA product normalized to the intensity of Ly49 mRNA amplified from each of the cultures for one of at least two replicate experiments is shown.

Results

Induction with anti-CD48

The apparent absence of induction of NK cell production of IL-13 in NK cultures despite the expression of both ligand and receptor could be attributed to inhibitory effect exerted by 2B4 due to its interaction with CD48 expressed on adjacent cells (11, 17, 18). To address the possibility that the density of CD48 may play a role in the inhibitory effect, we attempted to reduce the availability of CD48 on NK cells by masking the ligand with anti-CD48. We found that addition of soluble anti-CD48 to propagated NK cells, which resulted in the masking of CD48 determinants (data not shown), did not induce activation of NK cells (Fig. 1A). However, the masking would have also prevented any interaction with the receptor. Therefore, to maintain the presence of the ligand, we altered the ligand by treating NK cells with immobilized anti-CD48. Notably, stimulation in this manner resulted in induction of IL-13 mRNA in a short time (Fig. 1B). The level of induction was found to be variable but usually within 2-fold of that induced by immobilized anti-NK1.1 or with a combination of IL-12 and IL-18. We first examined NK cells from IFN-γ KO mice to eliminate possible inhibition of this Th2 cytokine by NK cell secretion of IFN-γ. Moreover, similar level of induction of IL-13 mRNA was found for NK cells from BALB/c mice and C57BL/6 mice. Because anti-FcR was added to all cultures in saturating concentrations, it is unlikely that the induction occurred via the Fc portion of the Ab. Furthermore, another hamster Ab, anti-TCR, was not effective.

Co-clustering of CD48 with 2B4

Because CD48 is anchored to the membrane via glycosidic linkage and therefore should not be able to signal on its own, we probed possible mechanisms of induction by examining the effect of anti-CD48 modulation at the cellular level by confocal microscopy. Fig. 2A shows that the immobilized anti-CD48 induced clustering of the determinant within an hour of incubation and was significantly greater than that found for nontreated cells (p < 0.001). We used either immobilization of cells on anti-CD48–coated coverslips or, alternatively, PE-anti-CD48–coated cells were cross-linked with...
anti-PE Image beads (Fig. 2A,2D). The clustering was similar in both cases. Incubation of NK cells on anti-TCR–coated cover slips did not result in a significant level of clustering (Fig. 2D). Interestingly, staining for 2B4 on these cells indicated significant coclustering and colocalization of receptor with the ligand, 2B4, on the same cells (Fig. 3A). Control staining with anti-NKp46 did not show a similar level of colocalization (Fig. 3B). To elucidate further the significance of CD48 colocalization with 2B4, we performed solid-phase signalosome precipitation for the receptor complex. Using a procedure that does not solubilize the raft, it was possible to visualize components in the detergent-insoluble raft complex (16). As shown in Fig. 3C, activation of IL-2–propagated NK cells by immobilized anti-CD48 brings 2B4 into the CD48 complex. Together with the colocalization data (Fig. 3A), these results suggest a cis interaction between CD48 and 2B4 in the same cell to form a signaling complex.

2B4 is required for activation of IL-13

The coclustering of CD48 with 2B4 by incubation with immobilized anti-CD48 suggested that the signal imparted by anti-CD48 could be mediated by 2B4. We therefore examined the effect of anti-CD48 activation on NK cells propagated from mice with a deletion of the 2B4 gene (2B4 KO) mice. It was found that the induction of IL-13 mRNA was significantly compromised in these cells (Fig. 4A). In contrast, activation of NK cells from CD2 KO mice was not affected (Fig. 4A) despite the fact that CD2 is also known to bind CD48. In the absence of 2B4, the lower affinity of CD2 for CD48 may be the reason for limited activation in some experiments (Fig. 4A). Therefore, to ensure that no residual signaling can occur via either the CD2–CD48 or 2B4–CD48 interaction, NK cells from CD2/2B4 double-KO mice were examined. Fig. 4B and 4C indicates that IL-13 mRNA in cells from these mice cannot be induced to any measurable extent by immobilized Abs in wt B6 NK cells. PE–anti-CD48 stained cells were incubated with anti-PE Image beads for sample labeled CD48-2 in D. E, B6 wt versus 2B4/CD2 KO CD48 clustering in control or immobilized anti-CD48–treated cells is shown. Statistically significant difference between experimental and control determined by two-tailed paired t test: *p ≤ 0.001.

FIGURE 2. Cross-linking of surface CD48 leads to clustering. IL-2–propagated NK cells from wild-type (wt) C57BL/6 or CD2/2B4 KO mice were stained with PE–anti-CD48 and then incubated without (control) or with immobilized Abs as indicated for 60 min at 37°C. Cells were then fixed and stained for other surface markers followed by confocal microscopy and image analysis. Images were captured using a ×60 objective with numerical aperture of 1.4. A. Representative confocal images showing surface CD48 distribution in cells treated with immobilized anti-CD48 or untreated (control) from wt and CD2/2B4 KO mice. B. Intensity was measured along the cell membrane using “membrane profile,” an ImageJ macro, as described in Materials and Methods, to obtain a distribution profile of CD48. C. A representative profile for control and anti-CD48–treated B6 wt cells is shown. D. The average (n = 60) clustering was assessed by measuring the ratio of maximum and median intensity in the membrane profile of individual cells from untreated or treated with respective immobilized Abs in wt B6 NK cells. PE–anti-CD48 stained cells were incubated with anti-PE Image beads for sample labeled CD48-2 in D. E, B6 wt versus 2B4/CD2 KO CD48 clustering in control or immobilized anti-CD48–treated cells is shown. Statistically significant difference between experimental and control determined by two-tailed paired t test: *p ≤ 0.001.

FIGURE 3. Colocalization and coprecipitation of 2B4 with CD48 in IL-2–propagated NK cells after treatment with immobilized anti-CD48. A and B, NK cells were incubated with immobilized anti-CD48 (CD48) or without (control) for 60 min at 37°C then fixed and stained with anti-2B4 (4) or anti-NKp46 (B). Overlay and product of difference from mean (PDM) shows colocalization and clustering of CD48 and 2B4 in the same cell to form a signaling complex. C, NK cells were incubated with indicated PE-conjugated Abs at 4°C for 30 min then transferred to 37°C for 5 min after washing. The detergent-insoluble raft complex was precipitated as described in Materials and Methods. Immunoprecipitates were analyzed by Western blot.
of CD2 as well as 2B4, the growth of ALAK cultures from these mice was not impaired, suggesting that if NK–NK fratricide occurred in the absence of these receptors (17), the effect was not significant in these cultures.

Although similar level of induction of IL-13 mRNA was found among different strains, it is possible that the interaction between CD48 and 2B4 may be modulated differently in different genomic contexts. Therefore, we also examined the B6.Sle1b congenic strain, which carries the Sle1b susceptibility locus from NZM2410, on the C57BL/6 background. Fig. 5 shows that induction of IL-13 mRNA by anti-CD48 in this strain, in which the 2B4 gene is derived from NZM2410, which is identical to that of BALB/c, does not differ from other strains. Activation signal mediated via CD248 requires the adapter molecule SAP, therefore we hypothesized that removal of SAP should affect the induction of IL-13 mRNA by anti-CD48 if it is mediated via 2B4. Having at hand a strain of B6.Sle1b that carries an inactivation of the sap gene, it was possible to compare the effect of SAP deletion on the induction of IL-13 mRNA by anti-CD48. Fig. 5 shows that in the absence of SAP, the induction of NK cells from Sle1b mice was severely compromised, further indicating that signaling from 2B4 is required for activation by anti-CD48.

**Correlation of anti-CD48 stimulation with B cell induction**

We had shown previously that the B cell induction of IL-13 mRNA expression is mediated by interactions between CD48 on B cells and 2B4 on NK cells (7). Because B cells do not express 2B4, it is possible that CD48 is in a different spatiotemporal configuration than that of NK cells. In light of the requirement for anti-CD48–induced clustering for activation of NK cells in the absence of other stimuli, it is likely that activation by B cells also induces similar changes in the CD48–2B4 complex on NK cells. However, we were unable to detect coclustering of CD48 with 2B4 after coinoculation of NK cells with B lymphocytes (data not shown). It is nonetheless possible, however, that in the absence of 2B4 on B cells, CD48 induces conformational changes in another way. Because CD2/2B4 double-KO NK cells also do not express 2B4, it is possible that this absence of cis interacting receptors might keep CD48 in a conformation similar to that of B cells and thus be able to stimulate IL-13 expression in wild-type NK cells. To address this possibility, B6 NK cells were cultured with double-KO NK cells. However, as shown in Fig. 6, only minimal extent of IL-13 mRNA was induced in these cultures compared with that in the same NK cells cultured with B cells. Therefore, induction of CD2 as well as 2B4, the growth of ALAK cultures from these mice was not impaired, suggesting that if NK–NK fratricide occurred in the absence of these receptors (17), the effect was not significant in these cultures.

Although similar level of induction of IL-13 mRNA was found among different strains, it is possible that the interaction between CD48 and 2B4 may be modulated differently in different genomic contexts. Therefore, we also examined the B6.Sle1b congenic strain, which carries the Sle1b susceptibility locus from NZM2410, on the C57BL/6 background. Fig. 5 shows that induction of IL-13 mRNA by anti-CD48 in this strain, in which the 2B4 gene is derived from...
by B cells may involve help from additional coactivators not expressed by NK cells. The inability of B cells to induce IL-13 mRNA expression by CD2/2B4 KO NK cells (Fig. 6) further confirms that the NK cell activation by B cells requires the ligation of 2B4. Furthermore, in the absence of CD2 and 2B4, no detectable cell death was observed in cocultures, and MHC expression of B cells was also diminished indicating that the absence of these receptors does not remove possible inhibitory signals that prevent NK-mediated killing of B cells.

Discussion

We have previously shown that B cells can activate NK cells via the interaction between CD48 present on B cells and 2B4 expressed by NK cells (7). To understand further the mechanism of this activation, we investigated whether IL-2–activated NK cells can be activated by the manipulation of these two determinants, which are also expressed on NK cells themselves, in the absence of other cell types. We found that immobilized anti-CD48 can effectively stimulate NK cells to initiate IL-13 mRNA expression. CD48 is a GPI-linked glycoprotein that cannot signal on its own. Therefore the signal must be imparted by another molecule associated with it, most likely in a lipid raft (19–21). Our results show that activation by immobilized anti-CD48 is correlated with clustering of CD48 and its colocalization with 2B4. Soluble anti-CD48 could not induce similar clustering nor could it induce IL-13 mRNA expression. CD48 has been reported to be found clustered with TCR in the lipid raft at the immune synapse upon stimulation by anti-CD3 (20). Furthermore, CD48 cross-linking can induce clustering, cytoskeletal reorganization, and tyrosine kinase activation in T cells (19, 21). Therefore, this ligand may cluster with other molecules expressed on NK cells. On NK cells, 2B4 has been found to be located in the lipid raft with the help of a conserved cysteine motif CXC in its transmembrane region (22, 23). Anti-CD48–induced clustering and colocalization of 2B4 may further stabilize these molecules in the lipid raft and activate the receptor signaling by enhancing phosphorylation and recruitment of adaptor molecules, such as SAP. The virtual absence of IL-13 mRNA in IL-2–propagated NK cells despite the presence of both 2B4 and CD48 suggests that the spatiotemporal conformation of the ligand and receptor must be altered before an activation signal can ensue. Thus, in the absence of other stimulatory signals, it appears that the anti-CD48–induced cis interaction between the receptor and ligand pair on the same cell allows the receptor to impart an activation signal. An alternative mechanism may be attributed to an alteration of inhibitory signals imparted by 2B4–CD48 interactions that is disrupted by the clustering with CD48. However, the significantly reduced level of activation in NK cells from 2B4 KO mice indicates that despite the removal of such inhibitory signals, activation cannot occur via the CD2 receptor even though it can bind to CD48. Thus, it seems that in the context of induction of the cytokine, IL-13, 2B4 serves only as an activating signal. The requirement for the presence of the adaptor protein SAP, necessary for positive signaling by 2B4 (1, 2, 24), further supports the notion of 2B4 functioning only as an activating receptor in this system. This dissection of the activation function of 2B4 is consistent with findings in human NK cells where it has been shown to mainly function as an activating receptor. In fact, CD48 engagement on human NK cells by 2B4–expressing cells has been shown to activate NK cells for cytotoxicity and IFN-γ production (25). The apparent requirement for cis-activation between CD48 and 2B4 for activation of NK cells also brings into question conclusions from experimental models using transfections of T cells that do not coincidentally express CD48 or 2B4 (9). Therefore, the conditions under which 2B4 can function as an inhibitor of NK activation may require further analyses. It should also be noted that these findings by Chlewicki et al. (27) are also in conflict with our previous findings showing that IFN-γ as well as IL-13 mRNA can be induced in C57BL/6 NK cells by saturating amounts of immobilized anti-2B4 (12).

What then is the in vivo significance of anti-CD48 stimulation of activated NK cells? In addition to the possible stimulation of NK cells by other NK cells expressing 2B4, regulation of CD8 T cells, the only other cell type known to express 2B4, comes to mind. Although it has recently been shown that the inhibitory activity of 2B4 on NK cells can play a role in the regulation of activated CD8 T cells in some infections, the effect may be virus strain specific (28). Furthermore, regulation of NK cell cytotoxic secretion by CD8 T cells may reveal a different pathway. It should also be noted that we have not been able to induce IL-13 expression from freshly isolated NK cells with immobilized anti-CD48 even in the presence of 100 U IL-2 (Yuhong Guo, unpublished observations). Thus, the induction of cytokine expression in vivo may require prior activation of NK cells by agents, such as polyinosinic-polycytidylic acid.

Finally, how is induction of NK IL-13 mRNA expression by B cells correlated with induction by NK cells? Both of them express CD48, which has the potential to activate 2B4. The inability of CD2/2B4 double-KO NK cells to induce wild-type NK cells (Fig. 6) suggests that the mere absence of 2B4 on the inducer cells does not account for the inductive ability of B cells. Therefore, it is likely that stimulation of NK cells by B cells, as well as by other cell types, require an additional costimulator(s) that has yet to be identified. This induction may also involve a conformational change of 2B4 similar to that induced by immobilized anti-CD48.

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Disclosures

The authors have no financial conflicts of interest.

References


