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NK Cells Inhibit T Cell Proliferation via p21-Mediated Cell Cycle Arrest

Prachi P. Trivedi, Paul C. Roberts, Norbert A. Wolf, and Robert H. Swanborg

NK cells have been shown to influence immune responses via direct interaction with cells of the adaptive immune system, such as dendritic cells, B cells, and T cells. A role for NK cells in down-regulation of T cell responses has been implicated in several studies; however, the underlying mechanism of this suppression has remained elusive. In this study we show that dark Agouti rat NK cells inhibit syngeneic T cell proliferation via up-regulation of the cell cycle inhibitor, p21, resulting in a G0/G1 stage cell cycle arrest. The inhibition is cell-cell contact dependent, reversible, and Ag nonspecific. Interestingly, NK cells do not inhibit IL-2 secretion or IL-2R up-regulation and do not induce T cell death. Thus, our results show that NK cells do not affect early T cell activation events, but specifically inhibit T cell proliferation by direct interaction with T cells. Our findings suggest that NK cells may play an important role in maintaining immune homeostasis by directly regulating clonal expansion of activated T cells. This novel mechanism of T cell regulation by NK cells provides insight into NK cell-mediated regulation of adaptive immunity and provides a mechanistic link between NK cell function and suppression of T cell responses. The Journal of Immunology, 2005, 174: 4590–4597.

N atural killer cells are important effectors of the innate immune system, and their role in antiviral and antitumor immunity has been well researched over the years. More recently, however, a previously unappreciated, immunoregulatory role for NK cells has emerged. It is well documented that NK cells can influence adaptive immune responses by their ability to secrete immunomodulatory cytokines and chemokines (1). Recent studies indicate that NK cells may influence adaptive immune responses by a more direct effect on activation and function of other immune cells, such as dendritic cells (DCs), NKT cells, B cells, and T cells. Activated human NK cells were shown to influence the function of autologous DCs via direct cell-cell interaction, suggesting that they may have a role in regulating Ag presentation and T cell priming (2–7). NK cells have been shown to play an immunomodulatory role in maternal-fetal tolerance (8) and to regulate proliferation and Ab production by B cells (9).

Studies in several disease models indicate that NK cells may play an important role in down-regulating T cell responses. For example, it has been reported that NK cells negatively regulate T cell responses to viral infection in mice (10). Secondly, studies in humans and animal models have clearly demonstrated a regulatory role of NK cells in the initiation and progress of autoimmune disorders (11, 12). Decreased NK cell activity and numbers are found in patients with multiple sclerosis (MS) (13, 14), rheumatoid arthritis (15), and systemic lupus erythematosus (16). In the animal model of MS, depletion of NK cells from C57BL/6 (B6) mice with anti-NK1.1 Ab resulted in a more severe form of T cell-mediated experimental autoimmune encephalomyelitis (EAE) with relapse (17). Exacerbated EAE was also observed in B6 β2-microglobulin−/− mice treated with anti-NK1.1 Ab. These mice lack NKT cells, thus implicating conventional NK cells in regulation of EAE. Similar results were obtained in a study performed in Lewis rats, in which depletion of NK cells resulted in a more severe form and an increased incidence of EAE (18). Although these studies have provided strong correlative evidence for a role of NK cells in down-regulating T cell responses, the molecular mechanisms involved in such regulation are not yet fully understood.

In an attempt to understand the role of NK cells in T cell regulation, previous studies from our laboratory showed that dark Agouti (DA) rat bone marrow-derived NK cells could inhibit syngeneic T cell proliferation in response to the self-Ag, myelin basic protein, as well as the mitogen Con A in a dose-dependent manner (19). Furthermore, using highly purified (>97%) NKRP1A+CD3− cells, we were able to show that the inhibition of T cell proliferation was indeed mediated by conventional NK cells and not by NKT (NKRP1A+CD3−) cells (20). The aim of the present study was to further define the molecular mechanisms involved in inhibition of syngeneic T cell responses by NK cells. We demonstrate in this study that NK cells induce a p21-mediated cell cycle arrest of activated T cells in a contact-dependent, reversible, and Ag nonspecific manner. These results indicate a novel mechanism of regulation of T cells by syngeneic NK cells that may be of importance in understanding the role of NK cell-T cell interactions in autoimmunity, viral infections, maternal-fetal tolerance, etc.

Materials and Methods

Animals

DA rats were purchased from Harlan Sprague Dawley and maintained in our American Association for Accreditation of Laboratory Animal Care-accredited animal facility. Naïve rats were used as a source of splenic T cells, thymocytes, and bone marrow-derived NK cells. The rat protocol was approved by the institutional animal care and use committee at Wayne State University.

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Antibodies
The following Abs and fluorochromes were used in the dilutions recommended by the manufacturer: FITC-labeled anti-CD3, PE-labeled anti-NKRP1A, PE-labeled anti-CD4, PE-labeled anti-CD25 (IL-2Ra), PE-labeled anti-keyhole limpet hemocyanin, FITC-labeled annexin V, 7-aminoactinomycin (7-AAD), anti-STAT5, anti-p21 (all from BD PharMingen), anti-phosphorylated STAT5, anti-β-actin, anti-p27, anti-cyclin D3, goat anti-rabbit IgG-HRP conjugate, goat anti-mouse IgG-HRP conjugate (all from Cell Signaling), anti-phosphorylated retinoblastoma (anti-pRb; Santa Cruz Biotechnology), Alexa 647-labeled chicken anti-goat IgG (Molecular Probes), 4',6-diamidino-2-phenylindole, and propidium iodide (PI; Sigma-Aldrich).

Preparation of cell suspensions
T cells were isolated as previously described (20, 21). Briefly, spleens were homogenized, erythrocytes were lysed using Tris-NaHCl (pH 7.2), and macrophages were depleted by adherence on plastic tissue culture flasks (Falcon; BD Biosciences). T cells were then enriched using T cell purification columns (Cedarlane Laboratories). Syngeneic thymocytes were irradiated at 2000 rad and used as APCs.

Preparation of bone marrow-derived NK cells
Bone marrow was obtained from naive DA rats by flushing the cavities of the femur and tibia bones with cold RPMI 1640 supplemented with 5% FCS (Invitrogen Life Technologies) using an 18-gauge needle. Single-cell suspensions were made using needles of increasingly smaller gauge (19). The bone marrow cells were treated with Tris-NaHCl (pH 7.2) to remove erythrocytes and then depleted of macrophages by adherence on plastic tissue culture flasks (175 cm²; Falcon) for 2 h at 37°C. Nonadherent cells were passed through nylon filters, collected in 50-ml conical tubes, washed with complete medium, and then layered onto Percoll (Pharmacia Biotech) density gradients consisting of 75, 65, 55, and 45% Percoll and HBSS in 15-ml conical centrifuge tubes. The cells were centrifuged at 2000 rpm for 15 min, and in the 45 and 55% Percoll fractions (fraction 1) were pooled, washed twice with HBSS, and resuspended in RPMI 1640 with 5% FCS. This fraction, previously found to be enriched in NK cells (19), was further purified using FACS to obtain NK CD3⁻ cells as described below.

FACS for NK⁺ and CD3⁻ cells
Fraction 1 cells were suspended at 1 x 10⁴ cells/ml in PBS containing 1% BSA, 0.02% sodium azide, and 5% normal rat serum and were incubated on ice for 20 min to block nonspecific binding sites. Cells were washed with PBS containing 1% BSA and 0.02% sodium azide, then stained with PE-labeled anti-rat NKRP1A and FITC-labeled anti-rat CD3 Abs. PE-labeled anti-keyhole limpet hemocyanin Ab was used as an isotype control Ab. Staining was performed in the dark on ice for 30 min with intermittent shaking. The cells were washed and resuspended in PBS with 0.02% sodium azide and sorted using the FACSVantage cell sorter (BD Biosciences). The NK⁺ CD3⁻ fraction was collected (20). The purity of the NK⁺ CD3⁻ cells exceeded 97%.

Proliferation and Transwell assays
To determine whether cell-cell contact is necessary for inhibition of T cell proliferation by NK cells, proliferation assays were conducted in 24-well culture plates with transmembrane inserts (Costar Corning). Transmembrane inserts of 0.4-μm pore size were used to prevent direct cell-cell contact and to allow free diffusion of soluble mediators. T cells were added to the bottom wells at 1 x 10⁵ cells/well along with irradiated thymocytes as APCs, also at 1 x 10⁵ cells/well. Sorted NK cells (NK⁺ CD3⁻) were added, at a ratio of 1:10 (NK/T cells), either directly to the bottom chamber or on the Transwell insert separated from the T cells by the transmembrane. For regular proliferation assays, 0.5 x 10⁶ T cells/well were cultured with 0.5 x 10⁵ APCs/well in a 96-well plate. The cells were cultured in RPMI 1640 containing 5% FCS and were stimulated for 72 h with Con A (2.5 μg/ml; Pharmacia Biotech) or for 48 h with PMA (10 ng/ml) and ionomycin (0.4 μg/ml; Sigma-Aldrich). No APCs were added when PMA and ionomycin were used to activate T cells. T cell proliferation was determined by incorporation of [³H]thymidine, as previously described (21). The cells were then collected, washed once with PBS, and fixed with 3% paraformaldehyde. For permeabilization, cells were treated with 0.1% Triton X-100 in PBS for 10 min at room temperature. The cells were washed twice with PBS, blocked with 2% chicken serum in PBS for 20 min, washed twice with PBS, and incubated with the indicated Abs for 30 min on ice. After staining, cells were washed, mounted onto slides in a drop of Mowiol mounting medium (Calbiochem), and allowed to dry at room temperature for 10 min. Cells were examined with a Nikon E800 Epifluorescence microscope equipped with dual excitation/emission filter wheels and z-axis controls. Images were captured using a Roper CoolSnap FX digital camera and were analyzed using MetaMorph Imaging software (Universal Imaging).

ELISA and CTLT-2 assay for IL-2 activity
Culture supernatants from T cell/NK cell cocultures were analyzed for the presence of IL-2. Supernatants were collected at 24 and 72 h and were analyzed using IL-2-specific ELISA kits (BioSource International) according to the manufacturer’s instructions. To determine the biological activity of IL-2, the CTLT assay was used (22). T cell/NK cell coculture supernatants were added to CTLT cells in a 96-well microtiter plate and cultured with RPMI 1640 containing 10% FCS. A standard curve was plotted from proliferation induced by standard murine rIL-2. The amount of active IL-2 present in the coculture supernatants was then determined by comparing it to the standard curve.

Flow cytometric analysis
Flow cytometric analysis was used to determine the expression of cell surface markers on T cells, e.g., IL-2R, and to determine cell size by forward scatter (FSC). Cells were prepared for flow cytometry as follows. Cells were incubated with PBS containing 1% BSA, 0.02% sodium azide, and 5% normal rat serum for 20 min on ice as a blocking step; washed with PBS containing 1% BSA and 0.02% sodium azide; and stained with the appropriate Ab for 30 min on ice. The cells were then washed and resuspended in PBS with 0.02% sodium azide and analyzed using the FACSscan flow cytometer and CellQuest software (BD Biosciences). For analysis of apoptosis, cells were stained with annexin V-FITC and the vital dye, 7-AAD, for 15 min at room temperature, then analyzed by flow cytometry. Activated T cells treated with 2.5 μM dexamethasone (Immuno-blot PVDF; Bio-Rad) for 20 min were used as a positive control for apoptosis. To determine the cell cycle progression of activated T cells in the presence or the absence of NK cells, cells were fixed with 70% ethanol, then stained with PI at 37°C for 15 min. Cells were analyzed immediately by flow cytometry.

MACS
PMA-ionomycin-activated T cells were cocultured with NK cells for 48 h in RPMI 1640 containing 5% FCS, then washed with PBS containing 1% BSA and 0.02% sodium azide, and stained with PE-labeled anti-NKRP1A Ab. NK cells were then depleted using anti-PE microbeads and MACS depletion columns (Miltenyi Biotec) according to the manufacturer’s instructions. Cells obtained after MACS were analyzed by flow cytometry for purity (97% of recovered cells were T cells).

Western blot analysis
Cells were cultured for the indicated periods of time, then lysed by adding Laemmli sample buffer (Bio-Rad) and boiling for 5 min in a water bath. The proteins were resolved by 10 or 12% SDS-PAGE, then transferred to a nitrocellulose membrane (Immun-blot PVDF; Bio-Rad). Nonspecific binding sites were blocked by incubating the blot in blocking buffer (5% nonfat dry milk in wash buffer) for 1 h at room temperature. The blot was washed three times for 5 min each time with wash buffer (0.5% Tween 20, 200 mM NaCl, and 50 mM Tris, pH 7.5), then incubated with the appropriate primary Ab overnight at 4°C. The blot was then washed three times for 5 min each time and incubated with the corresponding secondary Ab-HRP conjugate for 1 h at room temperature. The blot was washed three times for 5 min each time, and proteins were detected by ECL (Western blotting Lumion reagent; Santa Cruz Biotechnology) and autoradiography. After staining for the protein of interest, the blot was stripped in stripping buffer (100 mM 2-ME, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7), washed three times for 5 min each time, then probed with anti-β-actin Ab.

Immunofluorescence staining and microscopy
T cells (1 x 10⁵/well) were cultured in 24-well plates with or without PMA-ionomycin in the absence or the presence of NK cells (1 x 10⁵/well) for 24 h. The cells were then collected, washed once with PBS, and fixed with 3% paraformaldehyde. For permeabilization, cells were treated with 0.1% Triton X-100 in PBS for 10 min at room temperature. The cells were washed twice with PBS, blocked with 2% chicken serum in PBS for 20 min, washed twice with PBS, and incubated with the indicated Abs for 30 min on ice. After staining, cells were washed, mounted onto slides in a drop of Mowiol mounting medium (Calbiochem), and allowed to dry at room temperature for 10 min. Cells were examined with a Nikon E800 Epifluorescence microscope equipped with dual excitation/emission filter wheels and z-axis controls. Images were captured using a Roper CoolSnap FX digital camera and were analyzed using MetaMorph Imaging software (Universal Imaging).
Results

NK cells inhibit T cell proliferation via a cell-cell contact-dependent mechanism

We previously showed that DA rat bone marrow-derived NK \(^*\) CD3\(^*\) cells inhibit Ag- or Con A-induced T cell proliferation in a dose-dependent manner (20). To determine whether this inhibition was contact dependent or mediated through a soluble factor(s), we performed a Transwell assay. T cells were stimulated with Con A in the presence of irradiated APCs. NK \(^*\) CD3\(^*\) cells were added to the T cell cultures, either in direct contact with the T cells or separated by a transmembrane at a ratio of 1:10 NK:T cells, which was determined to give optimal results in our previous studies (19, 20). As shown in Fig. 1a, we observed that NK cells inhibited Con A-stimulated T cell proliferation only when they were in direct contact with the T cells. However, NK cells were not able to inhibit T cell proliferation when separated by a transmembrane, indicating that cell-cell contact is required for NK cell-mediated inhibition of T cell proliferation. To further confirm whether a soluble factor(s) may be involved, we tested T cell/NK cell coculture supernatants for the presence of a soluble inhibitory factor(s). Con A-stimulated T cells were cultured in the presence of NK cells. Culture supernatant was collected after 48 h of incubation and added to fresh T cell cultures. Addition of the supernatant to fresh T cell cultures did not inhibit Con A-stimulated T cell proliferation (Fig. 1b), confirming that cell-cell contact is required for the inhibition of T cell proliferation by NK cells.

To determine whether the lack of T cell proliferation in the presence of NK cells was due to increased T cell death, we determined the extent of apoptosis in T cell/NK cell cocultures. T cells were cultured with APCs in the presence of Con A. NK cells were added to the T cell cultures either directly or separated by a transmembrane. Cells were collected at 24 and 48 h, stained with annexin V-FITC and the vital dye 7-AAD, and analyzed by flow cytometry (Fig. 2). Dexamethasone-treated T cells were used as a positive control for apoptosis. There was no increase in T cell apoptosis due to NK cells, indicating that NK cells did not affect IL-2R expression, we next determined whether the T cells were able to up-regulate the IL-2R on their surface in the presence of NK cells. Cells from T cell/NK cell cocultures were stained with PE-labeled anti-CD25 (IL-2R\(\alpha\)) and FITC-labeled anti-CD3 Abs and analyzed by flow cytometry. IL-2R expression on Con A-stimulated T cells in the presence or the absence of NK cells was compared with expression on unstimulated T cells. As shown in Fig. 3c, we found that CD25 (IL-2R\(\alpha\)) was up-regulated on Con A-stimulated T cells to the same extent in both the absence and the presence of NK cells, indicating that NK cells did not affect IL-2R up-regulation. Moreover, T cells showed an increase in cell size, as reflected by FSC (Fig. 3c). Thus, based on up-regulation of IL-2R and increase in cell size, Con A-stimulated T cells expressed an activated phenotype despite their failure to proliferate in the presence of NK cells.

NK cell-mediated inhibition of T cell proliferation is reversible and independent of TCR engagement

PMA and ionomycin induce T cell activation by directly activating protein kinase C and increasing intracellular calcium levels, respectively. Hence, T cell activation by PMA-ionomycin is independent of TCR/CD3 engagement and can be induced in the absence of APCs. We postulated that if NK cell-mediated inhibition was dependent on TCR/CD3 engagement, then stimulation with PMA-ionomycin should overcome the inhibition. However, we observed that NK cells inhibited PMA-ionomycin-induced T cell proliferation in a contact-dependent manner (Fig. 4a), and the inhibition was accompanied by accumulation of high levels of biologically active IL-2 in coculture supernatants (Fig. 4b). These results indicate that the inhibition is independent of Ag presentation and TCR/CD3 engagement, and that NK cells may affect later events in the T cell activation process.

To test whether inhibition of T cell proliferation by NK cells was a reversible phenomenon, we asked whether PMA-ionomycin-stimulated T cells, cocultured with NK cells, would regain the capacity to proliferate upon removal of NK cells from the culture. Thus, PMA-ionomycin-stimulated T cells were cocultured with NK cells for 48 h, then NK cells were removed from the culture using magnetic cell separation. The T cells were cultured in fresh medium with PMA-ionomycin, and proliferation was evaluated by \(^{3}H\)thymidine incorporation. As shown in Fig. 4c, upon depletion of NK cells from the cultures, T cells proliferated vigorously, indicating that the NK cell-induced inhibition of proliferation was indeed reversible.
NK cells do not inhibit IL-2R signaling in T cells
Signaling through the IL-2R is one of the crucial steps in triggering T cell proliferation upon activation. To determine whether NK cells inhibited T cell proliferation by interfering with signaling through IL-2R, we studied the effect of NK cells on IL-2 induced STAT5 activation. Upon binding IL-2, the IL-2R phosphorylates...

FIGURE 2. NK cells do not induce T cell death. Cells were collected at 24 and 48 h, stained with annexin V-FITC and vital dye 7-AAD, and analyzed by flow cytometry to determine the extent of cell death in the indicated T cell cultures. Cells treated with 2.5 μM dexamethasone were used as a positive control for apoptotic cell death. Results are representative of two independent experiments.

FIGURE 3. IL-2 secretion and IL-2R up-regulation by activated T cells are not inhibited in presence of NK cells. a, IL-2 ELISA. Supernatants were collected from the indicated cell cultures at 24 and 72 h. The amount of IL-2 in the supernatants was determined using ELISA. b, CTLL bioassay for IL-2. Supernatants were collected from the indicated cell cultures at 24 and 72 h and added to fresh CTLL cells. CTLL proliferation was measured by [3H]thymidine incorporation. A standard curve was plotted from CTLL proliferation induced by standard rIL-2. The amount of biologically active IL-2 in the supernatants was determined by comparison with the standard curve. c, IL-2R expression on T cell surface and increase in T cell size upon activation. Cells from the indicated cultures were collected at 72 h, stained with anti-CD3-FITC and anti-CD25 (IL-2Rα)-PE Abs, and analyzed by flow cytometry. CD3+ T cells were gated on, and IL-2R up-regulation (left panel, green line) and increase in cell size, as determined by FSC (right panel), were studied. Cells stained with PE-labeled isotype Ab were used as a control (left panel, red line). Results are representative of two independent experiments.
JAK1, which, in turn, phosphorylates the transcription factor STAT5 (23). Western blot analysis using anti-STAT5 and anti-phosphorylated STAT5 (pSTAT5) Abs showed that activated pSTAT5 was not detectable in unstimulated T cells, but was present in PMA-ionomycin-activated T cells in both the absence and the presence of NK cells (Fig. 5). Thus, NK cells did not affect STAT5 activation, indicating that IL-2-IL-2R interaction and signaling were functional.

In the presence of NK cells, T cells are arrested in the G0/G1 phase of the cell cycle

NK cells inhibited T cell proliferation, but did not inhibit early activation events such as IL-2 secretion and signaling via the IL-2R, suggesting that NK cells may be interfering specifically with the cell cycle progression of T cells. Hence, we analyzed the cell cycle status of Con A-stimulated T cells in the absence and the presence of NK cells using PI staining. As shown in Fig. 6, most unstimulated T cells (A) were in the G0/G1 phase, with only 9% of the cells in S phase. Upon stimulation with Con A (Fig. 6B), ~45% of the cells were in S phase, indicating that they were actively synthesizing DNA and undergoing cell division. In the presence of NK cells (Fig. 6C), however, only 11% of the cells progressed into S phase, whereas most of the T cells remained in G0/G1 phase. Again, when NK cells were separated from T cells by a transmembrane (Fig. 6D), ~41% of the cells were in S phase, indicating that cell proliferation was occurring. These results show that Con A-stimulated T cells, cultured in direct contact with NK cells, are arrested in the G0/G1 phase of the cell cycle.

G0/G1 arrest of T cells is induced by p21

The progression of T cells from G0/G1 to S phase of the cell cycle is controlled by the activity of cyclins (types D and E) in complex with G1 phase cyclin-dependent kinases (cdk2, cdk4, and cdk6). These cyclin-cdk complexes induce phosphorylation of the Rb protein, resulting in the release of transcription factor E2F, which is required for the transcription of S phase genes (24). The activity of the cyclin-cdk complexes, in turn, is tightly regulated by inhibitor proteins such as p27 and p21 (25). To determine the molecular basis of the NK cell-induced G0/G1 arrest of T cells, we examined the status of these cell cycle regulatory proteins. As shown in Fig. 7a, immunofluorescence staining with anti-pRb Ab revealed that nuclear pRb could be detected in PMA-ionomycin-activated T cells in both the absence (B and C) and the presence (D) of NK cells. As expected, pRb staining was not detected in unstimulated T cells (Fig. 7a, A). Moreover, Western blot analysis showed up-regulation of cyclin D3 in stimulated T cells in the absence and the presence of NK cells (Fig. 7b). These data indicate that in the presence of NK cells, the up-regulation of cyclin D3 and the phosphorylation of Rb were not affected.
We then analyzed the levels of the G1-S cell cycle checkpoint inhibitors, p27 and p21, using Western blot analysis. As shown in Fig. 7b, p27 protein was detected in unstimulated, nonproliferating T cells, but was down-regulated in stimulated T cells in the absence as well as the presence of NK cells. Thus, NK cells did not affect regulation of p27 upon T cell activation. In contrast, p21 was detected at low levels in stimulated T cells, but in the presence of NK cells, p21 levels were highly elevated. Thus, the significantly high level of p21 induced in T cells in the presence of NK cells may account for the Go/G1 arrest of T cells.

Levels of p21 decrease after removal of NK cells

Because removal of NK cells from NK/T cell cocultures results in restored T cell proliferative responses, it was important to determine whether a concomitant decrease in p21 levels occurred. Accordingly, we cultured PMA-ionomycin-stimulated T cells with NK cells for 48 h, removed NK cells by magnetic cell separation, and recultured T cells with PMA-ionomycin for an additional 48 h. Western blot analysis revealed that the levels of p21 in T cells from which NK cells had been removed approached the levels of p21 present in PMA-ionomycin-activated T cells that were cultured for the entire 96-h period without NK cells (Fig. 8). Thus,
removal of NK cells from the cocultures leads to basal levels of p21 that correlate with restored proliferative responses.

Discussion
The aim of our study was to define the molecular events underlying the inhibition of T cell proliferation by NK cells. Our findings show that rat bone marrow-derived NK cells inhibit the proliferation of syngeneic T cells by inducing a G_{0}/G_{1} stage cell cycle arrest via up-regulation of the cell cycle inhibitor protein, p21. The inhibition is cell-cell contact dependent, Ag nonspecific, and reversible. NK cells do not affect early events in T cell activation, including IL-2 secretion, IL-2R up-regulation, and increase in T cell size, but specifically inhibit T cell division. Our study has unraveled a unique mechanism of T cell regulation by NK cells that could be of potential importance in understanding NK cell-mediated regulation of T cell function during various immune responses.

The role of NK cells in regulating T cell responses has been emphasized in several in vivo studies. For example, it has been observed that T cell proliferation and IFN-γ expression in response to murine CMV infection in mice are enhanced upon in vivo depletion of NK cells (10). Similar depletion studies in animal models of several autoimmune diseases, including EAE (18) and CD4^{+} T cell-mediated colitis (26), have indicated that NK cells may actively suppress autoreactive T cell responses. It has been suggested that NK cells may mediate this regulatory function via secretion of immunomodulatory cytokines and chemokines or via interaction with other immune cells, such as NK-T cells, DCs, and macrophages (12). In this study we show that NK cells have the potential to inhibit T cell proliferation via direct cell-cell interaction, thus emphasizing a more direct role for NK cells in the down-regulation of T cell responses.

Surprisingly we observed that although NK cells inhibit T cell proliferation, they do not inhibit IL-2 secretion, suggesting that they do not induce classical T cell anergy. Because T cells do not proliferate in the presence of NK cells, they do not consume IL-2, thus leading to its accumulation in the culture medium. Moreover, the up-regulation of IL-2R α-chain (CD25) upon T cell activation is not affected in the presence of NK cells. Upon binding to IL-2, the IL-2R induces phosphorylation of JAK1, which, in turn, phosphorylates the transcription factor STAT5 (23). Using Western blot analysis, pSTAT5 could be detected in T cells in the presence of NK cells, indicating that the IL-2-IL-2R complex was functional.

We found that NK cells inhibit PMA-ionomycin-induced T cell proliferation, indicating that the inhibition was independent of TCR/CD3 engagement and thus was Ag nonspecific. This would be expected, because, unlike T or B cells, NK cells do not express Ag-specific receptors. NK cells do express receptors that interact with MHC class I molecules on target cells (27). However, the addition of anti-MHC class I mAb (OX-18) did not restore proliferation, suggesting that this receptor-ligand interaction was not involved in mediating the inhibition (data not shown). Second, although we have established that cell-cell contact is necessary for NK-mediated inhibition of T cell proliferation and that soluble factor alone could not mediate this inhibition, NK cells have been implicated in CD8^{+} T cell-mediated suppression of B cell activity via secretion of the cytokine, TGF-β (28). However, the addition of anti-TGF-β-blocking Ab to our NK cell-T cell cocultures did not overcome the inhibition of T cell proliferation, indicating that the mechanism of inhibition was TGF-β independent (data not shown). Additional attempts to identify other soluble factors as well as the receptor-ligand pair(s) involved in this NK-T cell interaction are currently ongoing in our laboratory.

Interestingly, although NK cells prevented T cell proliferation, they did not induce T cell death. The T cells were arrested in G_{0}/G_{1} stage of the cell cycle, but showed an activated phenotype and, upon removal of NK cells, could revert back to proliferation, suggesting that although NK cells inhibit T cell proliferation, they do not eliminate the T cells. In the presence of NK cells, there was a significant increase in the level of the cell cycle inhibitor protein, p21, in T cells. Upon removal of NK cells from the cocultures, p21 expression returned to a basal level that correlated with restored T cell proliferation. p21 has been shown to prevent cell cycle progression by several mechanisms, including blocking formation of active cyclin-cdk complexes, inhibiting DNA replication through proliferating cell nuclear Ag, and interfering with c-Myc function (29, 30). Interestingly, p21 has also been shown to enhance the survival of several cell types via inhibition of apoptosis (30). Thus, p21 inhibits cell cycle progression, but prolongs cell survival. This is consistent with our observations that in the presence of NK cells, T cells did not proliferate, although they did not die. Based on these findings, we hypothesize that, via p21 up-regulation, NK cells may prolong the survival of activated T cells, but limit their clonal expansion, thereby maintaining immune homeostasis.

The precise physiological conditions under which this NK-T cell interaction may be of relevance must still be determined. For example, this negative regulation of T cell activity by NK cells could provide a mechanistic link between reduced numbers and function of NK cells and the exacerbation of autoreactive T cell responses observed in several autoimmune disease models (17, 18, 26). Another possible outcome of inhibition of T cell proliferation by NK cells is that NK cells may prevent the proliferation of memory T cells, thereby dampening the progress of certain autoimmune disorders. In support of this hypothesis, it was recently reported that depletion of NK cells led to increased memory T cell responses to the self-Ag myelin basic protein, suggesting that NK cells may be responsible for maintaining clinical remission in some MS patients by inhibiting the memory T cell pool (31).

The final outcome of an encounter between a T cell and an NK cell is probably influenced by several external factors, such as the cytokine milieu, extracellular matrix interactions, and the activation state of the NK cell and the T cell. Although there are several reports supporting the role of NK cells in down-regulating T cell responses and suppressing autoimmunity, other studies suggest a role for NK cells in the initiation of autoimmune diseases and activation of T cells (11, 12). This suggests that NK cells have the potential to exert both a stimulatory and an inhibitory influence on T cell responses.

These seemingly contradictory findings are also reflected in in vitro studies on NK cell-T cell interactions. For example, in contrast to our study, it was recently shown that murine splenic NK cells stimulate the proliferation of T cells via 2B4/CD48 interaction (32). Furthermore, activated human NK cells were shown to stimulate CD4^{+} T cell proliferation via costimulation through OX40-OX40 ligand interaction (33). We believe that these contrasting observations could be due to differences in the source and treatment of the purified NK cells used in these different reports. In the above-mentioned studies, the interaction of activated, peripheral (blood or spleen) NK cells with T cells resulted in enhanced T cell proliferation. In contrast, we observed that interaction of naive, unstimulated, bone marrow NK cells with T cells resulted in inhibition of T cell proliferation. Activation of NK cells with cytokines such as IL-2, IL-15, IL-18, or anti-CD16 Ab (human) results in up-regulation of surface receptors or ligands, such as 2B4 (murine) (32) or OX40 ligand (human) (34) on the NK cells and may confer the ability to enhance T cell proliferation rather than suppress it. Secondly, the bone marrow and splenic NK cells may
represent two different subsets of NK cells. The bone marrow NK cell subset may have an immunomodulatory capacity that spleen NK cells may lack and vice versa. For example, human decidual, but not peripheral, NK cells can express proteins that have immunomodulatory function and are postulated to play an important role in maternal-fetal tolerance (8). We are currently performing experiments to characterize the bone marrow NK cell subset more thoroughly and compare it to the splenic NK cells to determine the phenotypic and functional differences in these subsets.

In conclusion, our results show that NK cells inhibit T cell proliferation in an Ag-non-specific and reversible manner, suggesting that they may play an important role in maintaining general T cell homeostasis. This study provides a possible mechanistic link between decreased NK cell function and enhanced T cell responses, observed especially in several autoimmune disease models. Our findings have uncovered a novel mechanism of T cell regulation by NK cells, providing insight into the regulatory role of NK cells. Understanding the molecular mechanism of suppression of T cell responses by NK cells provides a potential target for therapeutic intervention in several human autoimmune diseases.

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Disclosures
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References