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Receptors and Counterreceptors Involved in NK-B Cell Interactions

Ning Gao,* Tam Dang,* Wesley A. Dunnick,† John T. Collins,‡ Bruce R. Blazar,‡ and Dorothy Yuan*‡

In addition to the well-documented effect of NK cells on B cell differentiation via their ability to secrete IFN-γ, NK cells can also induce, via direct cell-cell interactions, germline transcripts (Iγ2a) necessary for switch recombination to IgG2a. Analysis of the ligand-receptor pairs that could be involved in this induction revealed that the expression of CD48 on B cells is crucial for the induction. NK cells from mice with targeted deletions of either the CD2 or the CD244 gene, both of which encode ligands for CD48, are compromised in their ability to induce B cell Iγ2a expression. Interestingly, although CD244 can bind to CD48 with a higher affinity, the ability of NK cells from CD244−/− mice to stimulate Iγ2a is not as compromised as NK cells from CD2−/− mice. Despite the difference between cell surface receptors that are stimulated by NK cells vs those stimulated by the combination of LPS and IFN-γ, we show in this study that the initiation of γ2a germline transcription is regulated by similar cis-acting elements located at the 3′ end of the IgH locus. However, NK cells cannot induce the final steps of switch recombination resulting in the production of mature mRNA from recombinant DNA. Our findings suggest that these different signaling pathways converge on regulatory elements that are common to germline transcription; however, because NK induction does not result in the final steps of switch recombination, some signals initiated by LPS plus IFN-γ are not induced by NK cells. The Journal of Immunology, 2005, 174: 4113–4119.

Natural killer cells constitute one of the key components of the innate immune system in that they can be rapidly activated without the need for expansion of Ag-specific clones. There is increasing awareness of the importance of their role in modulation of the immune system via their ability to produce a number of cytokines as well as nonlytic interactions with target cells (1–3). B lymphocytes, by contrast, are important constituents of the specific immune system due to the presence of clonally distributed Ag receptors and the persistence of the memory of specific clones that can continue to produce the relevant Abs. An understanding of the interactions between these two cell types should provide important insights into the influence of the rapid but transient response to pathogens on the specific immune response that takes longer to initiate but is longer lasting.

Studies in both human and murine systems have shown that NK cells, on their own, exert minimal effects on IgM secretion of resting B cells. Only B cells that are preactivated in vivo can be enhanced by NK cells to increase both IgM and IgG synthesis. Furthermore, activated B cells can induce NK cells to up-regulate IFN-γ secretion (reviewed in Ref. 4). We have previously shown that, although NK cells cannot induce IgM secretion, they can initiate limited differentiation of resting cells as detected by the induction of germline transcripts for IgG2a (Iγ2a) (5), a necessary first step for switch recombination to IgG2a (6, 7). The Ig locus is the site of two types of rearrangements: V(D)J assembly that generates the V region exons at the H and L chain loci during B cell development and class switch recombination (CSR) at the H chain (IgH) locus after antigenic stimulation of specific B cell clones. CSR specifically alters C region genes through a deletional process, whereby a downstream C region gene is brought to proximity of a rearranged VDJ gene, allowing expression of one of the downstream isotypes (IgG, IgE, or IgA). CSR is preceded by germline transcription of target switch sequences located upstream of all of the C region genes except the Cδ. Activation and targeting of CSR is correlated with the ability of certain mitogens and cytokines to induce or suppress germline transcription of each specific C region genes (8, 9). Accurate splicing of germline transcripts is also critical for the efficiency of CSR (10). In addition to the cis-regulatory elements located upstream of the I promoters, a series of DNase I hypersensitive sites located 2–32 kb 3′ of the Cε gene and downstream of the IgH locus, also affects both germline transcription as well as the process of switch recombination itself (8, 9, 11, 12). Following the initiation of germline transcription, switch recombination involving breakage and joining of DNA segments requires additional factors, the most important of which is activation-induced cytidine deaminase (AID) (13), a member of the RNA-editing deaminase family. However, neither the target(s) of AID nor its potential cofactor(s) are precisely known and the exact role of AID is still under investigation. Interestingly, despite the ability of NK cells to stimulate production of appropriately spliced Iγ2a transcripts as well as AID mRNA, they do not stimulate production of γ2a transcripts from recombinated DNA (5). Therefore, NK cells induce the germline transcripts that precede switch recombination, but not the recombination event itself. Other inducers, such as LPS plus IFN-γ or activated T cells, induce both

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Abbreviations used in this paper: CSR, class switch recombination; AID, activation-induced cytidine deaminase; BAC, bacterial artificial chromosome; FL, fluoroscein; PI-PLC, phosphatidylinositol-specific phospholipase C.
germline transcription and switch recombination. To test a potential mechanistic basis for this difference, we examined the dependence of γ2a germline transcription induced by NK cells and by LPS plus IFN-γ on a set of regulatory elements 3′ of the Cε gene.

In human systems, both CD11a (14, 15) as well as CD40-CD40L (16) interactions have been shown to be important for NK induction of B cell responses; however, the interaction molecules for mouse cells have remained largely unknown. Some indicators of possible NK-B cell interaction molecules are suggested by studies in which cells transfected with B cell ligands can be shown to elicit cytotoxic activity of NK cells (17, 18). Because the induction of IFN-γ transcription by NK cells can occur without the addition of other factors, it is a sensitive measure that can be used for the identification of the ligand-receptor pairs that may be involved in the NK-B cell interaction. We will show in this report that the CD48-CD2 interaction is important for the activation of B lymphocytes by NK cells.

**Materials and Methods**

**Transgenic mice**

We prepared mice with a 230-kb transgene (Fig. 1A) of the entire H chain C region locus from strain 129 mice (Ight) (19). The transgenic mice were generated using the BALB/c × C57BL/6F1 fertilized eggs, and are maintained by backcrossing to C57BL/6. Thus, the germline locus is Ight. By homologous recombination in *Escherichia coli* (20), we inserted an assembled VDJH2 segment that encodes anti-arsonate binding activity (21) into the germline JH region of the 230-kb construct. We named this parent germline JH region of the 230-kb construct. We named this parent.

**Cell culture and preparation**

For B cell preparations, T lymphocytes were depleted from splenocytes of CD2−/− (Ref. 22; bred and kindly provided by Dr. M. Bennett, University of Texas Southwestern Medical Center), and BALB/c-Igf<tm1> (IFN-γ−/−) (Ref. 23) mice (The Jackson Laboratory), or B cell transgenic lines and fractionated by Percoll gradient centrifugation as previously described (24). The high density fraction (25) was further purified by binding to fluorescein-conjugated (FL)-CD43 (BD Biosciences) and F(ab′)2 of FL-goat anti-mouse IgG (Southern Biotechnology Associates) before incubation with anti-fluorescein-conjugated magnetic beads (Miltenyi Biotec), for depletion of remaining non-B cells and IgG+ cells, respectively. B cells were found routinely to be >90% positive for the CD19 gene marker. In vivo modulation of CD48, IFN-γ−/− mice were injected twice, on days −5 and −3, with 300 μg/animal of an ammonium sulfate cut of CD48 (HM48-1) (26). On day 0, B cells were isolated from these mice. NK cells were prepared by passage of spleen cells from BALB/c IFN-γ−/−, C57BL/6 (The Jackson Laboratory), CD2−/− or CD42−/− (27) mice over nylon wool columns to remove adherent cells, and depleted of T cells by complement-mediated lysis. NK cells were then isolated by positive selection using anti-DX5 Abs and magnetic beads (Miltenyi Biotec). Purified cells were cultured for 3–4 days in 1000 U/ml IL-2 as described previously (5), at which time the nonadherent cells were discarded, and the adherent cells were propagated for another 4–7 days. B lymphocytes were cultured either alone (1 × 10^6/ml), or together with NK cells (0.5 × 10^6/ml) in the presence of 100 U/ml IL-2 with or without other additives in 24- or 48-well Falcon tissue culture plates (BD Biosciences).

**Abs and reagents**

Hamster anti-CD40L (CD154; Ref. 28) Abs were kindly provided by Dr. R. Noelle (Dartmouth Medical School, Dartmouth, NH). Ammonium sulfate fractional preparations of hamster anti-CD48 (BCM1) were prepared as described (26). Rat anti-B220, hamster anti-CD48 (BCM1), goat anti-IgM F(ab′)2, and mouse anti-DX5 were purchased from BD Biosciences. Rat anti-CD28 (29), anti-IFN-γ (2.4-G2; Ref. 30), hamster anti-TCRβ (31), rat anti-IFN-γ (R4-6A2; Ref. 32), and rat anti-CD48 (1G10) Abs (originally obtained from Dr. J. Bromberg, Mount Sinai School of Medicine, New York, NY) were purified from hybridoma culture supernatants using Gammabind (Pharmacia Fine Chemicals) according to the manufacturer’s suggestions. The F(ab′)2 of rat anti-CD28 and the rat anti-CD48 Ab, 1G10, were prepared as previously described (33). Recombinant IFN-γ was purchased from Biosource International. Phosphatidylinositol-specific phospholipase C (PI-PLC) was purchased from Sigma-Aldrich.

**Semiquantitative RT-PCR analysis**

RNA was prepared using the TRIzol reagent (Invitrogen Life Technologies), and RT-PCR was performed as previously described (34). Primers for assessment of γ2a germline transcription (γ2a) and μm mRNA abundance have been described (5). The primers for assessment of cDNA from switched RNA (Sγ2a) were 5′-aagaaggaagtggatg-3′ and 5′-GGATGGAAGTGCGGCTTC-3′ corresponding to the V region of the AJR-specific gene used for the BAC transgenic mice, and the reverse primer (5′-GGCCAGGTGTCCGAGGTT-3′) located in the first exon of the γ2a gene. The 635-bp RT-PCR product derived from appropriately spliced mRNA was confirmed by restriction enzyme mapping. L449 primers are consensus sequences for most Ly49 family members including both BALB/c and C57BL/6 strains (S. Anderson, unpublished observations) (forward, 5′-ATCACCACAAAGTGAATGC-3′; reverse, 5′-TATGGGGCCCATTTTCAATC-3′). All primers were ascertainment to span intronic regions. To quantify RT-PCR products, at least one of each primer pair was 3′-end labeled with [γ-32P]ATP and used to spike reaction mixtures. Amplified products were quantified using the ImagQuant software package (Molecular Dynamics). 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.

**FACS analysis**

Cultured cells were stained and analyzed using the FACScan flow cytometer (BD Biosciences) as previously described (35). PE-conjugated (PE)-mouse anti-IgM, FL-mouse anti-IgM, PE-rat anti-CD2, PE-mouse anti-CD24, PE-rat anti-CD48, FL-rat anti-CD19, PE-mouse anti-IgD, FL-anti-hamster Ig, and PE-isotype control Abs were purchased from BD Biosciences.

**Results**

**Role of 3′ enhancer region in the regulation of induction of γ2a germline transcripts by NK cells**

Although IFN-γ is an important cytokine in driving preferential switch recombination to the γ2a exons, we have shown that both T and NK cells from IFN-γ−/− mice can induce γ2a germline transcripts that necessarily precede switch recombination. In contrast to stimulation by LPS plus IFN-γ or by T cells, however, NK cells cannot induce the final step of gene rearrangement required for expression of switched transcripts (2). The failure of NK cell stimulation to induce switch recombination might be due to the fact that NK cells induce factors in B cells that act through different cis-acting elements than do those induced by LPS plus IFN-γ. It is known that LPS plus IFN-γ-induced germline transcription and switch recombination to γ2a depends, to a large extent, on the set of enhancers 3′ of Cy (8, 9, 11, 12). We have studied transgenes of the entire H chain C region locus that undergo germline transcription and switch recombination (19). We recently identified transgenes that lack all four regulatory elements 3′ of Cε (HS3b, HS1/2, HS3b, and HS4). Mice expressing these transgenes express <5% the amount of LPS plus IFN-γ-induced γ2a germline transcripts compared with transgens with an intact 3′ regulatory region. As illustrated in Fig. 1B, the various lines established with the ARS/Igh transgene display two patterns of allelic exclusion. In about one-half of the lines, 30–50% of B cells

4 W. A. Dunnick, J. Shi, K. A. Graves, and J. T. Collins. Deletion of the 3′ end of the murine heavy chain constant region locus results in dramatically reduced germline transcription and switch recombination of the four y genes. Submitted for publication.
express transgenic IgM\textsuperscript{a} and not IgM\textsuperscript{b} of the endogenous (C57BL/6) genes, and the remainder of the B cells express the endogenous IgM\textsuperscript{b} and not the transgenic IgM\textsuperscript{a} (lines 847 and 822). Other lines exhibit virtually complete allelic exclusion by the transgenes and all of the B cells express IgMa (lines 858).

The lines with truncated transgenes allow us to test the dependence of the NK cell-induced \gamma2a germline transcription on the 3’ enhancer region (Fig. 1). To distinguish \gamma2a germline transcripts of the endogenous locus from \gamma2a germline transcripts of the transgenic locus, we digested the amplified product with \textit{Ase}I, which cuts only in the products derived from the transgene. As demonstrated by the intact, 399-bp band, all mice express \gamma2a germline transcripts; all of the \textit{IgMa} transcripts detected were resistant to \textit{Ase}I digestion. Identical results were obtained with a second transgenic line (line 1001), which also lacks the 3’ end of the \textit{H} chain locus (data not shown). Thus, like \gamma2a germline transcripts induced by LPS plus IFN-\gamma, germline transcripts induced by NK cells depend on elements in the 3’ end of the \textit{H} chain locus.

Fig. 1 also shows that mature, switched transcripts, which can be detected by primers specific for the transgenic V region, are also induced by LPS plus IFN-\gamma activation of cells carrying the intact transgenes. Such postswitch transcripts are not induced in cells carrying the transgene with the deleted 3’ enhancer (Fig. 1C).\textsuperscript{4} In contrast, activation by NK cells induced low to nondetectable levels of switched transcripts from cells carrying the intact transgenes despite the induction of \textit{IgMa} transcripts. These results are similar to our previous findings using nontransgenic B cells (5). Therefore, it is possible that other \textit{cis}-acting elements, perhaps located in the promoter region of the \gamma2a germline gene, bind transcription factors that are differentially stimulated by LPS plus IFN-\gamma vs NK cells.

\textbf{Inhibition of induction of \textit{IgMa} transcription by Abs against determinants expressed by B and NK cells} To identify the ligand/receptors that may be involved in the NK cell induction of B cell \textit{IgMa} transcription, we first used a number of Abs against possible candidates for the NK-B cell interaction in an attempt to block the induction. NK cells propagated from IFN-\gamma\textsuperscript{-/-} mice were used to ensure that the induction does not involve IFN-\gamma. Fig. 2A shows representative semiquantitative RT-PCR analyses of RNA obtained from cocultures in the presence of various Abs. The relative level of \textit{IgMa} induction in each coculture

\begin{figure}[h]
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\caption{Induction of \textit{IgMa} transcription by NK cells is regulated by elements in the 3’ end of the \textit{H} chain \textit{C} region locus. \textit{A}, Schematic of the transgenes in the 847, 858, and 822 lines. \textit{B}, The three lines of transgenic mice were stained, along with the parental strain, C57BL/6, with allotypespecific anti-IgM, as indicated, to assess the extent of allelic exclusion. C, Resting B cells from each of the indicated strains were cultured for 48 h either with LPS plus IFN-\gamma or with NK cells from IFN-\gamma\textsuperscript{-/-} mice. RNA extracted from each culture was analyzed by RT-PCR assay using specific primers for transcripts derived from germline (\textit{IgMa}) or the productively rearranged gene (\textit{Sy2a}, 635 bp). The \textit{IgMa} PCR product (399 bp) from the transgene was distinguished from that from the endogenous locus by digestion with \textit{Ase}I, which yielded a smaller species of 319 bp.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{Requirement for CD48 expression for induction of \textit{IgMa} transcription by NK cells. Column-purified, resting B cells from IFN-\gamma\textsuperscript{-/-} BALB/c mice were cultured for 2 days with or without day 7–10 IL-2-propagated NK purified from the same mice (NK:B ratio = 0.5:1) in the presence of various additives as indicated. \textit{A}, \textit{Left} and \textit{right} panels show the semiquantitative RT-PCR analysis of two representative experiments using \gamma2a \textsuperscript{-32P}-labeled primers. \textit{B}, Products fractionated on agarose gels were visualized, and band intensities were quantified on the ImagQuant. The relative level of amplified \textit{IgMa} cDNA was determined by normalization to that of amplified UM cDNA for each sample. Percent change was calculated by dividing the relative \textit{IgMa} level in B cells cultured with NK cells in the presence of additives by the relative level obtained from cultures without Abs. The average values obtained from three to four experiments are shown together with the SEM.}
\end{figure}
was determined as a function of the level of mRNA for membrane IgM (μM mRNA). The effect of the inclusion of Abs are compared in Fig. 2B in which the relative levels of induced Iγ2a transcripts is shown as a percentage of the levels induced in the absence of inhibitors. Thus, neither anti-CD28 nor anti-CD40L, both hamster Abs directed against ligands previously reported to be on NK cells (16, 29, 36), altered the level of induction of Iγ2a transcription nor did a hamster Ab against TCRαβ chains, added as a specificity control, because they are not expressed on the purified, IL-2-propagated NK cells. Therefore, it is unlikely that cross-linking of NK cells to B cells by virtue of the binding of the Ig Fc portion to the B cell FcRII affected the induction. The addition of anti-B220 Abs, which can recognize determinants on both B and NK cells, also had no effect, further indicating that binding to FcRs alone does not affect Iγ2a induction. In contrast, two different mAbs against CD48 significantly inhibited the interaction. Although the extent of inhibition by BCM1 (hamster Ab) was greater than that by 1G10 (rat Ab), the inhibition was partially reversed by the inclusion of anti-FcR Abs in the cocultures. Therefore, ligation of FcR on NK cells may have augmented the inhibition by these Abs. However, the F(ab')2 of the anti-CD48 Ab also significantly inhibited the induction, whereas neither the F(ab')1 of anti-CD28 nor that of anti-IgM caused significant decreases.

**Induction of Iγ2a requires the presence of CD48 on B cells**

A problem with inhibition studies with anti-CD48 Abs is that this receptor is expressed by both B and NK cells; thus it is not clear which cell type is affected. We therefore sought an alternate method to remove CD48. Because CD48 is known to be a GPI-linked protein (37), we attempted to remove it from B cells by enzymatic treatment with PI-PLC. The number of known GPI-linked B cell surface Ags is limited (38). Other than CD48, those expressed on B cells include CD24 and CD14, the LPS receptor, and a minor subset of IgD (39). Fig. 3A shows that treatment with 0.5 U of PI-PLC for 2 h effectively removed all of the CD48 but did not affect the expression of IgD or CD19. Furthermore, CD48 was not regenerated even after an incubation period of 24 h. The induction of B cell Iγ2a transcripts by IL-2-propagated NK cells was significantly reduced when the B cells were first treated with PI-PLC (Fig. 3B). Induction of Iγ2a transcripts by LPS and IFN-γ was also reduced, probably due to the removal of the CD14 portion of the functional LPS receptor by PI-PLC (40); however, the retention of substantial induction by this pathway suggests that the B cells were not rendered totally inert by the treatment with PI-PLC.

It is also possible to modulate CD48 in vivo by the injection of anti-CD48 Abs (26). Examination of the PBL from animals injected with anti-CD48 showed that virtually all of the detectable CD48 Ag on all cells was eliminated by this treatment (Fig. 4A). Furthermore, as indicated by staining with an anti-hamster Ab, very little of the Ab remained on the cell surface (3.6%) of the modulated cells. We therefore prepared resting B cells from animals treated in this manner. FACS analysis after purification...
showed that, although 5% of the CD19-positive cells remained resistant to the modulation (Fig. 4B), the B cells were dramatically compromised (78% inhibition) in their ability to express Ifn-γ transcripts in response to stimulation by NK cells (C). In comparison, the response to LPS plus Ifn-γ induction was affected to a much lesser degree (36% inhibition). Thus, removal of CD48 from only B cells by two alternative methods resulted in a compromised response to NK cells. Fig. 4C also shows that the low level of Ifn-γ expressed by B cells cultured on their own was not increased when the B cells were obtained from anti-CD48-treated mice, indicating that the in vivo encounter with the Ab did not enhance Ifn-γ transcription.

**Ligands on NK cells involved in the induction of Ifn-γ transcripts**

There are at least two receptors expressed on NK cells for CD48, CD2 and CD244. To determine whether either could be involved in the NK-B cell interaction, we prepared NK cells from animals with deletion mutations of the genes encoding CD2 and CD244. Because the induction of Ifn-γ by NK cells is amplified by the addition of Ifn-γ, we also included anti-Ifn-γ in the cocultures to restrict the effect to that mediated by cell-cell interactions. Fig. 5A shows that, in comparison to induction by NK cells propagated from B6 cells, the level of Ifn-γ transcripts induced by NK cells from CD2−/− animals was significantly compromised. Induction by NK cells propagated from CD244−/− animals was also reduced, although not to an equivalent extent. The titration of Ly49 mRNA levels expressed in NK cells in the cocultures did not reveal significant differences between the strains, indicating that the lower level of induction by CD2−/− NK cells was not due to reduced viability of these cells. Fig. 5B summarizes results of numerous experiments performed with NK cells obtained from various strains. Although accompanied by some variability due probably to the use of different preparations of propagated NK cells, the mean level of Ifn-γ transcription derived from CD2−/− NK cell stimulation was consistently lower than that from B6 NK cells both in the presence ($p \leq 0.0003$) and absence of anti-Ifn-γ ($p \leq 0.002$). In the presence of anti-Ifn-γ, the stimulatory activity of CD2−/− NK cells was also significantly lower than that by CD244−/− NK cells ($p \leq 0.003$). Although the stimulatory activity of CD244−/− NK cells was in general lower than that by B6 NK cells, the difference did not achieve statistical significance ($p \geq 0.065$). As shown previously, NK cells propagated from Ifn-γ−/− mice in general induced somewhat lower levels of Ifn-γ transcripts and approximated that induced by B6 NK cells in the presence of anti-Ifn-γ. Induction by either CD2−/− or CD244−/− NK cells in the presence of anti-Ifn-γ was significantly decreased ($p \leq 0.0003$ and $p = 0.007$) compared with induction of Ifn-γ−/− cells. Because absence of either CD2 or CD44 reduced the level of induction to below background levels, it is possible that both ligands play a role. However, CD2 appears to be the major inducer.

There is a possibility that deletion of either CD2 or CD244 altered the level of expression of the alternate receptor for CD48 in each strain. Therefore, we evaluated the propagated NK cells from each of the mutant strains for the expression of CD244 and CD2. Fig. 6 shows that, when compared with C57BL/6 NK cells, no significant difference in the expression of CD2 on CD244−/− cells or CD244 on CD2−/− cells was found. The lack of expression of the appropriate Ag on each mutant strain was also confirmed. Because only CD2, but not CD44, is expressed on B cells there is a possibility that the differential response to the CD2 vs CD244 ligand is modulated by the expression of CD2 on B cells. However, we found that the level of induction of Ifn-γ was not affected when B cells from CD2-deficient mice were cocultured with NK cells (data not shown). Therefore, CD2 on B cells does not play a role in the interaction.

**Discussion**

We have previously shown that both NK and activated T cells can induce B cell expression of γ2a germline transcripts in the absence of Ifn-γ (5). Thus, despite the requirement for Ifn-γ in the induction of IgG2a by LPS, stimulation by other Ags may not require Ifn-γ (41). LPS stimulates B cells via TLR, but the nature of the surface ligand(s) involved in NK stimulation of B cells was, until now, not clear. By the use of a number of Abs, including many that are directed toward determinants expressed on NK and B cells, we determined that the only reagents that consistently

**FIGURE 5.** The absence of CD2 expression on NK cells reduces their ability to stimulate B cell-Ifn-γ transcription. IL-2-propagated NK cells from CD2−/−, CD244−/−, or C57BL/6 mice were cultured with column-purified, resting B cells from BALB/c Ifn-γ−/− mice in the presence of polyI:C. A. A representative ethidium bromide-stained gel of 2-fold stepwise dilutions of CDNA obtained from each culture amplified with γ-32P-labeled primers specific for Ifn-γ, μM, or Ly49 sequences. B. The relative mean level (±SD of the mean) of at least three experiments from cells cultured either with or without anti-Ifn-γ, as indicated, were quantified by ImagQuant. Experiments from Ifn-γ−/− NK cells were also added as additional controls. Two-sample t tests assuming unequal variances yielded two-tailed $p$ values testing for the probability that the two samples are the same.

**FIGURE 6.** Expression of ligands for CD48 on propagated NK cells from mutant strains. Day 7 IL-2-propagated NK cells from C57BL/6, CD244−/−, or CD2−/− mice were stained with either PE-rat isotype control, PE-rat anti-CD2, or PE-mouse anti-CD244 Abs and analyzed by flow cytometry.

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demonstrated inhibition of the induction of IgY2a by NK cells are two anti-CD48 Abs (Fig. 2). The use of Abs to inhibit cell-cell interactions can be problematic due to interactions with the FcR of either B or NK cells that can result in either inhibition or stimulation of the response. Therefore, it is significant that the inhibition of the response was also apparent when F(ab’)2 of anti-CD48 Abs were used although the extent of decrease was somewhat less. The reduced level of inhibition may be attributed to anti-CD48 stimulation of NK cells that also express the ligand.

The removal of CD48 from B cells by either PI-PLC treatment (Fig. 3) or by in vivo modulation with anti-CD48 Abs (Fig. 4) significantly reduces the level of induction of IgY2a transcription by NK cells. This is the best indication that the CD48 on B cells (not that on NK cells) is critical for the induction. Whereas the reduction of LPS responsiveness in B cells treated with PI-PLC can most likely be attributed to the removal of the LPS receptor, CD14, the reason for the lower LPS response of the anti-CD48-modulated B cells is not clear. Although the B cell response to LPS was only minimally compromised in the CD48-/- mouse, other aspects of B cell function have not been examined (42).

Further evidence for the importance of CD48 in the NK stimulation of B cells is derived from our finding that the induction of IgY2a was significantly compromised when NK cells from mice defective in expression of CD2, the murine ligand for CD48. CD48 is anchored to the membrane via a GPI linkage. In the absence of the cytoplasmic domain, how the signal arising from interaction with the CD2 ligand is transmitted to B cells is not clear. However, GPI-anchored receptors have been shown to coimmunoprecipitate with various members of the SRC family of tyrosine kinases in glycolipid-enriched microdomains (43) as well as G protein subunits (44). Inasmuch as the removal of CD48 from B cells by either Ab modulation or PI-PLC treatment did not reduce the induction to background levels, other compensatory interactions may be involved. Considering the weak charge-charge interactions of CD2 with its ligands, which nonetheless exhibit strong specificity (45), one possibility is that any encounter between CD2 and CD48 only serves to initiate the interaction between the two cell types such that another receptor-ligand pair can provide the final stimulation. In the absence of CD48 or its ligand, other interaction molecules may continue to function, albeit much less efficiently. For example, CD48 has been shown to deliver an accessory signal for CD40-mediated activation of human B cells (46). The need for costimulation is further suggested by the observation that stimulation of B cells by anti-CD48 or F(ab’)2 of anti-CD48 was not sufficient for the induction of IgY2a transcription (data not shown). Furthermore, the inclusion of anti-CD48 in B cell cultures stimulated with LPS plus IFN-γ did not result in any augmentation of the induction (Fig. 2A). Finally, it is interesting that, although CD244 has been shown to have a higher affinity for CD48 than CD2, the absence of this receptor did not exert as profound an effect as the absence of CD2. This was not attributed to a compensatory increase in CD2 expression in the NK cells from this mutant mouse, nor is the expression of CD244 decreased in CD2-/- cells (Fig. 6). Therefore, the B cell signaling pathways and/or coreceptors induced by the two ligands may differ. Another possibility is that, because of its higher affinity, the interaction of CD244 with CD48 on NK cells in some way reduced the fraction of functional ligands remaining for interaction with CD48 expressed on B cells. CD48 has been shown to be important for the inhibitory function of CD244 on NK cells (27); however, how this factor could affect the role of CD244 as a ligand for activation of B cells is not clear.

The activation of IgY2a transcription despite the engagement, by LPS vs NK cells of different cell surface receptors on B cells, and possibly different intracellular signaling pathways, must ultimately result in induction of common transcription factors necessary for binding to the cis-regulatory sequences located in the 3’ end of the H chain locus (Fig. 1). In contrast to induction by LPS plus IFN-γ or activated T cells, however, NK cell induction does not result in productive switch recombination to IgG2a. The absence of switched γ2a sequences in the presence of increased abundance of germline transcripts further confirms that the induction by NK cells is not attributed to expansion of small numbers of preactivated B cells in the cocultures. The finding that NK cells, by themselves, can only stimulate the first few steps of switch recombination without inducing the terminal steps is not unreasonable, because one would not expect any encounter of NK cells with B cells in vivo to result in IgG2a secretion. It is likely that this interaction only skews the B cells to this response upon interaction with cognate Ag. Using monoclonal B cells from the quasi-monoclonal mouse (48), we have recently obtained evidence for this hypothesis (unpublished observations). This priming step may explain in vivo findings that many viral infections tend to result in Ig responses that are skewed toward IgG2a (49). Thus, in vivo stimulation of NK cells by poly(I:C), which results from stimulation by similar cytokines as those resulting from viral infections, also causes preferential increases in Ag-specific IgG2a production (50). Now that we have some information regarding the receptor-ligand pairs involved in the interaction between NK and B cells, it may be possible to determine their relative importance in the in vivo response to antigenic stimulation.

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Disclosures
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References
12. Pinaud, E., A. A. Khamlichi, C. Le Morvan, M. Drouet, V. Nalesso, M. Le Bert, and M. Cogne. 2001. Localization of the 3’ transcribed leader in IgY2a by NK cells is derived from our finding that the induction of IgY2a transcription despite the engagement, by LPS vs NK cells of different cell surface receptors on B cells, and