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Inhibition of Autoimmune T Cell Responses in the DA Rat by Bone Marrow-Derived NK Cells In Vitro: Implications for Autoimmunity

Ronald B. Smeltz, Norbert A. Wolf, and Robert H. Swanborg

Regulation of the immune response is critical to homeostasis. While innate immunity can influence the development of adaptive immune responses, its role in regulation is less well understood. Recently, NK cells have been implicated in the control of experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis. In this report, we show that rat bone marrow-derived NK cells exhibited potent inhibitory effects on T cell proliferation to both Con A as well as the central nervous system Ag myelin basic protein. There was also a significant decrease in both IFN-γ and IL-10 production in vitro, whereas levels of the β-chemokine monocyte chemoattractant protein-1 were significantly elevated. Flow cytometry studies suggest that the NK cells may play an important role in regulating both normal and autoimmune T cell responses by exerting a direct effect on activated, autoantigen-specific T cells. The Journal of Immunology, 1999, 163: 1390–1397.

The regulation of Ag-specific immune responses, both their induction and termination, is critical for immune system homeostasis. Dysregulation can lead to conditions, such as allergy and autoimmune disease. The role of adaptive immunity in regulation has been the focus of many studies, while the role of innate immunity is less well characterized.

Cells of the innate immune system (i.e., NK cells), for example, can affect the differentiation of naive T cells (reviewed in Ref. 1). IFN-γ is secreted by NK cells, and IFN-γ, in turn, drives CD4+ T cells to differentiate into Th1 cells. NK cells can also influence B cell function and Ab secretion (2). Conversely, production of IL-13 by NK cells can influence macrophage function and Th2 differentiation (3). Clearly, NK cells play a crucial role in both innate and adaptive immune responses.

Recent data have suggested that NK cells may play an important role in experimental autoimmune encephalomyelitis (EAE), 3 a rodent model for the human demyelinating disease multiple sclerosis (4, 5). While these studies showed that NK cells might play an important role in recovery from EAE, they did not investigate direct NK cell–T cell interactions. We chose to study NK cell regulation of T cell responses in the DA rat strain, which is highly susceptible to various autoimmune diseases, including EAE and collagen-induced arthritis (6–8). However, DA rats do not develop spontaneous autoimmune disease, suggesting that autoreactivity is strictly regulated. The goal of the present study was to investigate the ability of NK cells to regulate encephalitogenic T cell responses in vitro. We demonstrate that bone marrow-derived NK cells (BMNK) inhibit proliferation, and IFN-γ and IL-10 production by encephalitogenic Th1 cells at low effector to target ratios. BMNK inhibition of T cell responses was associated with a dramatic increase in levels of the β-chemokine monocyte chemoattractant protein (MCP)-1. These results have important implications with respect to how NK cells might regulate adaptive immune responses, particularly autoimmune diseases.

Materials and Methods

Animals and immunization

DA rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN), maintained in our American Association for the Accreditation of Laboratory Animal Care accredited facility, and used at 10–20 wk of age. Naive rats were used as a source of bone marrow (BM) cells. For studies of myelin basic protein (MBP)-primed T cells, rats were immunized s.c. with 25 μg of guinea pig MBP or MBP peptide 63–81 emulsified in IFA supplemented with 200 μg Mycobacterium butyricum (Difco, Detroit, MI) as described previously (9).

Preparation of cell suspensions

BM was obtained from naive rats by flushing the cavities of the femurs and tibias with cold RPMI 1640 supplemented with 5% FCS (Life Technologies, Grand Island, NY) using an 18-gauge needle. A single cell suspension was prepared by gentle aspiration of the marrow with needles of increasingly smaller gauge. BM cells were treated with Tris-NH4 Cl (pH 7.2) to remove erythrocytes, then depleted of macrophages by adherence on plastic (250-cm2 tissue culture flasks; Falcon, Lincoln Park, NJ) for 2–3 h at 37°C. Nonadherent BM cells were poured through nylon filters and collected in 50-ml conical centrifuge tubes, washed with complete medium, then layered onto Percoll (Pharmacia, Uppsala, Sweden) gradients to enrich for NK cells.

Responder T cells were obtained by preparing single cell suspensions of spleen or draining inguinal lymph nodes, depleted of macrophages by a 1-h adherence on plastic, followed by enrichment on T cell columns (Cytovax Biotechnologies, Edmonton, Alberta, Canada). T cells were cultured with irradiated syngeneic thymocytes (2000 rads) as APC at a 1:1 ratio (9).

Percoll fractionation

Nonadherent BM cells were layered onto Percoll density gradients consisting of 75, 65, 55, and 45% Percoll, and HBSS in 15-ml conical centrifuge tubes (Falcon; Becton Dickinson, Franklin Lakes, NJ) (10). Cells

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were centrifuged at 2000 rpm for 15 min. Cells in the 45 and 55% Percoll fraction were pooled as Fraction 1, and the 65% Percoll fraction was designated Fraction 2. The Fraction 1 and 2 cells were washed twice with cold HBSS and were resuspended in RPMI 1640 medium containing 5% FBS. Effector cells (BM cells) and target cells (T cells) were combined in different proportions (E:T) for use in proliferation assays.

**Proliferation assay**

T cell proliferation assays were performed as previously described (9). Briefly, lymph node cells were depleted of adherent cells, enriched for T cells on CytoVax columns, and plated in microtiter wells. Each well received 5 × 10^3 T cells and 5 × 10^3 syngeneic, irradiated thymocytes (2000 rads) as APC. Cells were cultured in RPMI 1640 containing 5% FCS. The cells were stimulated either with 5 μg/ml MBP, which was found optimal in previous studies, or with Con A at a concentration of 2.5 μg/ml. The cultures were incubated for either 72 h (Con A), or 96 h (MBP), and pulsed with 0.5 μCi of [3H]thymidine for the last 18 h. The plates were harvested using a Tomtec Harvester 96 and counted in a 1450 Microbeta Plus liquid scintillation counter (Wallac, Gaithersburg, MD). Results are presented as cpm ± SD.

**Cytokine analysis**

We focused on one proinflammatory Th1 cytokine that is also secreted by NK cells (IFN-γ), two Th2 cytokines (IL-4 and IL-10), and one chemokine (MCP-1). The latter was selected because it has been reported that in vivo neutralization of MCP-1 reverses the protective effect of oral tolerization on EAE (11). Culture supernatants from MBP or Con A-stimulated T cells were evaluated for IFN-γ, IL-4, IL-10, and MCP-1, using rat-specific commercial ELISA kits (Biosource International, Camarillo, CA; Life Technologies) according to the manufacturers’ instructions. Recombinant rat MCP-1, and anti-rat/mouse cross-reactive MCP-1 mAb (clone 2H5) were purchased from Biosource International and PharMingen (San Diego, CA), respectively.

**Flow cytometry**

Cells were prepared for flow cytometry by first incubating 1 × 10^6 Percoll-isolated BM cells with PBS containing 0.02% sodium azide, 1% BSA (PBS-A-BSA) and 1% normal rat serum for 30 min as a blocking step, as previously described (12). The cells were subsequently washed in PBS-A-BSA and incubated with the appropriate chromogen-labeled Ab for 20 min. Abs used were: FITC- or PE-labeled R73 (anti-rat αβ TCR), PE-labeled G4.18 (anti-CD3), FITC-labeled anti-rat NKR-P1 (NK cells), FITC-labeled anti-rat B220 (B cells), and FTC-anti-rat DNP as an isotype control (all purchased from PharMingen). Cells were analyzed in a FACScan flow cytometer using PC LYSIS software (Becton Dickinson, Mountain View, CA).

**Cytotoxicity assay**

Cytotoxicity assays were performed using the flow cytometric method of Slezak and Horan (13). Briefly, 2 × 10^6 column-enriched T cells were combined with 1 × 10^6 syngeneic thymocytes, and the cells were labeled with the lipophilic green dye PKH26 (Sigma, St. Louis, MO). We did not irradiate the thymocyte APCs because irradiated thymocytes might not have survived for the 72-h culture period, and would therefore have taken up the propidium iodide (PI) used to delineate the nonviable cell population. Uniform labeling of cells was confirmed by visualization using a fluorescent microscope. Labeled target cells were then incubated with 2.5 × 10^5 nonlabeled Percoll-enriched BMNK cells (1:8 E:T) and 2.5 μg/ml Con A in 24-well plates for a total of 72 h. Cells were then collected, stained with PI, and analyzed by flow cytometry. Controls consisted of Con A- or MBP 63–81-stimulated T cells alone (no BM), as well as a BM fraction lacking NK cells. Cytotoxicity was determined by calculating the percentage of cells positive for both PI (red) and PKH (green).

**Results**

**Procurement of BMNK cells**

To study the regulatory effects of BMNK cells on T cells, it was important to utilize an appropriate source of NK cells. BM has traditionally been a source of NK cells. Percoll density gradients were therefore utilized to separate and enrich for these cells, which are characterized as large granular cells. Plastic nonadherent BM cells from naive rats were applied to gradients consisting of 75, 65, and 45% Percoll, overlayed with HBSS. After centrifugation, cells from the 45–55% interfaces (designated as Fraction 1) as well as the 65% interface (Fraction 2) were harvested, washed, and evaluated for suppressive activity.

In two initial experiments, we evaluated Fraction 1 and Fraction 2 BM cells obtained from Lewis (LEW) and DA rats. As shown in Fig. 1, the Fraction 1 BM cells suppressed Con A-induced proliferative responses, whereas the Fraction 2 BM cells were not suppressive. Since DA Fraction 1 cells appeared to exert a more profound inhibitory effect on DA T cells relative to that achieved with LEW Fraction 1 cells on the LEW T cell proliferative response, we decided to focus on the DA strain for this investigation.

Flow cytometric analyses were conducted to characterize the cells present in the two fractions (Figs. 2 and 3). As shown in Fig. 2, two populations of cells can be discerned for each BM fraction (Fraction 1 and Fraction 2), and these are gated R1-R4. R1 and R3 represent small cells, and include a majority of B cells (B220^+ cells, Fig. 3B), and a very small percentage of αβ TCR^+ T cells (TCR^+ cells, Fig. 3C). R2 and R4 represent large granular cells. In four independent experiments, an average of 27 ± 0.5% of the cells in Fraction 1, region R2 express NKR-P1 (the NK cell marker), whereas only 5% of the R4 cells in Fraction 2 are NKR-P1^+ (Fig. 3D). Thus, the NK cells are enriched in BM Fraction 1, and more specifically, in region R2 (Fig. 3D). Employing two-color flow cytometric analyses with FITC-anti-NKR-P1 and PE-anti-αβ TCR, ~1% of the NKR-P1^+ cells in Fraction 1 expressed αβ TCR (Fig. 4). Similar results were obtained when the BM Fraction 1 cells were stained with FITC-anti-NKR-P1 and PE-anti-CD3 mAb (data not shown). Therefore, it is unlikely that NK-T cells play a major role in suppression of the T cell proliferative responses (Fig. 1, and data presented below). Moreover, we failed to detect IL-4 in the Fraction 1-containing supernatants, and NK-T cells reportedly produce IL-4 upon stimulation (14).

**Inhibition of T cell proliferation by BMNK cells**

To determine how NK cells might regulate T cell responses, BMNK cells were added at various ratios to T cells, and proliferative responses were evaluated. In the first group of experiments, splenic T cells were stimulated in vitro with Con A and syngeneic APCs in the presence of either Fraction 1 or Fraction 2 cells. BM cells were added at E:T ranging from 1:4 to 1:128. As shown in Fig. 5, only Fraction 1 cells (BMNK^+ ) were capable of inhibiting...
T cell proliferation to Con A. This inhibition was observed even at an E:T of 1:32; however, there was no significant inhibition of T cell proliferation by Fraction 2 cells, which do not contain BMNK cells (Fig. 5).

Next, we determined whether BMNK cells could inhibit the proliferation of T cells specific for MBP. T cells obtained from MBP-immunized DA rats were cultured with BM cells from naive DA rats. As shown in Fig. 6, only BMNK^1 (Fraction 1) cells were capable of inhibiting T cell proliferation to MBP, and significant inhibition was observed at an E:T of 1:64. No inhibition was observed with Fraction 2 cells. Similar results were obtained in seven independent proliferation experiments (four with Con A, and three with MBP).

**Effect of BMNK cells on cytokine production**

To correlate BMNK inhibition of Con A- and MBP-induced T cell proliferation with cytokine secretion, culture supernatants were analyzed for IFN-γ, IL-4, and IL-10 production by ELISA. IL-4 was not detected in any of the supernatants (data not shown). IFN-γ and IL-10 production, Th1 and Th2 cytokines, respectively, was
significantly reduced in response to either Con A or MBP in the presence of either unfractionated BM cells or Fraction 1 cells (Table I). However, no decrease in IFN-γ or IL-10 production was observed in cultures containing Fraction 2 cells (Table I). Note that IFN-γ is expressed in ng/ml, and IL-10 and MCP-1 (see below) are expressed in pg/ml (Table I).

**MCP-1 levels are significantly elevated in inhibited cultures**

The decrease in IFN-γ production in cultures with BMNK cells was not surprising since proliferation was also inhibited. To extend our analysis of cytokine production, we also evaluated production of the β-chemokine MCP-1 in the culture supernatants from Con A- and MBP-activated T cells. This chemokine has been implicated in the regulation of EAE (11). We found that MCP-1 levels were dramatically elevated in cultures that contained the Fraction 1 BMNK cells. The results of one experiment are presented in Fig. 7. Table I summarizes the results of three experiments, and includes data from Fig. 7. A fourth experiment gave similar results (data not shown). The supernatants that contained high levels of MCP-1 (152–411 pg/ml) were from T cell cultures that exhibited suppressed proliferative responses (compare Table I with Figs. 5 and 6). MCP-1 levels were low in wells not containing BMNK cells.

### Table 1. IFN-γ, IL-10, and MCP-1 production in cultures containing BMNK cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>IFN-γ (ng/ml)</th>
<th>IL-10 (pg/ml)</th>
<th>MCP-1 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T, APC, Con A</td>
<td>35</td>
<td>639</td>
<td>84</td>
</tr>
<tr>
<td>+BM (1:1)</td>
<td>4</td>
<td>111</td>
<td>381</td>
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<tr>
<td>BM + Con A</td>
<td>ND</td>
<td>ND</td>
<td>29</td>
</tr>
<tr>
<td>Expt. 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T, APC, fraction 1 (1:8)</td>
<td>ND</td>
<td>ND</td>
<td>94</td>
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<tr>
<td>T, APC, fraction 2 (1:8)</td>
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<td>11</td>
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<tr>
<td>T, APC, Con A</td>
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<td>+Fraction 2 (1:16)</td>
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<td>34</td>
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<td>+Fraction 1 (1:32)</td>
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<td>432</td>
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<td>652</td>
<td>30</td>
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<td></td>
<td></td>
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<tr>
<td>T, APC, fraction 1 (1:8)</td>
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<td>ND</td>
<td>54</td>
</tr>
<tr>
<td>T, APC, fraction 2 (1:8)</td>
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<td>ND</td>
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<tr>
<td>T, APC, MBP</td>
<td>6.6</td>
<td>76</td>
<td>25</td>
</tr>
<tr>
<td>+Fraction 1 (1:8)</td>
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<td>78</td>
</tr>
<tr>
<td>+Fraction 2 (1:32)</td>
<td>5.7</td>
<td>52</td>
<td>33</td>
</tr>
</tbody>
</table>

a T cells were stimulated for 72 h with Con A (expts. 1 and 2) or MBP (expt. 3) and APCs, with/without BM cells. Supernatants were tested by ELISA.
b SDs were <10% for all samples.
c BM cells were not fractionated in this experiment.
d ND, not detected (OD < control without cytokine reference standard).
e Background values (no Con A or MBP).
f NT, not tested.
supernatant were incubated overnight at 4°C with 100, 200, or 0.15–1.2 ng/ml.

**FIGURE 9.** Recombinant rat MCP-1 suppresses rat T cell proliferative responses to MBP (A) and Con A (B). Stock MCP-1 concentrations were 0.15–1.2 ng/ml.

**FIGURE 8.** Competitive inhibition of binding of MCP-1 to the capture Ab coated on the ELISA plate by anti-MCP-1 mAb. This supernatant is from culture of T cells + APCs + BMNK Fraction 1 (E:T, 1:8) + Con A. Aliquots of supernatant were incubated overnight at 4°C with 100, 200, or 500 μg/ml anti-MCP-1 (V:V), then tested for MCP-1 by ELISA.

**Inhibition of T cell proliferation is associated with cytotoxicity**

Because BMNK cells were potent inhibitors of T cell proliferation, it was important to determine whether the NK cells had a direct effect on the T cells. This was investigated using a flow cytometric assay in which T cells were labeled with the lipophilic green dye PKH26, then stimulated with Con A in the presence or absence of APCs and Fraction 1 BMNK cells. PKH26-labeled target T cells stained only with PKH26. Dead BM effector cells take up PI (red), while live T cell targets stain only with PKH26. Dead BM effector cells take up only PI.

To confirm that MCP-1 plays a role in the inhibition of proliferation, exogenous recombinant rat MCP-1 was added to microtiter wells in MBP- and Con A-induced proliferative responses. As shown in Fig. 9, MCP-1 dramatically suppressed Ag- and mitogen-stimulated proliferative responses.

**Discussion**

It is increasingly clear that cells of the innate immune system, especially NK cells, can have a substantial influence on adaptive immune responses, and that this influence extends to both humoral and cellular arms. There is substantial data to support the hypothesis that NK cells play an important role in the control of autoimmune disease. NK cell defects have been shown in autoimmune oophoritis (15) and anti-DNA Ab production in C57BL/6 lpr mice (2). The development of autoantibodies in C57BL/6 lpr mice correlated with the disappearance of NK1.1+ cells. Furthermore, a regulatory role for NK cells was shown in the development of
inflammatory bowel disease in B6 mice via a perforin-dependent mechanism (16). Perforin was shown to protect animals from autoimmunity in Lupus-prone MRL/\textit{lpr} mice, although the role of NK cells was not specifically addressed (17). In EAE, one of the first studies to suggest a regulatory role for NK cells in EAE was the administration of Linomide, a compound known to have NK cell-stimulating functions (18). SJL/J mice administered Linomide were significantly protected from EAE induced with spinal cord homogenate (18). Depletion of NK1.1 cells in the B6 mouse led to exacerbations of EAE, and higher mortality (4). A similar study in the Lewis rat by Matsumoto et al. (5) revealed that depletion of NKR-P1\textsuperscript{+} cells in vivo led to an increase in disease severity and reduced incidence of recovery. While these studies strongly suggest a role for NK cell-mediated regulation of EAE, none specifically address NK cell effects on T cells during an immune response.

In this report, we investigated NK cell regulation of T cells. We used a more traditional source of NK cells (BM rather than splenic NK cells). BMNK cells were potent inhibitors of T cell proliferation to both Con A and MBP, a CNS autoantigen. Inhibition was evident at low E:T ratios (E:T < 1:16) and was accompanied by a corresponding decrease in levels of IFN-γ and IL-10. Interestingly, we found high levels of MCP-1 in cultures containing BMNK cells. We consider it likely that one source of the MCP-1 is the BMNK cells because maximal levels were present in the cultures containing Fraction 1 BMNK cells (Table I). However, we cannot exclude the possibility that T cells or APCs also secrete MCP-1, or that the BMNK cells indirectly induce T cells or APCs to produce this chemokine. Recombinant MCP-1 appears to inhibit T cell proliferative responses (Fig. 9), which is consistent with a previous report that this chemokine can have protective effects on the development of EAE in mice orally tolerized with encephalitogenic peptide (11). It has also been reported that MCP-1 acts as a lymphocyte chemoattractant for memory T cells (19). Therefore, one might speculate that the MCP-1 is produced by the NK cells, which, in turn, attract the activated T cells.
Moreover, as shown in Figs. 10 and 11, inhibition of T cell proliferation by BMNK cells is associated with a significant increase in the number of PI-positive (i.e., dead) T cells when Con A-activated T cells are cultured with BMNK cells for 72 h. This suggests that one mechanism by which the BMNK cells inhibit proliferation may involve cytotoxic activity directed against the autoreactive T cells. The possibility that perforin- and/or Fas/FasL-mediated cytotoxicity is involved is currently under investigation. However, the mean fluorescence intensity of the cells that did not stain with PI was not decreased, as would be expected if the cells had proliferated (Figs. 10 and 11). This might suggest that cytokines also play a role in BMNK-mediated inhibition of T cell responses by suppressing proliferation. We previously found that cytokines also play a role in BMNK-mediated inhibition of T cell responses in EAE (20). This issue is also under investigation.

There is evidence that NK cells play an important role in autoimmune disease. In the report by Matsumoto et al. (5), an increase in peripheral blood NK cells in Lewis rats was observed during both clinical EAE and recovery, but NK cells were present in the spinal cord only during clinical disease. Despite the increase in peripheral blood NK cells, there was no corresponding change in the percentage of splenic NK cells. Thus, it is possible that BMNK cells are recruited to the CNS via the bloodstream, participate in recovery from EAE, and exit from the CNS after the disease-inducing effector T cells have been lysed. Furthermore, depletion of NK cells in both rats and mice resulted in disease exacerbation and relapse (4, 5). These results suggest that NK cells play a role in recovery from EAE, and perhaps multiple sclerosis (21), by inhibiting the induction of new T cell responses within the CNS, e.g., via epitope spreading (22, 23). Mice depleted of NK cells developed a more severe form of colitis, which is correlated with the expansion and increase in the number of donor T cells that mediate colitis (16). Studies are in progress to elucidate the mechanism by which BMNK cells inhibit T cell responses and to ascertain the role chemokines play in the activation and function of NK cells.

References


