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Chronic alcohol consumption decreases the percentage and number of NK cells in the peripheral lymph nodes and exacerbates B16BL6 melanoma metastasis into the draining lymph nodes

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Abstract

NK cells in the lymph nodes play important roles in inhibiting tumor metastasis into draining lymph nodes. Previously, we reported that chronic alcohol consumption interferes with NK cell trafficking from the bone marrow to the spleen. Herein, we found that alcohol consumption decreases the numbers of NK cells in lymph nodes. Adoptive transfer experiments indicated that continued exposure of donor splenocytes to alcohol inhibits NK but not T cell trafficking to lymph nodes. Alcohol did not negatively affect CCR7⁺ and CXCR3⁺ NK cells, but decreased the percentage and number of CD62L⁺ NK cells in the spleen, which are an important source of NK cell trafficking into the lymph nodes. These data suggest that modulation of the microenvironment associated with alcohol consumption impairs the trafficking of NK cells to lymph nodes. The decreased number of NK cells in the lymph nodes was associated with increased melanoma metastasis into the draining lymph nodes.

Keywords

NK cells; trafficking; lymph nodes; CD62L (L-selectin); melanoma metastasis

1. Introduction

NK cells are important components of the innate immune system. These cells kill virus-infected and tumor cells by identifying the absence or low expression of MHC molecules on target cells [1]. NK cells can also be activated by the pathogen-associated molecular pattern molecules through Toll-like receptors to initiate a series of immune responses [2]. Thus, NK cells are at the forefront of host defense. The majority of NK cells originate in the bone marrow (BM). After maturation these cells are released, circulate in the blood, and traffic to peripheral organs. Recent research indicates that NK cells are recirculating cells. They recirculate among the NK cell-containing organs including lymph nodes (LN) [3–6]. The

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main pools for NK cells in the periphery are the spleen, blood, liver and lung; however, some NK cells also are produced in the thymus [7,8] and in the LN [9]. NK cells from these pools contain different phenotypes and have different functions. BM-derived NK cells have a mature phenotype, which is characterized by surface expression of Mac-1^{hi}CD43^{hi}CD127⁻ and Ly49 receptors. Functionally they exhibit strong cytolytic activity, but weak IFN- γ production. Thymus-derived NK cells express an immature phenotype and are CD127⁺. Most do not express Mac-1, CD43 or Ly49 receptors. These cells are good IFN- γ producers but express weak cytolytic activity [7].

The population of NK cells in the LN is very small, and comprises only 0.5% of the total cell population in LN [3,10]. Although the numbers are low, these NK cells play an important role in metastasis of B16 melanoma and other tumors into the LN [10]. Once a tissue is populated by tumor cells, NK cells are recruited into the inflammatory site and into the draining LN where they interact with dendritic cells. By producing TNF- α , IFN- γ , and other factors, NK cells accelerate dendritic cell maturation [11]. In addition, these NK cells also kill the immature dendritic cells, which improves the efficiency of antigen presentation by mature dendritic [12,13]. The IL-12 produced by dendritic cells activates NK cells that subsequently produce IFN- γ , which in turn primes and polarizes T cells toward Th1 functionality [4,14]. Failure to recruit NK cells into the LN compromises T cell priming and polarization and also increases melanoma metastasis into the LN [10].

Like T, B and dendritic cells, the recruitment of NK cells into the LN is controlled by the cell adhesion molecules, chemokines and their receptors. Trafficking of T, B, and a subpopulation of NK cells to LN under steady state conditions is primarily dependent on the chemokine receptor, CCR7, and its ligands CCL19 and CCL21 [4]. CXCR3 and its ligands, CXCL9, CXCL10, and CXCL11, and CD62L (L-selectin), a cell adhesion molecule expressed on NK cells, control trafficking of NK cells into LN in response to inflammation as well as melanoma stimuli [10,15].

Recruitment of NK cells into the liver is impaired by chronic alcohol consumption, and this worsens liver injury during murine cytomegalovirus infection or Con-A induced inflammation [16]. We found that chronic alcohol consumptions also decreases the percentage and number of NK cells in the spleen [17,18], and increases the percentage and number of NK cells in the BM [17]. We recently found that chronic alcohol consumption inhibits tumor-specific CD8⁺T cell expansion in B16BL6 melanoma-bearing mice [19]. Since NK cells in the LN are crucial to T cell priming and polarization [4,13], the impaired expansion of melanoma-specific CD8⁺T cells could be related to the decreased number of NK cells in response to chronic alcohol consumption. Thus, we examined the hypothesis that chronic alcohol consumption decreases the number of NK cells in the LN during the steady state. We found that chronic alcohol consumption, representative of the intake associated with human alcoholics, decreases NK trafficking into the LN without increasing the rate of NK apoptosis or decreasing the rate of NK cell proliferation. Impaired NK trafficking was not associated with alterations in CCR7 or CXCR3; however, it was correlated with decreased percentage and number of splenic CD62L⁺ NK cells. The decrease in the numbers of NK cells in the LN correlated with enhanced B16BL melanoma metastasis into the LN.

2. Materials and methods

2.1. Animals and alcohol administration

Female C57BL/6 mice 6–7 weeks of age were purchased from NIH Charles River Laboratories (Wilmington, MA). Mice were housed in the Wegner Hall Vivarium at Washington State University (Pullman, WA). The experimental protocols were approved by

the Institutional Animal Care and Use Committee and followed previously described procedures [20]. Briefly, mice were housed singly after one week of acclimation in plastic cages with filter tops. There were given free access to Purina 5001 laboratory chow, filter sterilized double-distilled deionized water, or 20% w/v alcohol Everclear™ as the sole drinking fluid throughout the experimental period from three to six months independent of whether the mice were inoculated with melanoma tumors. Similar results were obtained in replicative experiments conducted within this time frame of alcohol feeding, which is consistent with other findings in a previous report [17].

2.2. Tumor cell culture, inoculation and metastasis to LN

The B16BL6 mouse melanoma cell line, a derivative of B16F10 that is more invasive and metastatic, was cultured in high glucose DMEM (Thermo Scientific HyClone, Logan, UT) supplemented with 10% fetal bovine serum (Equitech-Bio, Inc. Kerrville, TX) 100 µg/ml streptomycin and 100 U/ml penicillin in a 5% CO₂ incubator at 37°C. Cells were harvested at 70% confluence after aspirating the culture medium, and washing the cells with calcium- and magnesium-free phosphate buffer (CMF). Cells were detached with trypsin (Invitrogen, Carlsbad, CA), washed with culture medium, resuspended in CMF and then counted with a Beckman Vi-CELL XR cell viability analyzer (Beckman Coulter, Fullerton, CA). Mice were inoculated subcutaneously into the right hip region with 2×10^5 B16BL6 melanoma cells in an injection volume of 200 µl cells. Mice were euthanized three weeks later and the right inguinal LN removed and photographed. The relative tumor burden was determined and expressed as the percentage of tumor area divided by the greatest cross-sectional area of each LN. The areas were measured using NIH ImageJ software.

2.3. Reagents

The following fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein-cyanine 5.5 (PerCP), and allophycocyanin (APC) or biotin-labeled anti-mouse antibodies were obtained from BD Biosciences Pharmingen (San Diego, CA) or BioLegend (San Diego, CA): anti-NK1.1 (PK136), anti-CD3 (145-2C11), anti-CD62L (MEL-14), anti-CXCR3 (CXCR3-173), anti-CCR7 (4B12), and anti-5-bromo-2-deoxyuridine (BrdU) (3D4). Streptavidin-PE and Annexin-V-FITC were ordered from BD Biosciences. BrdU was purchased from Sigma-Aldrich (St. Louis, MO). Carboxyfluorescein succinimidyl ester (CFSE) was purchased from Invitrogen.

2.4. Isolation of splenocytes, BM cells and LN cells

Spleens from mice were removed and transferred into tissue culture dishes containing 10 ml of phosphate buffered saline (PBS) + 0.1% bovine serum albumin (BSA). Single cell suspensions were created by pushing individual spleens through a stainless steel wire screen with a syringe plunger and then passed through a 100 µm BD Falcon strainer (BD Biosciences, San Jose, CA) to remove debris. The cells were pelleted by centrifugation, washed with 50 ml of PBS + 0.1% BSA, and centrifuged again. The cell pellet was resuspended in 5 ml of red blood cell lysis buffer at room temperature for 5 min. Then, 30 ml of PBS + 0.1% BSA were added to terminate the lysis. The cell suspension was filtered through a 40 µm cell strainer (BD Falcon, BD Biosciences) into a fresh 50 ml tube, centrifuged, and the cell pellet was washed twice with 50 ml of PBS + 0.1% BSA. The cells were counted with the Beckman CELL XR cell viability analyzer and resuspended in an appropriate amount of PBS + 0.1% BSA or in serum-free RPMI 1640 medium to a final concentration of 10^8 cells/ml.

Two femurs were collected for BM cell isolation. The BM was flushed out using a 12 ml syringe attached with a 25 ½ G needle filled with 10 ml PBS. BM cells were washed once with PBS and then centrifuged. The cell pellet was resuspended in 5 ml of red blood cell

lysis buffer at room temperature for 5 min. Then 30 ml PBS + 0.1% BSA were added to terminate the lysis, and the cell suspension was filtered with a 40 μ m cell strainer (BD Falcon). Cells pelleted by centrifugation were washed twice with 50 ml of ice-cold PBS + 0.1% BSA and then counted with the Beckman CELL XR cell viability analyzer. Cells were resuspended in an appropriate amount of PBS + 0.1% BSA to a final concentration of 10^8 cells/ml.

For cell isolation from LN, inguinal and axillary LN were collected and transferred to a tissue culture dish with PBS + 0.1% BSA. Single cell suspensions were created by forcing the LN through a 100 μ m cell strainer with a syringe plunger. The cell suspension was filtered with 40 μ m nylon mesh to remove debris. Cells were washed with PBS + 0.1% BSA, and adjusted to a concentration of 10^8 cells/ml with PBS + 0.1% BSA as indicated above.

2.5. CFSE labeling and adoptive cell transfer

Splenocytes (5×10^7) from water drinking mice 8–10 weeks of age (donor cells) were prepared as described above and suspended in 500 μ l of serum-free RPMI 1640 medium mixed with 4.5 ml of 5 μ M CFSE in PBS. The cells were incubated at 37°C for 8 min. CFSE labeling was stopped by adding 30 ml of RPMI 1640 + 10% FBS to the cell suspension. Labeled cells were washed twice with RPMI 1640 + 10% FBS and resuspended in 500 μ l of PBS. One water drinking and one alcohol consuming recipient mouse were injected with 2×10^7 in 200 μ l of the CFSE labeled donor cells (isolated from one water drinking mouse) via the lateral tail vein. In total 5 individual donor mice were used for the adoptive transfer experiments. Recipient mice were euthanized at the indicated time points. Cells from the spleen, femurs and LN were prepared as described above. Transferred cells in each organ were determined by flow cytometry analysis as described previously [20].

2.6. Flow cytometry analysis

General flow cytometry analysis followed previously described methods [20]. For CCR7 analysis, cells were incubated with anti-mouse CD3-APC, anti-mouse NK1.1-PerCP and biotin conjugated anti-mouse CCR7 at room temperature for 30 min. Cells were washed once with PBS+0.1%BSA+0.09%NaN₃ [fluorescence-activated cell sorting (FACS) buffer], incubated with streptavidin-PE at room temperature for 30 min, washed once with FACS buffer, and then resuspended in FACS buffer for further analysis. Cell phenotypes were analyzed with FACScan (BD Biosciences) or FACSCaliber (BD Biosciences) flow cytometers and the results analyzed using CellQuest software (BD Biosciences). NK cells are defined as CD3⁻NK1.1⁺ cells.

2.7. BrdU in vivo incorporation assay

The BrdU incorporation assay followed the method described previously [20]. Briefly, BrdU (Sigma, St. Louis) prepared fresh daily in drinking water or 20% (w/v) alcohol at the concentration of 0.8 mg/ml was given to mice nine days before analysis. LN cells were isolated and stained as indicated above. Stained cells were washed with FACS buffer, and then fixed with Cytotfix/Cytoperm™ (BD Pharmingen) at 4°C for 20 min. The fixed cells were washed twice with Perm/Wash™ plus buffer (BD Pharmingen) and then incubated for 60 min at 37°C after with 30 μ g DNase I. Cells were pelleted, washed twice with Perm/Wash™ plus buffer, and then stained with anti-BrdU-FITC (BD Pharmingen) at room temperature for 30 min. The cells were washed and then analyzed by flow cytometry as described above.

2.8. Statistical analysis

Microsoft Excel was used to analyze the data. Student's t-test was used to test the difference between groups. Differences between groups were considered significant at $p < 0.05$.

3. Results

3.1. Chronic alcohol consumption decreases the percentage and number of NK cells but does not induce NK cell apoptosis or alter NK cell proliferation in the LN

Chronic alcohol consumption increases the percentage and number of NK cells in the BM and decreases the percentage and number of NK cells in the spleen by compromising NK cell release from the BM and inducing splenic NK cell apoptosis [17,18]. We also found that chronic alcohol consumption impairs tumor specific T cell expansion in mice subcutaneously inoculated with B16BL6 melanoma [19]. Inhibition of specific T cell expansion by chronic alcohol consumption has also been found during bacterial infection [21]. The quantity and quality of NK cells in the draining LN play an important role in priming T cells in an immune response [4,22,23]. Therefore, we examined if chronic alcohol consumption affected NK cells in the LN. We found that alcohol consumption did not affect the total number of lymphocytes in the LN (Fig. 1E); however, it decreased the number (Fig. 1F) and percentage (Fig. 1A, B, & G) of $CD3^+NK1.1^+$ cells in the LN compared to water drinking controls by about 2-fold. While the total number of lymphocytes in the LN was not significantly changed, this is not surprising since NK cells comprise only 0.5% of the total LN cell population. Thus, even the 2-fold decrease in the number of NK cells in the alcohol consuming mice might not be reflected in a statistically significant decrease in the number of total cells in the LN or in the numbers of T and B cells, the main cell populations in the LN. Indeed, we did not find significant changes in the percentage and number of T and B cells in the LN (data not shown). Chronic alcohol consumption did not differentially affect the constitutive rate of apoptosis (Fig. 1C & H) or proliferation (Fig. 1D & I) of NK cells in the LN. These data indicate that the decrease in the numbers of NK cells in the LN of alcohol consuming mice is not likely due to enhanced apoptosis or impaired proliferation.

3.2. Chronic alcohol consumption does not impair expression of $CCR7^+$ NK or T cells in the spleen

The majority of NK cells in the LN are circulatory cells from the BM and spleen. Since, chronic alcohol consumption did not alter the rates of apoptosis or proliferation of these cells in the LN, the decrease in number of NK cells in the LN could result from the impaired recirculation of these cells from the BM and spleen. The results in Fig. 1 indicating a major effect of alcohol consumption on the numbers of NK cells in the LN lead us to determine if migration of these cells from the BM and spleen was impaired. We previously reported that chronic alcohol consumption compromises NK cell release from the BM [17], which could at least partly contribute to the decrease in number of NK cells in the LN. $CCR7$ plays an important role in homing of lymphocytes to LN under steady state conditions, and results from splenocyte transfer experiments indicate that knockout of the $CCR7$ gene ($CCR7^{-/-}$) significantly decreases the homing of spleen cells (containing both T cells and NK cells) to the LN [4]. We found that the percentage of $CCR7^+$ NK cells in the spleen was slightly increased in the alcohol consuming mice compared to the water drinking mice (Fig. 2B & 2D). The percentage of $CCR7^{hi}$ expressing $CD3^+NK1.1^-$ T cells also increased slightly as a result of alcohol consumption while the percentage of $CCR7^{int}$ $CD3^+NK1.1^-$ T cells was slightly decreased (Fig. 2 C & E). These results suggest that the splenic $CCR7$ signaling pathway is intact and not likely associated with decreased numbers of NK cells in the LN in alcohol consuming mice.

3.3. Chronic alcohol consumption increases the percentage of CXCR3⁺ NK cells and decreases the percentage and number of CD62L⁺ NK cells in the spleen

Circulation of NK cells from the spleen and BM to the LN is controlled by cell adhesion molecules, especially CD62L [10,15]. During inflammation, CXCR3 as well as CD62L play important roles in the trafficking of NK cells to the LN from the BM and spleen [4]. We found that chronic alcohol consumption significantly increased the percentage of CXCR3⁺ NK cells in the spleen (Fig. 3A). The percentage of CD62L⁺ NK cells was significantly lower in the spleen and BM of alcohol consuming mice compared to the water drinking mice (Fig. 3B). This was reflected in a 40% lower number of CD62L⁺ NK cells in the spleen of alcohol consuming mice compared to their water drinking counterparts (Fig. 3C). The expression of CD62L on the CD62L⁺ NK cells in the spleen as determined by the mean fluorescence index was not different between water drinking and alcohol consuming mice (data not shown). The percentage of CD62L⁺ NK cells in the BM of alcohol consuming mice was slightly lower (Fig. 3B); however, the number was higher in the alcohol consuming mice than in the water drinking mice (Fig. 3C) because the total number of NK cells in the alcohol consuming mice is higher in this organ due to compromised release [17]. In the LN, neither the percentage of CD62L⁺ NK cells (Fig. 3B) nor the mean fluorescence index was different (data not shown). However, the number of CD62L⁺ NK cells in the LN was lower in alcohol consuming mice (Fig. 3C) as a direct result of fewer NK cells in the LN (Fig. 1F). Interestingly, chronic alcohol consumption did not affect the percentage of CD62L⁺ or CXCR3⁺ CD3⁺ T cells in the spleen, BM and LN (Fig. 3D & 3E). These results indicated that the CXCR3 signaling pathway is intact in alcohol consuming mice and they suggested that CD62L is the likely target of alcohol consumption resulting in impaired trafficking of NK cells into the LN.

3.4. The inhibition of chronic alcohol consumption on NK cell homing to LN is time-dependent

Cell transfer experiments were conducted to further determine if the decrease in numbers of NK cells in the LN was caused by impaired trafficking of these cells from the spleen to the LN as described in the Materials and Methods. Trafficking of CFSE-labeled donor splenocytes to the LN, BM and spleen was determined. No statistically significant differences between the two groups were observed in the number of transferred NK or CD3⁺ T cells in the LN, BM and spleen 18 hr after transfer (Fig. 4A–F). However, seven days after cell transfer, the number of transferred NK cells in the LN of alcohol consuming mice was significantly lower than in the LN of water drinking mice (Fig. 4A). There were no significant differences between the two groups in the number of transferred NK cells in the BM (Fig. 4B) or spleen (Fig. 4C). No differences in the numbers of transferred CD3⁺ T cells were observed in LN, BM, and spleen at either 18 hr or 7 days (Fig. 4D–F).

3.5. Chronic alcohol consumption decreases the percentage of transferred CD62L⁺ NK cells in the spleen

CD62L expression is key to NK cell recruitment into LN [10]. The spleen is one of the major sources for recruitment of NK cells to the LN. Thus, it is possible that the low number of adoptively transferred NK cells in the LN of alcohol consuming mice at 7 days after donor splenocyte transfer (see Fig. 4A) could result from the loss of transferred CD62L⁺ NK cells in the spleen induced by alcohol. Therefore, we examined the CD62L⁺ NK cells in the spleen utilizing adoptive transfer experiments as described in the Materials and Methods. The percentage of CFSE[−] and CFSE⁺ CD62L⁺ NK cells (donor derived) in the spleen of recipient mice was examined seven days after the cell transfer. The percentage of CFSE[−] CD62L⁺ NK cells in the recipient alcohol consuming mice was significantly lower in alcohol consuming mice (Fig. 5) than in their water drinking counterparts as expected from the results shown in Fig. 3B. However, the percentage of CFSE⁺ CD62L⁺ NK cells was also

lower in the alcohol consuming mice than in the water drinking mice (Fig. 5). The percentages of CD62L⁺ NK cells injected into water drinking and alcohol consuming mice were identical since they were derived from the same donor. The fact that we observed a decrease in the percentage of transferred CFSE⁺ CD62L⁺ NK cells in the alcohol consuming mice indicates that chronic alcohol consumption in some way specifically compromises CD62L expressing NK cells in the spleen possibly by altering the organ microenvironment.

3.6. Chronic alcohol consumption exacerbates B16BL6 melanoma metastasis into the draining LN

NK cells and not T cells play an essential role in the control of melanoma metastasis into the draining LN [10]. Since chronic alcohol consumption decreases the number of NK cells in the LN, we determined if this was reflected in increased metastasis into the draining LN after subcutaneous inoculation of B16BL6 melanoma. Melanoma growth was observed in all eight draining inguinal LN of alcohol consuming mice. Only three out of eight LN from water drinking mice contained visible melanoma growth (Fig. 6). No melanoma growth was evident in the left inguinal LN in either group (data not shown). The overall relative tumor burden also was greater in the alcohol consuming mice compared to the water drinking group.

4. Discussion

In the present study we demonstrated that chronic alcohol consumption decreases the number of NK cells in the LN by impairing NK cell trafficking from the spleen. The decrease in the numbers of NK cells in the LN correlated with increased metastasis of subcutaneously inoculated B16BL6 melanoma into the draining LN. These findings combined with our previous results clearly demonstrate that the modulation of NK cells by chronic alcohol consumption is organ specific [17,18]. We showed previously that chronic alcohol consumption did not significantly affect NK cells in the thymus; however, it increased the percentage and number of NK cells in the BM by compromising NK cell release. Similar to the LN, alcohol consumption did not alter NK cell proliferation and only moderately increased apoptosis of NK cells in the BM [17]. The decrease in numbers of splenic NK cells resulted from decreased release of these cells from the BM and from enhanced apoptosis of NK cells expressing an immature phenotype [18].

While the findings support the concept that chronic alcohol impairs homing of NK cells to the LN, the origination of NK cells in the LN is not completely understood. It is generally believed that the majority of NK cells in the LN are derived from the BM; however, we previously found that alcohol consumption compromises NK cell release from the BM. Once BM NK cell release is impaired, splenic NK cells become the most important source for the replenishment of LN NK cells. In addition, around 15–30% of NK cells in the LN can be thymus-derived [7]. LN also have the potential to produce their own NK cells [9]. Thus, the decrease in number of NK cells in the LN associated with chronic alcohol consumption could occur from one or all of the following three possibilities: a) increased NK cell apoptosis in the LN, b) impaired proliferation of NK cells in the LN, and c) compromised recirculation of NK cells from other NK cell pools such as the BM and spleen to the LN. The results of this investigation ruled out the first two possibilities, since the degree of NK cell apoptosis and proliferation is not altered in the LN. These findings further indicate that the effects of chronic alcohol consumption are organ specific. Therefore, it is likely that the decrease in the numbers of NK cells in the LN was associated with compromised recirculation from NK cell pools.

The majority of cells in the LN are T cells and B cells. The fact that we did not observe a decrease in the total numbers of cells in the LN during chronic alcohol consumption

suggests that trafficking of T cells and B cells is not impaired in the alcohol consuming mice at steady state. This further suggests that the machinery that determines lymphocyte trafficking to LN, which includes CD62L and CCR7 and their ligands, is not altered in the LN of alcohol consuming mice. Indeed, we found no difference in the percentage of CD62L⁺ T cells and a slight increase in the percentage of CCR7^{hi} T cells. In the steady state, NK cell homing to LN is also controlled by CD62L, CCR7, and their ligands [4,10]. CD62L, CXCR3, and their ligands control NK cell homing to LN in response to inflammation and the presence of tumors. Alcohol consumption did not adversely modulate the percentage of cells expressing CCR7 and CXCR3; therefore, it is not likely that these molecules are involved in the impaired homing of NK cells to LN.

The data herein indicate that CD62L is an important molecular target by which chronic alcohol consumption modulates NK cell homing to LN. Alcohol consumption decreases the percentage of CD62L⁺ NK cells in the spleen and BM, but not in the LN. The overall decrease in the total numbers of NK cells in the spleen accentuates the deficiency of the splenic CD62L⁺ NK cells. While the number of CD62L⁺ NK cells is increased in the BM, this increase is caused by the general accumulation of NK cells in the BM [17].

Short term (18 hr) splenocyte transfer experiments revealed no differences in organ distribution between alcohol consuming and water drinking mice indicating that the lymphocyte homing and retention mechanisms in the LN as well as in the BM and spleen are intact. However, after the donor cells have been exposed to alcohol for 7 days, the continued recirculation of these cells to the LN is impaired. This finding is further supported by the results in Fig. 5 indicating a decrease in the percentage of transferred CD62L⁺ NK cells in the spleen. The mechanism underlying these later findings is not known. It is possible that alcohol facilitates shedding of CD62L from NK cells. Alcohol *in vitro* has been associated with increased shedding of CD62L from Jurkat T cells [24]. However, this is unlikely since the CD62L expression on spleen and LN T cells was not different between water drinking and alcohol consuming mice. Additionally, we found no impairment in trafficking of T cells to LN, which depends on CD62L [10]. It is also possible that CD62L⁺ NK cells are more prone to apoptosis in the blood and/or LN; however, this is unlikely for the following reasons. If CD62L⁺ NK cells were more prone to apoptosis in the blood, then we should have observed a decrease in the numbers of these cells in the spleen and BM as well as in LN. If CD62L⁺ NK cells entering the LN were more prone to apoptosis, then we would have expected to see an increased rate of apoptosis in alcohol consuming mice under steady state conditions. This was not the case (Fig. 1H). LFA-1 expression is essential to retention of NK cells in LN [3], and alcohol could impair expression of this adhesion molecule on NK cells. We examined this possibility; however, we found that the percentage and expression of LFA-1 were not diminished in chronic alcohol consuming mice (data not shown). While the data implicate CD62L as the molecular target of chronic alcohol consumption that modulates splenic NK cell trafficking to the LN, additional studies are needed to define the mechanism in more detail.

NK cell recruitment to LN is essential in preventing LN metastasis of B16 melanoma tumors [10]. Herein we found that the decreased numbers of NK cells in the LN are associated with increased metastasis of melanoma into the draining LN after subcutaneous inoculation into the hip region. This is in contrast to our previous findings that alcohol consumption inhibits metastasis to the lung [25,26] even when NK cells were depleted with anti-NK1.1 antibody [25] or when melanoma cells were inoculated into *beige* mice, which have compromised NK cell activity [27]. The exact mechanism underlying these regional differences is not known. Both NK cytolytic activity and IFN- γ production in the LN NK cells are essential to inhibition of melanoma metastasis into the LN [10]. We previously showed that NK cytolytic activity is impaired by alcohol chronic consumption [28]. We also showed that

chronic alcohol consumption decreases the percentage and number of IL-15 producing cells in the spleen [18], and it is possible that IL-15 production could be compromised in the LN as well. IL-15 is chemotactic for NK cells and facilitates the adhesion of NK cells to vascular endothelium, and it is also important to the production of IFN- γ [29]. Since alcohol consumption also reduces the numbers of NK cells in the LN by two-fold, this would accentuate the impact of any decrease in IFN- γ production in the LN.

In summary, the results indicate that chronic alcohol consumption decreases the percentage and number of NK cells in the inguinal and axillary LN. This effect is not due to increase in the rate of NK cell apoptosis or to decrease in NK cell proliferation. Previous findings indicate that decreased numbers of NK cells in the spleen are related to compromised release of these cells from the BM [17]. The data herein indicate that fewer splenic NK cells are available for homing to the LN. Exacerbation of metastasis into the draining LN of mice chronically consuming alcohol is likely due to an inability of NK cells to be recruited in response to subcutaneous melanoma inoculation along with other deficient NK cell responses in the LN.

Abbreviations used

BM	bone marrow
LN	lymph nodes
CMF	calcium- and magnesium-free phosphate buffered saline
FITC	fluorescein isothiocyanate
PE	phycoerythrin
PerCP	peridinin chlorophyll protein-cyanine 5.5
APC	allophycocyanin
BrdU	5-bromo-2-deoxyuridine
CFSE	carboxyfluorescein succinimidyl ester
PBS	phosphate buffered saline
BSA	bovine serum albumin
FACS	fluorescence-activated cell sorting

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Fig. 1.

Effects of chronic alcohol consumption on NK cells in the LN. (A) and (B) Representative dot plots of gated NK cells in the LN cell population of water drinking mice (A) and alcohol consuming mice (B), respectively. NK cells were identified by gating on the NK1.1⁺ cells that are CD3⁻ in this and subsequent figures. (C) Dot plot indicating annexin V stained cells in the LN from alcohol consuming mice. (D) Dot plot indicating BrdU⁺ LN cells from water drinking mice. (E) Total number of cells in LN. (F) Number of NK cells in LN. (G) Percentage of NK cells in the LN. (H) Percentage of annexin V⁺ NK cells. (I) Percentage of BrdU⁺ NK cells. Values are mean $\pm$ S.D. for groups containing 7–10 mice. The data are representative of three different experiments with similar findings. ETOH group different from Water group, ** $p < 0.001$. Water = Water drinking group. ETOH = Alcohol consuming group.

Fig. 2.

Effects of chronic alcohol consumption on the expression of CCR7 in splenic NK cells and NK1.1⁻CD3⁺ cells. (A) Dot plot indicating gated NK cells (upper left region) and CD3⁺ T cells (lower right region) in the spleen of alcohol consuming mice. (B) Dot plot indicating CCR7⁺ NK cells in gated splenic NK cells of alcohol consuming mice. (C) Histogram of CCR7⁺ gated CD3⁺ T cells in the spleen of alcohol consuming mice. M1: CCR7^{hi}, M2: CCR7^{int}. (D) Percentage of CCR7⁺ NK cells in the spleen. (E) Percentage of CCR7^{hi} and CCR7^{int} CD3⁺ T cells in the spleen. Values are mean $\pm$ S.D. for groups containing 7–10 mice. The data are representative of two different experiments with similar findings. ETOH group different from Water group, * $p < 0.05$. Water = Water drinking group. ETOH = Alcohol consuming group.

Fig. 3.

Effects of chronic alcohol consumption on CXCR3⁺ and CD62L⁺ NK cells. (A) Percentage of CXCR3⁺ NK cells in the spleen, LN, and BM NK cells. (B) Percentage of CD62L⁺ NK cells in the spleen, LN, and BM NK cells. (C) Number of CD62L⁺ NK cells in the spleen, LN, and BM. (D) Percentage of CXCR3⁺CD3⁺ T cells in spleen, LN, and BM CD3⁺ T cells. (E) Percentage of CD62L⁺ CD3⁺ T cells in the spleen, LN, and BM CD3⁺ T cells. Values are mean $\pm$ S.D. for groups containing 7–10 mice. The data are representative of two different experiments with similar findings. Open bars, Water drinking group. Cross-hatched bars, Alcohol consuming group. Alcohol consuming group different from Water drinking group, * $p < 0.05$. ** $p < 0.001$.

Fig. 4.

Effects of chronic alcohol consumption on the trafficking and distribution of transferred NK and NK1.1⁺CD3⁺ T cells into the spleen, LN, and BM. Splenocytes from one water-drinking mouse were isolated, labeled with CFSE. These cells divided into two parts, and 2×10^7 cells were injected intravenously into one alcohol consuming mice and one water drinking control mice via the lateral tail vein. In total 5 individual donor mice were utilized. Mice were euthanized at the indicated time points. Splenocytes, LN cells, and BM cells were isolated and stained with anti-mouse CD3-PE and anti-mouse NK1.1-PerCP and specific cell populations were analyzed by flow cytometry. (A) Number of CFSE⁺ NK cells in the LN. (B) Number of CFSE⁺ NK cells in the BM. (C) Number of CFSE⁺ NK cells in the spleen. (D) Number of CFSE⁺CD3⁺ T cells in the LN. (E) Number of CFSE⁺CD3⁺ T cells in the BM. (F) Number of CFSE⁺CD3⁺ T cells in the spleen. ETOH group different from Water group, *p<0.05. Water = Water consuming mice; ETOH = Alcohol consuming mice.

Fig. 5.

Effect of chronic alcohol consumption on the percentage of splenic CD62L⁺ NK cells before and after adoptive splenocyte transfer. Splenocytes from one donor mouse were isolated, labeled with CFSE, divided into two parts, and 2×10^7 cells in 200 μ l of PBS were injected intravenously into one alcohol consuming and one water drinking mice via the lateral tail vein. In total 5 donor mice were utilized. Splenocytes were isolated 7 days after cell transfer and analyzed by flow cytometry. CFSE⁻, Percentage of CD62L⁺ NK cells that comprise the host NK cell population; CFSE⁺, Percentage of CD62L⁺ NK cells that comprise the adoptively transfer NK cell population. Values are mean $\pm$ S.D. ETOH group different from

Water group, * $p < 0.05$, ** $p < 0.001$. Water = Water consuming mice; ETOH = Alcohol consuming mice.

Fig. 6.

Effects of chronic alcohol consumption on B16BL6 melanoma metastasis into the draining LN. Mice consumed alcohol for five months before tumor inoculation. B16BL6 melanoma cells then were inoculated subcutaneously into water drinking and alcohol consuming mice into the right hip area at a concentration of 10^5 cells in 200 μ l of CMF. Mice were euthanized three weeks after the tumor cell inoculation. The right inguinal LN were removed and evaluated for the presence of metastasis. The relative tumor burden was expressed as the percentage of tumor areas divided by the greatest cross-sectional area of the LN. The areas were measured using NIH ImageJ software. Each group contained 8 mice. ETOH group different from Water group, * $p < 0.03$. Water = Water drinking group; ETOH = Alcohol consuming group.