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NK Cell Enhancement of Antigen Presentation by B Lymphocytes

Paula Jennings*† and Dorothy Yuan2†

Ag presentation to CD4 T cells can be mediated by a number of cell types depending on the anatomical site in which Ag is first encountered. For blood borne Ags, cells localized in situ in the spleen should be major players. There is now much evidence that B cell Ag presentation may be particularly important in the priming of memory T cells. The majority of NK cells are also localized in the spleen. Inasmuch as we have previously shown that NK cells can modulate various aspects of B cell differentiation, we entertained the possibility that NK cells can also influence Ag presentation by B cells. By specific depletion of NK cells before immunization, we show herein that NK cells play an important role in modulating the ability of B cells to process and present Ag to T cells. These effects are particularly important in the generation of memory T cells. The findings are further substantiated by in vitro experiments showing that the enhancement does not require IFN-γ but is mediated by direct cell-cell interaction. These results show, for the first time, that the rapid activation of a component of the innate response can even exert effects on the Ag-specific memory response. The Journal of Immunology, 2009, 182: 2879–2887.

NK cells as part of the innate system can influence the Ag-specific responses mediated by the adaptive immune system. There is much evidence that this modulation is dependent on activation of dendritic cells (DC) or macrophages that are stimulated by pathogens to initiate cytokine circuits that can in turn activate NK cells (1–3). The signal given to accessory cells by pathogens can be substituted by Poly(I:C) or adjuvant stimulation, which induces a cytokine circuit that can activate NK cells resulting in the enhancement of Ag specific switching to both IgG1 and IgG2c in responses to model T cell independent Ags (4, 5). In addition to effects resulting from NK cell production of cytokines, there is also in vitro evidence that NK cells can activate B cells via direct cell contact even in the absence of IFN-γ (6, 7).

To assess the relevance of these findings to Ag-specific responses in vivo, we have examined the role of NK cells on B cell responses to stimulation by T cell dependent (TD) Ags. Our results show that depletion of NK cells resulted in minimal effects on the primary B cell response. Interestingly, however, Ag-specific CD4 T cell proliferation was significantly reduced. Furthermore, the absence of NK cells during priming reduced their ability to help B cells during a subsequent Ag-specific challenge. Thus, it is possible that NK cells play a role on memory T cell development. Because B cell Ag presentation may be necessary for optimal primary T cell proliferation (8–11) as well as subsequent expansion of memory T cells (12), we examined the effect of NK cells on B cell Ag presentation and showed that NK cells can indeed enhance both Ag processing and presentation by B lymphocytes; therefore, the effects of NK cells on memory T cell development in vivo may be due to a direct consequence of NK-cell augmentation of B cell Ag presentation. These findings were further substantiated by examining the effect of NK cell depletion on Ab responses in mixed bone marrow chimeras reconstituted with MHC class II (MHC II) negative B cells. The results of these experiments show that in addition to the amplification of the cytokine circuit, which directly affects Ig switch recombination, activated NK cells can also influence the ability of B cells to present Ag to T cells, resulting in altered extent of T cell help.

Materials and Methods

Mice

BALB/c, C57BL/6, JHD (13), B6.PK136 (14), OT-II (15), and D011 mice (16) were bred and maintained under specific pathogen-free conditions at the Utah Southwestern Animal Resources Center. Breeding pairs of BALB/c-IgG2c–Ifg–/H11002 and B6.129S2-Ciita+/40 (MHC II−/−, Ref. 18) mice were purchased from The Jackson Laboratory.

In vivo NK cell depletion

B6 and BALB/c mice were depleted of NK cells by i.p. injection of either 75 μg anti-NK1.1 Abs on days 4 and 2 before immunization or a single injection of 20 μl of anti-asialo GM1 Abs on day 1. This treatment did not deplete NKT cells and kept NK cells depleted for ~2 wk (anti-NK1.1) or at least 4 days (Fig. 1, E–G). On a routine basis, the presence of NK cells was assessed by staining PBLs with anti-NK1.1, and/or anti-DX5 and anti-CD3, collected on the FACScan flow cytometer and analyzed on CellQuest (BD Bioscience).

Bone marrow chimeras

Mixed bone marrow chimeras were generated according to Crawford et al. (12) to obtain mice that exhibited MHC II deficiency only on B cells (B(MHC II−/−)). Bone marrow cells harvested from B(MHC II−/−) and JHD mice were mixed at a ratio of 3:1 and 1–2 × 107 cells were transferred into lethally irradiated B6.PK136 (14) or B6 hosts (850 Gy 137Cs gamma-irradiation (γ Cell 40; Atomic Energy)). Chimerism was confirmed by staining with biotinylated anti-MHC II plus streptavidin (SA)-allophycocyanin and FITC-anti-CD19 or anti-IgM.
**Ab responses**

For primary Ab responses, mice were immunized i.p. with TNP-KLH in Ribi. One group was left untreated (open symbols) while the other was treated with anti-asialo GM1 on day −1 (closed symbols). A. After 10 days, sera were collected by tail bleeds and the primary response was analyzed by subclass-specific ELISA on TNP-BSA coated plates. After another 35 days (45 days after initial immunization), when the anti-asialo GM1-treated group was found to have recovered the NK cell compartment (data not shown), both groups were then challenged i.p. with FL-KLH given in PBS. Sera were collected after 10 days and analyzed by subclass specific ELISA on FL-BSA coated plates for the primary B responses in the context of primed T cell help (B) and on KLH coated plates for both the primary and secondary B cell responses (C). One representative of two different experiments is presented. D. One group of C57BL/6 mice were depleted of NK cells with anti-NK1.1 (gray symbols) as described in Materials and Methods before they were immunized with KLH in Ribi, along with control mice (open symbols) and with B6.PK136 mice (solid symbols). Forty-five days later, all mice were challenged with FL-KLH (i.p. in PBS) and Ab responses at day 10 were assessed. Each symbol represents one mouse except for the horizontal bars that indicate the average. Preimmunization levels were all below 0.15 and were subtracted from the values shown. Statistical significance is indicated when p values were ≤0.05, obtained by two-tailed unpaired Student’s t test. Liver monocytes isolated from mice treated with anti-asialo GM1 as in A or with anti-NK1.1 as in C were stained with the indicated Abs (E and F, respectively), and analyzed by FACS. G. FACS analysis of splenocytes obtained from the same animals in E.

**T cell responses**

For up-regulation of CD69 on T cells, 2 × 10^6 purified, CFSE-labeled T cells from OT-II mice were transferred by tail vein injection i.v. 1 day before immunization with 25 μg OVA in Ribi. Eight to 16 h later, spleens and mesenteric lymph nodes (MLNs) were analyzed by FACS. For in vivo T cell proliferation, 5 × 10^6 purified, CFSE labeled T cells from OT-II mice were transferred by tail vein injection (i.v.) 1 day before immunization with 25 μg OVA in Ribi. Spleens and MLNs were collected on D3 after immunization, the earliest time point at which proliferation was clearly detectable. Cells were then stained with PE anti-CD4 and the extent T cell proliferation was determined in the spleen and MLNs by quantifying the percent of CFSE cells in all of the gates that presented at least half of the fluorescent intensity of the cells present in the gate displaying the highest fluorescent intensity.

**CFSE labeling**

Cells were resuspended in PBS at 1 × 10^7 cells/ml and incubated for 15 min at 37°C with 10 μM or 5 μM carboxyfluorescein diacetate (Molecular Probes) for assessment of proliferation and CD69 up-regulation, respectively.
ELISA

Ig ELISA was performed as previously described (4). The isotypes and the subclasses of bound IgG were detected by HRP-conjugated, isotype-specific anti-mouse Ig Abs (Southern Biotechnology Associates) and developed with the substrate, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich). Results show represent dilutions that are most sensitive to changes in Ab levels. ELISA plates were read by an automated ELISA reader (Molecular Diagnostics) at OD 405 nm.

Preparation of cells

Resting B cells were prepared from C57BL/6 mice. After complement-mediated T cell depletion, splenocytes were fractionated on Percoll gradients by centrifugation (19). High-density cells were then labeled with biotinylated anti-CD43 followed by depletion of non-B cells using SA-coupled magnetic beads (BD Biosciences). Some experiments anti-CD3+ labeled cells were sorted using APC-conjugated SA on a FACS DIVA (BD Biosciences).

T cells were purified from OT-II B6 mice. Splenocytes were passed through a nylon wool column and then negatively selected using biotinylated anti-MHC II and PE anti-CD5 and magnetic beads (SA and anti-PE conjugated, respectively). The purified population contained CD8+ and CD4+ cells; the latter group being specific for the OVA derived peptide (aa 323-339, abbreviated as OVA3) in the context of MHC II.

NK cells were obtained from IFN-γ−/− BALB/c mice. Splenocytes were passed through a nylon wool column and then positively selected using anti-CD5 and magnetic beads. NK cells were then propagated in 10% fetal calf serum at 1,300 rad (137Cs gamma-irradiation), and 80% of the culture supernatant was removed and 0.1 ml of T cells at 1 each well. After 2 days, 0.5 ml cells were harvested the next day for determination of incorporated [3H]thymidine. Results show represent dilutions that are most sensitive to changes in Ab levels. ELISA plates were read by an automated ELISA reader (Molecular Diagnostics) at OD 405 nm.

In vitro T cell proliferation

Two × 10⁶ B cells were cultured overnight, in 96-well plates, alone or in the presence of cultured NK cells (1 × 10⁶) with or without OVA or OVA3, in a final volume of 0.1 ml (Ag concentrations were titrated so that the T cell proliferation was within the linear range). Cells were then irradiated at 1,300 rad (137Cs gamma-irradiation), and 80% of the culture supernatant was removed and 0.1 ml of T cells at 1 × 10⁶/ml were added to each well. After 2 days, 0.5 μCi of [3H]thymidine was added per well and cells were harvested the next day for determination of incorporated [3H]thymidine.

Antibodies

Mouse anti-NK1.1 (for in vivo injection), anti-CD43 (for B cell purification) and anti-thy 1.2 (for in vitro T cell depletion) were purified from hybridoma culture supernatants (PK136, S11 and J1.10, respectively) using GammaBind (PharmaCyt BioTech). Anti-asialo GM1 was purchased from WAKO). Anti-MHC II, anti-CD19, and anti-CD4 were purchased from eBioscience. Anti-CD5, anti-IgM, anti-CD3, anti-IL2rβ, and SA-APC were purchased from BD Biosciences. Polyclonal rabbit anti-NKp46 was purchased from R&D Systems. Anti-CD69 was purchased from Biolegend. HRP-conjugated Abs for ELISA were purchased from Southern Biotechnology Associates. All Abs were titrated before use. Goat anti-NKp46 and donkey anti-Goat Ig was purchased from R&D Systems.

Results

Effect of NK cell depletion on T cell help for the Ab response to a TD Ag

We have previously shown in C57BL/6 mice that the B cell response to T cell-dependent Ags is not affected by the presence or absence of NK cells (4). To confirm these results in BALB/c mice, which generally yield higher TD responses, we treated them with anti-asialo GM1 1 day before immunization with TNP-KLH in the presence of the adjuvant Ribi. As seen by the ELISA results (Fig. 1A), no apparent difference in Ag specific Ig titers was detectable between NK cell depleted and nondepleted mice. Therefore, NK cells also do not play a role in the Ab response to a TD Ag in BALB/c mice. To determine whether the depletion of NK cells during the initial priming of T cells affects the generation of T cell memory we assessed their ability to help B cells by examining the Ab response in the same groups of mice to the same carrier but conjugated to a different hapten, fluorescein (FL). At this time (45 days after the initial depletion), the NK cell compartment has returned to control levels. Immunization with the Ag in PBS ascertained that these NK cells would not be activated by adjuvant, and any response observed would be mostly due to help from memory T cells generated during the primary response to KLH. The responses in control animals (Fig. 1B) show that memory T cells were able to help B cells in the absence of adjuvant. Significantly, however, mice that were depleted of NK cells during priming mounted a reduced secondary IgG1 response but the Ig titers of other isotypes were not affected (even though the levels found for the other isotypes were lower, they were still well within detection limits). Because the effect of NK cell depletion was detected only during the secondary response, we infer a role for these cells in the generation of memory T cells. We also measured both the primary and secondary response to the carrier protein KLH in the same groups of mice. Fig. 1C shows that, as expected, total KLH specific IgG1 responses were greatly enhanced with the second immunization and depletion of NK cells during priming also decreased the secondary B cell response. Thus, whereas it is clear that NK cells play a role in the priming of T cells; due to the increased T cell help whether NK cells are also important for the generation of memory B cells cannot be assessed by this experimental protocol. Another group of animals that were previously primed with TNP-KLH were depleted of NK cells immediately before challenge with FL-KLH. The IgG1 responses of these animals were not affected (data not shown) confirming our previous results showing that once primed, depletion of NK cells does not affect the ability of the T cells to help (4). These results were reproduced in another experiment (data not shown). Assessment of the effect of NK cells in BALB/c mice necessitated the use of anti-asialo GM1, which may affect other cell types in addition to NK cells in unpredictable ways. Therefore, we repeated the experiment in C57BL/6 mice. In this case, a group of mice was depleted of NK cells by treatment with anti-NK1.1 Abs. In addition, the response of these animals was compared with another group of age-matched mice (B6.PK136) carrying a transgene which generally yields higher TD responses, we treated them with anti-NK1.1 Abs. In addition, the response of these animals was compared with another group of age-matched mice (B6.PK136) carrying a transgene that allows the continuous production of anti-NK1.1 Abs, resulting in the chronic depletion of NK cells (14). All three sets of animals were first primed with KLH in the presence of Ribi. Forty-five days later, they were challenged with FL-KLH in PBS. Fig. 1D shows that all three groups were able to mount FL-specific IgG3, but low IgG2c responses. However, in comparison with control animals, the IgG1 response was significantly reduced in both of the groups depleted of NK cells. Therefore, NK cells play a significant role in the development of memory T cell responses in both BALB/c and C57BL/6 mice.

Because the measured effect of NK cell depletion is primarily on the IgG1 responses, it is possible that these procedures depleted NKT cells. We and others (14, 21) have shown that NKT cells are not usually depleted by these treatments. However, due to the controversy surrounding this possibility (22), we ascertained that under our conditions of NK depletion, the NKT cells remain intact by assessing the representation of NKT cells in liver MNCs, which contains the largest population of NKT cells. Fig. 1E and F show that, despite the depletion of NK cells in BALB/c, C57BL/6, as well as B6.PK136 mice, the NKT cell compartments (CD4⁺, IL2rβ for NK1.1⁺) remain intact, nor did they appear to be expanded. Furthermore, staining with NKP46, another NK cell-specific Ab, shows that the absence of binding by anti-NK1.1 is not due to masking of the determinant by the Ab (Fig. 1G). In any case, even if a minor population of NKT cells were depleted, there is no evidence that NKT cells can affect the specific response to protein Ags.
mice (gray) are shown in cells after Ag injection into NK cell depleted mice (bold line) or control presence (bold line) or absence (gray line) of Ag are shown in FACS. Representative plots of OT-II T cells after injection of Ribi in the Ribi only. Spleen cells harvested on day 3 were directly analyzed by Materials and Methods.

dent’s t test. Considering the extent of CFSE dilution of T cells injected into MLNs were harvested and T cell proliferation was assessed by OVAp, in the context of MHC II. Three days later, the spleens and JHD mice were depleted of NK cells before T cell transfer and immunization. Fig. 3, B and C show that deletion of NK cells did not further reduce T cell proliferation. Because deletion of NK cells no longer altered the T cell response in the absence of B cells it appears that much of the effect of NK cells on T cell proliferation is dependent on B cells.

To measure T cell activation by means of an alternative assay, which is more proximal to the activation event, the effect of NK cell depletion was assessed by monitoring the up-regulation of the activation marker CD69 on Ag-specific T cells. T cells from OT-II

Effect of NK cell depletion on Ag dependent T cell expansion

The development of memory T cells has been shown to be correlated with the expansion of T cells during the primary response (23). To determine whether NK cells can affect initial T cell expansion, an assay to assess T cell proliferation was performed. One day before immunization with OVA, CFSE-labeled T cells from OT-II transgenic mice were transferred into control or anti-NK1.1-treated C57BL/6 mice. The CD4+ T cells from OT-II transgenic mice recognize the OVA-derived peptide (residues 323–339), OVAp, in the context of MHC II. Three days later, the spleens and MLNs were harvested and T cell proliferation was assessed by CFSE dilution by FACS. Fig. 2A confirms that OT-II T cell proliferation was dependent on the presence of Ag. Representative plots showing the extent of CFSE dilution of T cells injected into a NK cell-depleted mouse compared with that in the control mouse (Fig. 2B) indicate that, in the absence of NK cells, the level of T cell proliferation was reduced. The relative percent decrease from comparison of five pairs of individual animals (Fig. 2C) indicates an average of 20%. Thus, the presence of NK cells is required for optimal T cell expansion following immunization.

Depletion of NK cells does not affect T cell expansion in the absence of B cells

The reduction in T cell proliferation in the absence of NK cells could be attributed to a number of factors. However, there is a considerable amount of evidence indicating that B cells are required for optimal primary CD4+ T cell expansion as well as memory cell generation (8–11) and it is possible that NK cells affect this B cell function. To confirm the requirement for B cells CFSE-labeled T cells from OT-II mice were transferred into wild-type (WT) or JHD mice, which lack B cells. Indeed, primary T cell expansion in response to specific Ag was reduced in the absence of B cells (Fig. 3A). To determine whether the residual response was dependent on NK cells, JHD mice were depleted of NK cells before T cell transfer and immunization. Fig. 3, B and C show that deletion of NK cells did not further reduce T cell proliferation. Because deletion of NK cells no longer altered the T cell response in the absence of B cells it appears that much of the effect of NK cells on T cell proliferation is dependent on B cells.

To measure T cell activation by means of an alternative assay, which is more proximal to the activation event, the effect of NK cell depletion was assessed by monitoring the up-regulation of the activation marker CD69 on Ag-specific T cells. T cells from OT-II
mice were purified and labeled with CFSE. Two × 10^6 T cells were transferred i.v. into B6 and JHD mice that were either depleted or not depleted of NK cells followed by i.p. injection of OVA (25 μg per mouse in adjuvant). Eight to sixteenth hours later, the spleens were harvested and stained for CD69 expression on CD4^+ cells. Fig. 3, D and E shows that only CFSE-labeled CD4^+ cells exhibited up-regulation of CD69. As observed for T cell proliferation, depletion of NK cells reduced T cell activation to a significant extent in B6 mice but not in JHD mice (Fig. 3, E and F).

**NK cell effect on T cell proliferation requires B cell MHC II expression**

Previous reports have indicated that B cell deficient mice display some intrinsic complications such as defects in dendritic cell (DC) representation and in abnormalities in splenic architecture (24, 25) which may affect immune responses. To address the possibility that our results may have been skewed by these defects, mixed bone marrow radiation chimeras were generated, so that the only B cells lacked MHC II expression (B/(MHC II^-/-) mice; Ref. 12). The hosts were either B6 or B6.PK136 mice that constitutively secrete anti-NK1.1 Abs. Two months after reconstitution, FACS analysis of the PBLs confirmed that all animals were reconstituted with MHC II^+ cells, albeit at somewhat lower levels than that in WT mice. Importantly, however, the B cells remained MHC II^+, and the NK cells in the B6.PK136 hosts remained depleted (Fig. 4A). The proliferative response of transferred T cells from OT-II mice to OVA was then assessed in each animal. Fig. 4B shows that T cell proliferation was reduced in the chimeras compared with control B6 mice confirming that the T cell expansion is partially dependent on MHC II expression on B cells. If the effect of NK cells on primary T cell expansion requires B cell Ag presentation, the absence of NK cells should not affect T cell proliferation in these chimeras. Fig. 4C shows that indeed NK cell depletion caused no further reduction in the response of these chimeras, supporting our original conclusion that the effect of NK cells on T cell proliferation is dependent on B cells.

**Reconstitution of Ab response in B/(MHC II^-/-) chimeras**

To determine whether reintroduction of MHC II^+ B cells into the B/(MHC II^-/-) chimeras made in either B6 or B6.PK136 hosts can increase the T cell response, 1.5 × 10^7 resting B cells were injected into the mice. One day later T cells from OT-II animals were collected and injected with OVA in adjuvant. The T cell proliferative response observed in the reconstituted animals was not significantly higher than in nonreconstituted animals although FACS analyses indicated that MHC II^+ B cells could be detected in the spleens (data not shown). It is possible, however, that the extent of reconstitution was insufficient to influence the level of detectable T cell proliferation.

To test the functionality of the injected WT B cells by a more sensitive assessment, we immunized the mice with FL-KLH in adjuvant. Fig. 4D shows that, all of the mice, including hosts that were deficient in NK cells and regardless of whether they were injected with B cells, mounted detectable IgM and IgG3 responses. Thus, even in the absence of MHC II^+ B cells, Ag presentation by other APCs is sufficient to support these responses. However, with the exception of one mouse, the more T cell-dependent responses, IgG1 and IgG2, were very low, confirming that B cell reconstitution by the injected resting B cells was not optimal. To test whether these mice can, nevertheless, generate T cell memory, 30 days later, all mice, including those not originally reconstituted with B cells, were given another injection of 1.5 × 10^7 MHC II intact B cells, and then challenged with an alternative hapten on the same carrier, TNP-KLH in PBS. Due to the large variability of Ab responses from these reconstituted mice the difference between groups did not achieve statistical significance. However, when the values from two different independent experiments were combined as shown in Fig. 4E, the IgG1 response between groups were clearly different. Thus, the presence of B cells during priming increased the ability of T cells to help a primary B cell response (nine of nine reconstituted animals vs one of eight nonreconstituted animals (p ≤ 0.09) confirming previous results that B cell Ag presentation is optimal for T cell memory generation. In contrast, with the exception of one mouse, nine of ten B/(MHC II^-/-) chimeras made in B6.PK136 mice did not respond (p ≤ 0.03). Thus, despite the inability of the introduced B cells to drive a significant level of T cell proliferation, they were able to induce a population of memory T cells which may be at least partially dependent on the presence of NK cells.

**Enhancement of B cell Ag presentation by NK cells**

NK cells play an important part in the cytokine circuits induced during an immune response. These circuits could be directly responsible for the initial T cell proliferation and, thus, altered cytokines in the absence of NK cells could explain the reduced proliferation seen in Fig. 2. Additionally, as shown by Assarsson et al. (26), it is possible that NK cells can directly enhance T cell proliferation. Another possibility for the effect of NK cells on primary T cell expansion is that NK cells could be modulating Ag presentation by either professional APCs or by B cells. We have previously documented, by in vitro experiments, that NK cells can initiate various aspects of B cell differentiation, such as induction of germline transcription (6), as well as up-regulation of both CD86 and CD69 on resting B cells (27). Therefore, we directly assessed the role of NK cells on B cell Ag presentation in vitro. Ag-specific proliferation of T cells was examined as a reporter for presentation efficiency. CD4^+ T cells from D011 transgenic mice were incubated with B cells purified from BALB/c IFN-γ^-/- B cells and fractionated by Percoll density sedimentation (96–98% CD19^+ MHC II^+). Fig. 5A shows that prior incubation of the B cells with NK cells propagated from IFN-γ^-/- mice can increase presentation of OVA by resting B cells. Because NK cells were propagated from IFN-γ^-/- mice, the enhancement could not be attributed to IFN-γ secreted by NK cells. However, in this experiment, in which NK cells were sygenctic to the responding T cells, the possibility exists that the apparent enhancement came from residual APCs capable of presenting Ag which was retained in the propagated NK cell population. To eliminate this possibility additional experiments were performed with CD4^+ T cells from OTII transgenic mice (Fig. 5, B–E) and Ag presentation by resting B cells from C57BL/6 mice was examined. In addition to intact OVA, which requires processing before it can be presented by MHC II, the response to OVA, which can be directly loaded onto the MHC II, was also assessed. Fig. 5B shows a representative experiment and Fig. 5C shows the average fold increase of five additional experiments. Thus, even across allogeneic disparity, NK cells can enhance the ability of B cells to stimulate Ag specific proliferation. Interestingly, a moderate, albeit significant, increase was also detected when OVA was used (B and C). Therefore, it appears that NK cells can program B cells to enhance Ag presentation by mechanisms that increase both the expression of costimulatory molecules as well as processing of Ag by B cells. The resting, high density, B cells used in the experiments shown are a relatively homogeneous population, which should facilitate detection of any change caused by NK cells. In general, greater T cell proliferation was induced by B cells in the low-density fraction. It was still possible, however, to detect enhancement of presentation by coincubation with NK cells (data not shown). This
population is more heterogenous due to endogenous activation and the interpretation of the effect of NK cell stimulation is therefore, not as clear.

Of note, no effect was seen when freshly isolated NK cells were used (data not shown). Importantly, Fig. 5 shows that, under our conditions, there is no increase in T cell proliferation by the...
addition of NK cells to T cells without Ag or peptide. To ensure that NK cells are not acting on contaminating APCs in the B cell population the above experiments were repeated using highly purified B cells (99% CD19+ MHC II+) obtained by sorting on the FACS (Fig. SD). In addition, if NK cells are separated from B cells during preincubation the enhancement of Ag presentation was eliminated, showing that the effect is dependent on direct contact (Fig. 5E).

Discussion

We have shown that depletion of NK cells during priming does not affect primary Ab responses; however, the secondary T cell response as measured by their ability to help B cells via cognate recognition is reduced. Furthermore, the effect of NK cells being on IgG1, but not IgG2a, levels suggests a role for NK cells that is not totally dependent on enhanced IFN-γ production by these cells. The effect of NK cells was detected even when they were not depleted during the secondary challenge; therefore, the decreased response is not due to a direct effect of NK cells on B cell Ab production. These results are consistent with our previous findings showing that depletion of NK cells after T cell priming has no effect on the Ab response (4). In contrast, NK cells appear to exert their influence on the TD response by enhancing Th cell memory development during their primary encounter with Ag. Others have shown that the extent of T cell proliferation during priming correlates with the extent of T cell memory generation (23). Furthermore, CD4+ memory cell generation is optimal in the presence of Ag presentation by B cells (12, 28). Thus, when we examined a more proximal effect of NK cells on the response to TD Ags by assessing their effect on T cell up-regulation of CD69 expression at 8 h (Fig. 3, D–F) and on T cell proliferation at 3 days after immunization (Fig. 3, A–C), we found that NK cells seem to have an effect on T cell activation only in the presence of B cells and more specifically on B cells that can present Ag (Fig. 4). Importantly, our results also show that under these experimental conditions in which the only manipulation involved was the depletion of NK cells in situ, and which did not require the introduction of exogenously activated DCs (29), NK cells exert their effect primarily via Ag presentation by B cells and not via other APCs.

Despite the observations that NK cell depletion decreases T cell activation, this effect did not reduce the primary Ab response, probably due to T cell help that is independent of NK cells. Certainly, the adjuvant could stimulate various APCs including DCs that can affect T cell help both directly or via cytokines (30, 31). However, we have shown herein, for the first time, that NK cell depletion during priming reduced the Ab responses that is dependent on help from primed T cells. Thus, the expansion of T cells must have been influenced by the initial Ag presentation event, which in turn might be modulated by NK cells. Alternatively, it is possible that the subpopulation of APCs targeted by NK cells preferentially generates memory T cells. A distinction between these mechanisms of NK-cell enhancement will depend on further understanding of CD4+ memory T cell development. There is already evidence, however, that the generation requires specific receptors both during the initial priming and the subsequent expansion of the memory cells (28, 32–34). A prime candidate points to the interaction between OX40 interaction with its ligand, a costimulatory molecule, that can be expressed on both B and other APCs. Thus, one possibility is that NK cells alter the relative expression of this ligand on B cells (35) and thus favor their ability to present Ag.
Whether B cells are important for the generation of T cell responses has been a frequent subject of investigation. A number of studies (36–38) point toward a necessary role. However, other reports suggest that they can be substituted by other cell types (39, 40). Elegant demonstration of the need for B cell Ag presentation in the generation of memory T cells involved the use of B/MHC II−/− chimeras (12). We used a similar experimental scheme to examine the effect of NK cells (Fig. 5, D and E). The detection of IgM and IgG3 responses that are independent of reconstitution of MHC II−/− B cells suggests that the expression of these isotypes are less dependent on presentation by B cells. Importantly, however, despite the low primary IgG1 response it appears that the injected B cells are nonetheless sufficient for the priming of T cells such that they can provide significant help upon secondary challenge. It should be noted that for B cell reconstitution, we injected only resting B cells that had not previously been incubated with Ag. These reconstitution experiments, using resting B cells, while giving somewhat variable results, further extend previous findings (12), by examining the functional capacity of memory T cells generated upon reconstitution of B cells. More importantly, the finding that the depletion of NK cells affected priming in this system provides in vivo evidence that NK cells can enhance the ability of B cells to present Ag. Furthermore, the preferential effect on IgG1 responses suggests that the help from NK cells is unlikely to be solely attributed to their ability to secrete IFN-γ.

Due to the number of cell types involved in the in vivo response, it was not possible to evaluate whether NK cells directly affected B cell Ag presentation in vivo. However, by in vitro experiments we have shown that NK cells can indeed augment Ag presentation by resting B cells (Fig. 5). It should be noted that we verified that these IL-2–propagated NK cell cultures did not contain NKT cells. In addition, using the in vitro assay, we found that IL-2–propagated, but not freshly isolated, NK cells were able to augment presentation by resting B cells. This supports the in vivo findings because TD Ags require the presence of adjuvant to initiate immune responses and a likely pathway for the adjuvant effect is via activation of innate cells that can initiate the cytokine circuit that can in turn activate NK cells (14). Interestingly, greater enhancement by NK cells was observed when Ag but not peptide was used, suggesting additional programming of B cells which results in enhanced ability to process Ag, in addition to the increased expression of costimulatory molecules. Importantly, under our experimental conditions, we did not observe enhanced proliferation of T cells by NK cells alone. This discrepancy with a previous report (26) might be explained by the lower T:NK ratios we used as well as the fact that the NK cells were irradiated before the addition of T cells. We also determined that this enhancement was IFN-γ independent and contact dependent. At present, we have not yet uncovered the nature of the interaction molecules involved. Nonetheless, these results provide the first demonstration of a possible mechanism by which direct interactions between NK and B cells can affect the outcome of B cell responses to specific antigenic challenge.

We have shown that T cell proliferation in both spleen and lymph nodes is affected by the depletion of NK cells and that this effect may be due to enhancement of Ag presentation by B cells; however, the site of initial interaction between NK and B cells in vivo is not clear. The effects of NK cells on T cell proliferation as well as expansion of memory cells were observed only when mice were immunized in the presence of Ribi; however, we were unable to detect significant increases in NK cell representation in the MLNs after this immunization (data not shown). Therefore, NK cell recruitment due to activation by Ribi may be less extensive than that induced by the injection of DCs that have been activated in vitro (29). Furthermore, the interaction between DCs and NK cells can result in negative as well as positive sequelae (41–43). Clearly, however, mature NK cells are localized to the splenic red pulp (44–46) where, upon activation, they are likely to encounter B cells in the marginal zone. Marginal zone B cells have been shown to have enhanced Ag presentation ability (47). Although this B cell subset express CD1d and could present lipid Ags to NKT cells (48), the deficiency in response to the T-dependent protein Ag is unlikely to be due to depletion of CD1 restricted NKT cells. However, whether marginal zone B cells can also respond to NK cells for presentation of MHC class II restricted protein Ags requires further investigation.

Finally, it is clear that the conclusions reached herein are derived from the results of experiments using model Ags. In addition, adequate activation of T cells required the inclusion of adjuvant in the immunogen. Whereas the use of Ribi can certainly activate the cytokine circuit, the extent that it can mimic the effect of various infectious agents is not clear (49, 50). However, the use of this defined system permits the detection of relatively small, yet clear differences that can be attributed to NK cells, and which may play a more significant role in actual infections.

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References


