Introduction

B and T lymphocytes from healthy individuals are tolerant of self-antigens and are activated by foreign antigens. In contrast, although natural killer (NK) cells are also tolerant of normal cells that express self-ligands, NK cells are activated by cells that lack self-ligands. As described in the “missing-self” hypothesis, NK cells monitor for self–major histocompatibility complex (MHC) class I expression and eliminate tumor cells and virally infected cells that lack self-MHC class I. Consequently, healthy allogeneic cells are also targets for NK cells because they lack self-MHC. For self- or non–self-discrimination, NK cells express inhibitory receptors that recognize MHC class I, including members of the human killer cell immunoglobulin (Ig)–like receptor family, murine Ly49 family, and the conserved CD94/NK-cell group 2 (NKG2) receptors. MHC-binding receptors are on overlapping subsets of NK cells; it is thought that all NK cells must express at least one self-MHC–binding inhibitory receptor to achieve self-tolerance.

2B4 (CD244) is expressed by all NK cells, and the ligand for 2B4, CD48, is expressed broadly on hematopoietic cells. Human 2B4 is predominantly activating, although it is inhibitory on immature NK cells and those from patients with X-linked lymphoproliferative disorder. Surprisingly, NK cells from 2B4-deficient mice exhibit enhanced killing of CD48 targets, indicating that murine 2B4 acts as an inhibitory receptor. Differences in 2B4 signaling may stem from contextual influences of signaling lymphocytic activation molecule-associated molecule (src homology 2 domain protein 1A).

There are examples when self-MHC class I inhibition is absent, yet NK cells remain self-tolerant. Humans and mice that have low MHC class I expression due to transporter-associated with antigen processing 2 (TAP-2) protein or β2 microglobulin (β2m) deficiency, have self-tolerant NK cells. NK cells from MHC class I–deficient individuals may have decreased activating receptor function to account for self-tolerance, although other studies disagree, so the mechanism of tolerance remains unknown. Some NK cells in MHC class I–sufficient hosts lack any known self-MHC class I inhibitory receptors; in C57BL/6 H-2b mice, 10% of NK cells lack expression of the H-2b–binding inhibitory receptors, Ly49C, Ly49I, and CD94/NKG2A. Furthermore, there is a window during NK development in which MHC-binding inhibitory receptors are absent, but some effector functions are present, giving rise to a pool of seemingly unregulated immature NK cells.

Self- or non–self-discrimination is often manifest in transplantation settings, wherein T cells recognize allogeneic antigens as foreign, and NK cells recognize allogeneic MHC in terms of missing-self. However, even healthy, autologous cells must meet certain criteria to avoid elimination by immune cells, namely by not expressing foreign antigen and maintaining expression of self-MHC. Herein, we demonstrate that 2B4 represents a second system for murine NK-cell self-recognition in which the widely expressed ligand, CD48, acts as an autonomous marker of normal cells. Previously, we demonstrated that 2B4 inhibits NK-cell responses to tumor cells in vitro and in vivo. We also found that 2B4 inhibits killing of nontransformed allogeneic and syngeneic cells in vitro. The current study extends these observations by (1) comparing the relative contributions of MHC class I and CD48 to NK-cell self-tolerance, (2) determining whether this inhibitory...
system accounts for the self-tolerance of NK cells that are not regulated by MHC class I, and (3) demonstrating that 2B4 regulates NK-cell tolerance to normal, nontransformed cells in vivo.

Study design

Mice, cell lines, and NK-cell preparation

C57BL/6 (H-2b, B6, wild-type [WT]) and Rag-1^{-/-} mice were purchased from the Jackson Laboratories (Bar Harbor, ME) and Rag-1^{-/-} mouse from Taconic (Germantown, NY). Bone marrow rejection assay

Bone marrow rejection assay

For transplantation, bone marrow from WT B6 donor mice was dyed with 2 μM PKH26 (Sigma) for 5 minutes at room temperature, and β2m^{-/-} bone marrow was dyed with 5 μM CFSE (5,6-carboxyfluorescein diacetate, succinimidyl ester; Molecular Probes, Eugene, OR) for 10 minutes at 37°C. WT and β2m^{-/-} cells (10 × 10^5 each) were coinjected intravenously into WT and 2B4^{-/-} mice given 8.5 Gy irradiation the day prior. Mice were depleted of NK cells using 10^7 L-anti–asialo-GM1 (Wako Chemicals, Richmond, VA) intraperitoneally on days −3 and −1 prior to transplantation. After 48 hours, splenocytes were harvested and analyzed by flow cytometry.

Results and discussion

Given that both 2B4 and Ly49 molecules can inhibit NK cells, we wanted to determine whether these two act redundantly to inhibit NK cells. If the systems are redundant self-markers, then one alone would be sufficient to inhibit NK cells. Conversely, if the two systems are nonredundant, then a cell would require both for protection from NK cells. To test this, NK cells from B6 mice were tested for lysis of RMA variants. RMA cells are H-2b^+ and CD48^+; RMA-S cells are TAP-deficient RMA cells, and thus are H-2b^low and CD48^+ or CD48^- WT NK cells exhibited the lowest cytotoxicity against CD48^- RMA (H-2b^+) targets (Figure 1A). Absence of MHC relieved some inhibition as seen with CD48^- RMA-S (H-2b^-) targets. However, maximal cytotoxicity was seen only against the CD48^- H-2b^- targets. In comparison to WT, 2B4^- NK cells demonstrated greater killing of RMA cells (Figure 1A). Removal of MHC class I lead to maximal 2B4^- NK lytic activity, but CD48 had no effect. Over all, these data demonstrate that CD48 and MHC class I provide additive, nonredundant layers of protection from NK cells.

β2M-deficient mice have low MHC class I expression, yet NK cells from β2m^{-/-} mice are tolerant of autologous MHC class Ilow cells.20,21 NK cells of β2m^{-/-} mice express higher levels of Ly49 receptors and thus may be inhibited by the low levels of MHC class I found in such mice.22-24 However, increased sensitivity to low levels of residual MHC is unlikely to account for NK self-tolerance, and NK cells of β2m^{-/-} mice express higher levels of Ly49 receptors, which may be a self-MHC binding inhibitory receptor. Ly49C/I/NKG2A/C/E are nonredundant layers of protection from NK cells. Therefore, we determined whether 2B4 is the inhibitory receptor that maintains NK-cell tolerance in β2m-deficient mice. IL-2–stimulated β2m^{-/-} NK cells were tested for lysis of RMA variants. Like WT, β2m^{-/-} NK cells had the lowest lysis of CD48^- RMA (H-2b^+) (Figure 1B). In comparison, the CD48^- H-2b^- RMA-S targets were more susceptible to β2m^{-/-} NK lysis. But removal of CD48 inhibition permitted substantially more lytic activity by the β2m^{-/-} NK cells. This finding suggests that in the absence of MHC class I, 2B4-CD48 interactions play a dominant role in tolerance.

Recent studies indicate that a proportion of NK cells in C57BL/6 mice lack known class I inhibitory receptors.22 To determine whether 2B4 regulates NK cells lacking self-MHC-binding inhibitory receptors, Ly49C/I^- NKG2A/C/E^- NK cells were sorted from polyI:C-stimulated mice and tested for 2B4 function. If Ly49C/I^+/NKG2A/C/E^- NK cells are hypofunctional,
donor cells were detected. Representative dot plots are shown from experimental group. Error bars represent standard deviations of the pooled data.

marrow is depicted as the ratio of the percentage of potentially self-reactive, are not hypofunctional (Figure 1C).

RMA-S) targets. However, both Ly49C/I/NKG2A/C/E (CFSE-dyed) and Ly49C/I/NKG2A/C/E+ cells lysed CD48- RNA-S cells, indicating that NK cells lacking known inhibitory receptors for H-2h, and thus potentially self-reactive, are not hypofunctional (Figure 1C). Importantly, Ly49C/I- NKG2A/C/E+ cells were inhibited when the target expressed CD48. This finding is consistent with the hypothesis that 2B4-CD48 interaction contributes to the self-inhibition of NK-cell subsets that may be minimally regulated by MHC class I signals.

To investigate whether 2B4 inhibits NK-cell elimination of syngeneic cells in vivo, WT and 2B4-deficient mice were tested for rejection of bone marrow transplants. Mice were injected with CFSE-dyed β2m-deficient B6 bone marrow and an equal number of PKH26-dyed B6 WT bone marrow. Two days later, the spleens were harvested from WT and 2B4−/− recipients, and remaining donor cells were detected. Representative dot plots are shown from one experiment (Figure 2A) as well as the ratio of β2m−/− versus WT donor cells recovered (Figure 2B). WT NK cells rejected β2m−/− bone marrow, as the ratio of β2m−/− versus control WT bone marrow retained in unmanipulated hosts is significantly lower (0.23 ± 0.13) than the corresponding NK-depleted recipients (0.7 ± 0.20; Figure 2B). That this difference is due to NK-cell-mediated rejection of β2m−/− cells is supported by the fact that, in NK-depleted mice, the ratio of β2m−/− versus WT bone marrow cells retained in NK-depleted mice should be 1.0, small differences in counting of cells injected can readily influence this measurement. What is important to note is that the number of β2m−/− bone marrow cells retained in NK-depleted WT and 2B4−/− recipients (6.5% ± 1.8% versus 5.2% ± 0.6%, Figure 2A) is not significantly different.

2B4−/− mice exhibited significantly greater rejection of the β2m−/− cells as compared with WT mice (compare WT rejection ratio of 0.23 ± 0.13 with 2B4−/− rejection ratio of 0.06 ± 0.04, P = .001; Figure 2B). These results indicate that the 2B4-CD48 interaction protects autologous cells from NK-cell rejection in the absence of self-MHC class I.

In vivo we did not find evidence for killing of MHC class I+ syngeneic bone marrow by 2B4−/− mice (Figure 2A; 13.2% ± 1.7% WT cells recovered in WT hosts compared with 11.3% ± 2.1% recovered in 2B4−/− hosts). This may be due to compensation in 2B4−/− mice preventing in vivo autoreactivity, a phenomenon seen for the inhibitory receptor signal regulatory protein alpha (SIRPα). Indeed, it has been shown that anti-CD48 treatment of WT mice prevents engraftment of syngeneic bone marrow.

In summary, we found that NK cells insufficiently regulated by MHC are inhibited from killing syngeneic cells by 2B4-CD48 interaction. Most notably, our results demonstrate the first evidence for non–MHC-mediated NK tolerance in vivo. Because CD48, the ligand for 2B4, is widely expressed on hematopoietic cells, these findings have implications for NK-mediated rejection of normal and malignant hematopoietic cells.

Acknowledgment

IL-2 was obtained through the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (NIAID), NIH.

References

2B4 (CD244)-CD48 interactions provide a novel MHC class I-independent system for NK-cell self-tolerance in mice

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