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NK Cells Regulate CD4 Responses Prior to Antigen Encounter

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NK cells not only respond rapidly to infection, shaping subsequent adaptive immunity, but also play a role in regulating autoimmune disease. The ability of NK cells to influence adaptive immunity before Ag exposure was examined in a gender-dependent model of preferential Th1 and Th2 activation. The inability of young adult male SJL mice to activate Th1 cells was reversed via depletion of NK1.1+ cells, whereas the presence or the absence of NK1.1+ cells did not alter responses in age-matched females. Consistent with a gender-dependent role in regulating adaptive immunity, significantly more NK1.1+ cells were present in males compared with females, and this difference was reversed by castration. In contrast to NK1.1+ cells derived from C57BL/6 mice, no spontaneous cytokine secretion was detected in NK1.1+ cells derived from either male or female SJL mice, although an increased frequency of IL-10-secreting NK1.1+ cells was observed in males vs females following in vitro stimulation. Direct evidence that NK1.1+ cells in males influence CD4+ T cell activation before Ag exposure was demonstrated via the adoptive transfer of APC from control and NK1.1-depleted males. The absence of a functional NK T cell population in SJL mice suggests that NK cells influence adaptive immunity before Ag exposure via alterations in APC activity. The Journal of Immunology, 2003, 171: 234–239.

Natural killer cells are critical components of the innate immune system that respond rapidly to a variety of insults via cytokine secretion and cytolytic activity. Activation results in the secretion of multiple cytokines that play critical roles in modulating adaptive immune responses (1–6). Inbred strains of mice are classified based on NK cytolytic activity: high responder strains exhibit spontaneous cytolytic activity that is enhanced by IFN; intermediate responder strains, e.g., C57BL/6 and BALB/c, exhibit basal cytolytic activity that is readily increased by IFN; and low responder SJL mice exhibit little spontaneous cytolytic activity, which is very poorly augmented by IFN (7).

NK cells have been implicated in the regulation of a variety of both animal models and human autoimmune diseases, including lupus, diabetes, and multiple sclerosis. NK cell depletion worsens murine lupus, while adoptive transfer delays disease onset (8), suggesting that NK cells play a regulatory role. In the murine model, the severity of experimental autoimmune encephalomyelitis (EAE) is increased by NK cell depletion (9). During EAE, NK cells inhibit Ag- and cytokine-induced CD4+ T cell proliferation independent of CD8+ or NK1.1+ T cells (10). In support of a role for NK cells in autoimmune disease of the CNS, NK cells derived from multiple sclerosis patients express elevated IL-5 and IL-12Rβ2 during remission. During relapses, these NK cells lose their Th2 phenotypic properties (11), suggesting a role in regulating clinical disease.

SJL mice, which are sensitive to both active and passive EAE, are low NK responders due to a unique defect in thymic processing (7). This phenotype is altered to inducible by thymectomy, but not in chimeras prepared with bone marrow cells derived from either inducible or high responder phenotype donors (7). Consistent with a defect in thymic processing, reconstitution of irradiated high phenotype recipients with bone marrow cells derived from SJL donors results in an NK population exhibiting an inducible cytolytic phenotype. SJL mice have limited numbers of CD3+NK1.1+ cells and fewer CD3+NK1.1+ T cells, although a slightly larger population of NK T cells has been identified based on CD1d/α-galactosylceramide tetramer staining (12) or CD122 and TCR coexpression (13). In contrast to NK T cells in other strains of mice, NK T cells in SJL mice are unable to secrete IL-4 or IFN-γ following TCR ligation (14). Consistent with the inability to secrete IL-4, a unique IL-4 genetic defect in NK T cells derived from SJL mice has been described (13), independent of NKR-P1 gene expression. Together these data indicate that SJL mice not only have a reduced frequency of NK1.1+ T cells relative to other strains of mice, but these cells also appear to lack the functional capabilities ascribed to NK T cells. Furthermore, in many, but not all, strains of mice, NK and NK T cells express a surface Ag recognized by anti-NK1.1 mAb, encoded by the NKR-P1C gene, which transduces an activation signal (13). Recently it has been suggested that the unique low NK responder phenotype in SJL mice is due to the expression of the NKR-P1B gene product, also recognized by anti-NK1.1 mAb, which transduces a negative signal (15).

In addition to a paucity of NK cells (13, 16), CD5+ B cells (17), CD4+CD25+ T regulatory cells (18), a limited Vβ repertoire (19), and defective NK T cells (13, 14), SJL mice exhibit a gender-dependent preferential activation of Th1/Th2 cells (20). Differential responsiveness, resulting in an Ag-specific Th1 response in females and castrated males vs a Th2 response in males (21, 22), is controlled before Ag encounter by a subset of macrophages that function as APC (21, 23, 24). APC function in young adult SJL males is regulated by both IL-4 and IL-10 before Ag encounter.

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4 Abbreviations used in this paper: EAE, experimental autoimmune encephalomyelitis; DTH, delayed-type hypersensitivity; KLH, keyhole limpet hemocyanin; PEC, peritoneal exudate cells.
and stained with FITC anti-NK1.1 (mAb PK136), fixed and permeabilized for 15 min in Cytoperm/Cytofix, washed in Permwash, and stained with PE-anti-IL-10 (mAb JES5-2A5), PE anti-IL-4 (mAb 11B11), or PE anti-IFN-γ (mAb XMG1.2; all obtained from BD Pharmingen). Samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) using CellQuest Pro software.

Adaptive transfers

APC activity was examined using peritoneal exudate cells (PEC) as a source of APC (21, 23, 24). PEC were induced by i.p. injection of 2 ml of thioglycolate broth (Difco, Detroit, MI) and were harvested from the peritoneal cavity by lavage with 7–10 ml of Joklik-modified MEM (Life Technologies, Grand Island, NY) supplemented with 5 U/ml of heparin (Sigma-Aldrich) and 25 mM HEPES buffer, pH 7.2, 72 h after induction. PEC, containing <7% Ly-6G+ and >90% CD11b+ cells, were transferred i.p. at 5 × 10^6 to naive males on the day of immunization. KLH-specific DTH responses were determined as described above. The effect of NK cells on APC activity was examined with APC derived from mice depleted of NK cells via i.p. injection of 0.5 ml of PBS containing 250 µg of anti-NK1.1 (mAb PK136) on days −7, −4, and 0 relative to PEC induction. Control mice received 0.5 ml of PBS containing 250 µg of anti-β-galactosidase (mAb GL113). Depletion of NK cells was confirmed by flow cytometry.

Statistical analysis

Student’s t tests were used to analyze the significance of experiments with only two experimental groups. One-way ANOVA was used in experiments with three or more groups. Differences were considered significant at p ≤ 0.05. Data are expressed as the mean ± SE.

Results

NK cells regulate Th1 activation in male SJL mice

To determine whether NK cells play a role in the APC-dependent inability of young adult male SJL mice to mount a Th1-mediated CD4+ T cell response, Ag-specific Th1 activation, as measured by induction of DTH responses, was compared in NK-deficient and control mAb-treated young adult male SJL mice. Previous data demonstrated a direct correlation between Ag-specific secretion of Th1 cytokines and DTH responses as well as Ag-specific secretion of Th2 cytokines and a lack of measurable DTH responses (20, 25). Flow cytometric analysis of splenic populations on day 6 after primary immunization confirmed the absence of NK cells in anti-NK1.1-treated mice (data not shown). Age-matched female SJL mice responded regardless of the presence or the absence of NK cells (Fig. 1). Consistent with previous data (24, 25), Ag-specific Th1 CD4+ cells were not induced in control mAb-treated male SJL mice (Fig. 1). By contrast, young adult male SJL mice depleted of NK cells exhibited Ag-specific DTH responses identical to the responses in female SJL mice (Fig. 1) and castrated male SJL mice (22). Potential effects of cellular debris or Ab-bound
Based on the ability to activate Ag-specific Th1 cells in NK cell-deficient young adult males, the frequencies of NK1.1+ cells in low responder SJL mice of both genders were compared with those in age-matched DTH responsive male C57BL/6 mice. Similar to C57BL/6 mice, splenic NK1.1+ expression is predominantly restricted to the CD3−NK1.1+ population in SJL mice. However, consistent with previous reports, the frequencies of both NK1.1+ cells and NK1.1− cells are reduced in SJL mice compared with those in NK-inducible C57BL/6 mice. Comparison of the frequencies of NK1.1+ cells in age-matched 6-wk-old male and female SJL mice that have identical spleen cell numbers showed that Th1-responsive females had a significant reduction in both NK1.1+ NK cells and NK1.1− CD3− NK T cells compared with Th2 responder male SJL mice.

Although the reduced frequency of NK cells in SJL compared with C57BL/6 mice suggested a possible correlation between NK responder phenotype and frequency, age-matched male BALB/c mice (intermediate NK responders) contained ∼3% DX5+ NK cells (data not shown), similar to the frequency of total NK1.1+ cells detected in male SJL mice. Similar frequencies were detected in all three strains of mice using the DX5-pan-NK mAb (data not shown). This suggests the absence of a correlation between NK cell frequency and phenotypic designations based on cytolytic activity.

The gender-dependent inability of males to mount a Th1 CD4+ T cell response is reversed by castration. To determine whether the increased frequency of NK cells in male vs female SJL mice is a consequence of the hormonal environment, the frequency of total NK1.1+ cells in 6-wk-old castrated or sham-castrated males was compared with that in age-matched females. Sham-castrated males exhibited a frequency of splenic NK cells identical with that in untreated males. Castration reduced the frequency of NK1.1+ cells in males to approximately the frequency found in female SJL mice (i.e., ∼2%; Fig. 2B) without an alteration in splenic cell numbers. These data are consistent with a gender-dependent effect on NK cell frequency.

**Increased frequency of IL-10-secreting NK cells in male SJL mice**

The Th2-associated cytokines IL-4 and IL-10 influence APC activity in male SJL mice before Ag encounter. Although previous data suggested that IL-10 is derived from the APC population, the precise source of these cytokines is unknown. NK cells and NK T cells secrete a variety of cytokines, including IL-4 and IL-10. IL-4 secretion by NK T cells is undetectable in untreated males, but in vivo anti-CD3-induced activation was compared in age-matched male and female SJL mice, male BALB/c mice, and male C57BL/6 mice. Consistent with previous data, no IL-4 or IFN-γ generation was detected in male SJL mice (12, 13), but also demonstrate the absence of functional NK T cells in SJL mice (14). These data not only confirm the absence of functional NK T cells in male SJL mice but also demonstrate the absence of a gender-dependent differential expression correlated with the increased frequency of NK1.1+ cells in male SJL mice.
gender-dependent differential in cytokine secretion. In the absence of stimulation NK1.1<sup>+</sup> cells derived from neither male nor female SJL mice secreted detectable IFN-γ, IL-4, or IL-10. Following activation, a small percentage of NK1.1<sup>+</sup> cells from both male and female SJL mice secreted IL-4, IL-10, and IFN-γ (Fig. 4, A and B). Although both IL-4 and IL-10 have been implicated in the gender-dependent induction of Th1/Th2 cells in these mice, no difference in the frequency of NK1.1<sup>+</sup> cells secreting IL-4 or IFN-γ was detected. By contrast, an increased frequency of NK1.1<sup>+</sup> cells derived from 6-wk-old male SJL mice secreted IL-10 compared with age-matched female mice. It is not clear whether the small frequency of cytokine-secreting cells derived from SJL mice represents distinct populations secreting individual cytokines or, alternatively, the secretion of multiple cytokines by individual cells. The latter possibility is supported by analysis of NK1.1<sup>+</sup> cells derived from C57BL/6 mice. In contrast to SJL mice, in which no cytokine secretion was detected before activation, >40% of NK1.1<sup>+</sup> cells derived from male C57BL/6 secreted all three cytokines, and the frequency increased ~2-fold following stimulation (data not shown). These data demonstrate an increased frequency of IL-10-secreting NK cells in male SJL mice following in vitro stimulation and raise the possibility that IL-10 derived from NK<sup>+</sup> cells in males contributes to the inability to activate CD4<sup>+</sup> T cells that secrete Th1 type cytokines following protein Ag immunization.

**NK cell depletion alters APC activity**

The absence of induction of CD4<sup>+</sup> T cells expressing Th1 cytokines in male SJL mice is regulated via an APC (21, 24). The data suggest that the increased frequency of NK cells in male SJL mice correlates with the inability to activate Th1 effectors. To determine whether the increased frequency of NK cells in males correlated with changes in function, APC were isolated from NK cell-depleted age-matched males and females and transferred to naive male recipients before immunization. APC derived from age-matched females support Th1 activation in 6-wk-old male SJL recipients, but APC derived from male SJL donors do not, nor do they inhibit Th1 activation following transfer into female recipients (21, 23, 24). Depletion of NK1.1<sup>+</sup> cells from female donors before APC isolation did not alter their ability to support Th1 induction in immunized male recipients (Fig. 5). Similarly, APC derived from control mAb-treated male donors were unable to support induction of Th1 cells, consistent with previous data (21). By contrast, APC derived from NK1.1<sup>+</sup>-cell-depleted male SJL mice support the activation of Th1 cells following adoptive transfer into naive 6-wk-old male recipients (Fig. 5). To insure that specific cell depletion and not nonspecific activation altered APC function, frozen and thawed spleen cells, equivalent to ~20% of the splenic lymphocyte population, were transferred to 6-wk-old male and female recipients. Th1 cells were induced by subsequent immunization in females, but not in males (data not shown), consistent with a specific effect mediated by selective depletion of NK1.1<sup>+</sup> cells. These data suggest that NK cells in male SJL play an active role in regulating the induction of Th1 cells via regulation of the APC population.

**Discussion**

NK cells, including the CD3<sup>+</sup> subset of NK cells, play a critical role(s) in both the early response to infection and in immune homeostasis (1, 2). The data in this report support the concept that NK cells also play a role in regulating adaptive immunity before Ag encounter. SJL mice exhibit a unique gender-dependent differential response to immunization with protein Ag (23). Preferential activation of Th1 or Th2 cells is controlled via the effects of gonadal hormones (32–34) on APC activity (22). Immunization results in the induction of Ag-specific Th1 cells in young adult females and in the preferential activation of CD4<sup>+</sup> T cells expressing a predominantly Th2 cytokine secretion pattern in young adult male SJL mice (20). Preferential activation of Th2 cells in males can be shifted to the preferential activation of Th1 cells by adoptive transfer of APC derived from Th1-responding females (21) or by castration (21, 22, 32). However, the preferential activation of Th1 cells in females cannot be shifted by the transfer of APC derived from young male SJL mice (21). Furthermore, administration of testosterone to female SJL mice increases IL-10 secretion and decreases the severity of EAE (33). Inhibition of IL-4 or IL-10 before immunization in male SJL mice results in Th1 activation (25), demonstrating that these cytokines influence the environment before Ag exposure. Furthermore, the expression of transgenic IL-10 in EAE-susceptible mice renders them EAE-resistant (31, 35), similar to the resistance to active EAE induction observed in male SJL mice (20).

Previous data suggested that a macrophage APC was either the direct or indirect target of gonadal hormone activity (22); however,
gonadal hormone receptors have not been identified on macrophages (36). Analysis of the potential role of NK cells in the pathway linking the male gonads to APC activity was conducted based on the observations that NK cells influence APC maturation (37), and APC influence NK cell function (37–40). The demonstration that NK cell depletion allowed the activation of Th1 effectors in males without altering the response in female mice supports a role for NK cells within this hormone-dependent pathway. Although classified as a low responder strain based on cytolytic activity, the frequency of NK1.1+ cells in young adult male SJL mice, which is lower than that in intermediate responder C57BL/6 mice, is comparable to intermediate responder BALB/c mice. Surprisingly, the data demonstrate that male SJL mice have ~2-fold more NK1.1+ cells compared with age-matched female SJL mice. Castration, which reverses gender-dependent induction of Th2 cells in males, resulting in both activation of Th1 cells and susceptibility to active EAE (21, 22), reduces the frequency of NK1.1+ cells in males to approximately that in females. Although it has previously been suggested that increased levels of the female hormone 17β-estradiol selectively inhibits NK cytolytic function (41), these data suggest that in addition to altering the phenotype of CD4+ T cells (21), gonadal hormones play a role in regulating the frequency of NK1.1+ cells in SJL mice.

NK cells and NK T cells secrete a variety of cytokines, including IL-4 and IL-10 (2, 5, 6). Although SJL mice have a small population of NK1.1+ T cells (12, 13), these data extend previous observations (13, 14) and demonstrate the absence of a link between gender and either IL-4 or IFN-γ secretion by NK T cells in SJL mice. The absence of a functional response by NK T cells in this strain of mice suggests that the phenotype identified is likely to reflect a function of NK1.1+ NK cells in SJL mice. Cytokine secretion was examined to explore the possibility that the NK cells present in males contributed to the IL-4 and/or IL-10 that influence APC activity before Ag encounter (25). In contrast to NK1.1+ cells derived from the intermediate responder C57BL/6 mice, which secreted IFN-γ, IL-4, and IL-10 without stimulation, no spontaneous cytokine secretion was detected from NK cells derived from SJL mice of either gender. The absence of spontaneous cytokine secretion is consistent with the low responder designation based on limited cytolytic potential (27). Following stimulation, a small percentage of NK1.1+ cells derived from male and female SJL mice secreted cytokines. By contrast, stimulation of NK1.1+ cells derived from C57BL/6 mice resulted in the secretion of all three cytokines analyzed by a large percentage of cells. The frequency of NK1.1+ cells derived from C57BL/6 mice secreting cytokines, either spontaneously or following stimulation, exceeded 100%, suggesting that individual NK cells are capable of secreting multiple cytokines. In addition to an increased frequency in male SJL mice, the frequency of NK1.1+ cells secreting IL-10 following activation was increased in males compared with females. The observation that depletion of NK cells from males or inhibition of IL-10 before isolation (25, 30) results in an alteration of APC function suggests a causative relationship. An in vivo environment in which low levels of IL-10 alter APC function without dramatically altering the surface phenotype (30) coupled with the inability to detect NK cell cytosine secretion without stimulation support a potential IL-10-mediated interaction between NK cells and the APC population. However, due to the extremely low frequency of cells secreting IL-10 and the absence of a differential frequency in cells secreting IL-4, it is not possible to rule out a noncytokine, possibly cell-cell, interaction in this model. One possibility in support of a potential cell-cell interaction is recent data demonstrating that the molecule recognized by anti-NK1.1 expressed by SJL mice is encoded by a gene (NKR-P1B) (15) distinct from the gene encoding the NK1.1 epitope in other strains of mice (NKR-P1C) (42). The observation that NKP-P1B encodes a molecule delivering an inhibitory signal suggests the possibility that a gender-dependent increased frequency of NK cells in male SJL mice expressing an inhibitory molecule may modulate the immune response via a cell-cell contact mechanism in contrast to cytokine secretion.

Adaptive transfer of a limited number of APC derived from Th1-responsive females before immunization results in the activation of Th1 cells in males (21, 23, 24), providing a powerful tool to examine the regulation of APC activity. The correlation between gonadal hormones and NK frequency suggested that NK cell frequency and/or activity might correlate with the activation of Th2 cells in male SJL mice. To examine this possibility, NK1.1-depleted males were examined for Th1 activation. In contrast to NK cell-depleted females, which respond normally, Th1 cells were activated in NK-depleted, but not control, males. Furthermore, APC derived from NK cell-depleted males were as efficient as APC derived from females in supporting Th1 activation in naive male recipients in which a Th2 response is normally induced by immunization. These data suggest a correlation between the increased frequency of NK cells in young adult male SJL mice and preferential activation of Th2 cells. Potential effects of cellular debris or activation through FcRs expressed by the APC population (43, 44) were ruled out by transferring splenocyte-derived debris with or without prior absorption of anti-CD8 mAb. Although the present data do not provide an explanation for the interaction(s) of hormones with either the NK cells or the APC that ultimately affects the cytokine profile of Ag-specific CD4+ T cells, the data support the concept that NK cells may play a previously unrecognized role in regulating immune responses before Ag encounter.

References


