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Cigarette smoke alters the invariant natural killer T cell function and may inhibit anti-tumor responses

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Abstract Invariant natural killer T (iNKT) cells are a minor subset of human T cells which express the invariant T cell receptor V α 24 J α 18 and recognize glycolipids presented on CD1d. Invariant NKT cells are important immune regulators and can initiate anti-tumor responses through early potent cytokine production. Studies show that iNKT cells are defective in certain cancers. Cigarette smoke contains many carcinogens and is implicated directly and indirectly in many cancers. We investigated the effects of cigarette smoke on the circulating iNKT cell number and function. We found that the iNKT cell frequency is significantly reduced in cigarette smoking subjects. Invariant NKT cells exposed to cigarette smoke extract (CSE) showed significant defects in cytokine production and the ability to kill target cells. CSE inhibits the upregulation of CD107 but not CD69 or CD56 on iNKT cells. These findings suggest that CSE has a specific effect on iNKT cell anti-tumor responses, which may contribute to the role of smoking in the development of cancer.

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1. Introduction

Smoking is the main cause of preventable morbidity and mortality in the world [1]. Carcinogens from cigarette smoke can lead directly to lung cancer [2] and are implicated in several other malignancies such as bladder, cervix, stomach, liver and kidney [3]. In addition to the increased cancer risk, smokers have increased susceptibility to infections [4] and a marked increase in autoimmune disorders such as rheumatoid arthritis [5].

Abbreviations: α GalCer, α -Galactosylceramide, CSE, Cigarette smoke extract, iNKT, Invariant natural killer T cell, PBMC, Peripheral blood mononuclear cells

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Studies have shown that immunocompromised animals have an increased incidence of cancer [6]. Circulating natural killer (NK) cell number and anti-tumor activity are reduced in smokers compared to non-smokers [7,8]. We have previously demonstrated that cigarette smoke extract (CSE) reduces the NK cell function *in vitro* in a dose-dependent manner [9]. These studies suggest that cigarette smoke alters the host immunity, enabling tumor cells and pathogens to evade immune responses and increasing susceptibility to autoimmune conditions.

Invariant natural killer T (iNKT) cells are a rare subset of human innate T cells defined by their expression of the invariant T cell receptor (TCR) V α 24 J α 18 and NK-like surface molecules [10]. The invariant TCR recognizes glycolipid antigen such as the marine sponge-derived antigen α Galactosylceramide (α GalCer) [11,12]. Invariant NKT cells are potent immunomodulatory cytokine secreting cells, capable of simultaneously producing IFN- γ and IL-4 when stimulated [13,14]. Invariant NKT cells respond rapidly to antigen (Ag) or infection (<2 h) and can activate other cells of the innate and adaptive immune systems including NK cells [15]. Invariant NKT cells are implicated in the prevention of tumor growth and the eradication of existing tumors in mice [16,17]. In humans the number of circulating and tissue-resident iNKT cells are reduced in cancer [18–20]. Currently iNKT cells are the basis of numerous phase I and II oncology trials [21–24]. As well as their ability to rapidly produce immunomodulating cytokines, iNKT cells have been reported to lyse target tumor cells *in vitro* [25,26].

The objective of this study was to investigate if cigarette smoke affects the function of iNKT cells. We investigated the levels of circulating iNKT cells in a cohort of healthy subjects who smoke 20 cigarettes daily compared to age-matched non-smoking individuals. We also investigated both the iNKT cell production of immune modulating cytokines and their ability to efficiently lyse tumor cells before and after exposure to CSE.

2. Materials and methods

2.1. Ethics statement

The ethics committee at St. Vincent's University Hospital, Dublin granted approval for all aspects of this study. Blood samples were obtained with informed written consent from healthy individuals who smoked 20 cigarettes daily and age-matched controls.

2.2. Preparation of PBMC cells

100 mls of venous blood was collected in heparinized collection tubes. PBMCs were isolated by density centrifugation on Lymphoprep (Nycomed, Norway) at 400 g for 25 min. Cells were washed twice and cell pellets were re-suspended in 1 ml of RPMI 1640 medium, and cell yields and viability were assessed by ethidium bromide/acridine orange staining. The cell suspension was adjusted to 100×10^6 cells/ml in Miltenyi separation buffer (Miltenyi Biotech, UK).

2.3. Generation of iNKT cell lines

iNKT cells were isolated from PBMC cell suspensions by positive selection of anti-6b11 magnetic bead (Miltenyi Biotech, UK) labeled cells (as per manufacturer's instructions). The purified iNKT cell suspensions were stained with 6B11 PE and CD3 PE-CY5 monoclonal antibodies (mAb) (BD Biosciences, UK) for 30 min, before washing. The cells were then sorted by high speed FACS for double positive cells (6B11⁺ CD3⁺) using a MoFlo FACS (Dako Cytomation, USA). 1000 sorted cells were cultured per 96-round bottomed plate well in complete RPMI medium with 150,000 irradiated PBMC from 2 donors, 10 ng/ml of PHA (Sigma Aldrich, UK) and 250 U/ml of IL-2 (Immunotools, Germany). The PHA was diluted out over 48 h using a complete RPMI with 250 U/ml of IL-2. Media was replenished every 7–10 days and wells divided when required.

2.4. Flow cytometric staining and analysis

Cells were stained with optimal amounts of the previously titrated mAbs CD3, CD4, CD8, 6B11, CD56, CD69, CD107 and relevant isotype controls (BD Biosciences, UK) and incubated at 4 °C for 30 min. Cells were washed twice in pbs/pba and resuspended in 1% PFA and acquired after a 10 min incubation at 4 °C. Cells were analyzed using a FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences). Lymphocytes were gated ('lymphogate') by their density and granularity using forward scatter and side scatter parameters, which contained 95% of peripheral lymphocytes. All further analysis was performed on lymphocytes only. Thirty thousand lymphocyte events were acquired in each case. Frequencies were expressed as a percentage of lymphocytes (or iNKT cells where appropriate).

2.5. Cigarette smoke extract

A system for generating cigarette smoke was developed based upon a validated pump system described by Bernard and colleagues [27], with modifications described by Cawood et al. [28]. Smoke from four cigarettes was pumped through a 30 ml RPMI culture medium. Ten puffs were pumped for each cigarette, each puff contributing 35 ml of smoke, every 30 s, resulting in approximately 75% of the cigarette being consumed, and the 350 ml of smoke extract was generated per cigarette. Each milliliter of CSE contains 0.133 (4/30) cigarette's worth of smoke-derived constituents. The resultant CSE was sterilized by filtering through a 0.2- μ m filter, pH adjusted to 7.4, and then stored at -20 °C. The cigarettes used were Marlboro Reds, Class A, rated as tar 10 mg, nicotine 0.8 mg, and carbon monoxide 10 mg. Bernhard's validated volumetric calculations are based on the assumption that a human generates 350 ml smoke with each cigarette and has a blood volume of 6 liters. If that person smokes 20 cigarettes per day, then each 300 ml (6000/20) of blood contains the equivalent of one cigarette. Therefore, each milliliter of blood contains 0.0033 (1/300) cigarette's worth of smoke-derived constituents. By this calculation, CSE can be considered to contain 40 times (0.133/0.0033) the amount of smoke-derived constituents than would be expected in the blood of a smoker who smokes

20 cigarettes per day. A 2.5% (1/40) solution of CSE would equate to 20 cigarettes per day.

2.6. Effect of CSE on iNKT phenotype

iNKT cell lines were cultured with or without CSE, either alone or with CD1d transfected C1R cells at 1:1 ratio in the presence or absence of 100 ng/ml of α GalCer for 24 h in a 96-U bottom well plate at 37 °C. After 24 h cells were harvested and stained with the mAbs, CD3, CD4, CD8, 6B11, CD56, CD69 and CD107 and acquired by flow cytometry using a FACSCalibur and analyzed by Cell Quest

2.7. Cytokine production assay

iNKT cell lines with or without CSE, were cultured alone or co-cultured with CD1d transfected C1R cells at 1:1 ratio in the presence or absence of 100 ng/ml of α GalCer for 72 h in a 96-U bottom well plate at 37 °C. After 24 h 125 μ l of culture supernatant was removed and assessed by ELISA (R&D Systems, UK) and FlowCytomix (Bendermed Systems, UK) for cytokine secretion. At 72 h the remaining supernatant was assessed by ELISA.

2.8. Cytotoxicity assay

CD1d transfected Hela target cells loaded with 100 ng/ml of α GalCer were labeled with carboxyl fluorescein succinimidyl ester (CFSE) and then added to the iNKT cell populations at effector/target ratios 10:1. Cell co-cultures were incubated at 37 °C for 4 h, in the absence or presence of CSE. Killing of CFSE-labeled target cells was measured by staining with 7-aminoactinomycin D (7-AAD) immediately after incubation and by immediate analysis by flow cytometry (as per manufacturer's instructions for the flow cytometry-based Total Cytotoxicity & Apoptosis Detection Kit, Immunochemistry, USA).

3. Results

3.1. Enumeration of iNKT cells in cigarette smoking individuals

The frequency of circulating iNKT cells in cigarette smoking subjects was assessed by flow cytometry using a combination of the mAbs 6B11 PE and CD3 Per-Cp and compared to age matched non-smoking cohort (Fig. 1). The frequency of iNKT cells was significantly lower in the smoking cohort (mean of 0.22%) compared to the non-smoking cohort (0.33%) ($P < 0.05$) the frequency of conventional T cells was unchanged between smokers and non-smokers (data not shown).

3.2. Cell yields and viability

There were no significant differences in cell viability after exposure to cigarette smoke extract as determined by EB/AO staining, in all cases viability exceeded 90% (data not shown).

3.3. Generation of iNKT cell lines

Five iNKT cell lines were generated and were assessed by flow cytometry to determine purity and phenotype of each (data not shown). Each line was >99% positive for 6B11, CD3 illustrating an enrichment >200-fold. The expression of the invariant TCR was also examined using the monoclonal antibody pairing of V α 24 and V β 11, all cells were positive for V α 24 and for V β 11. Of the 5 lines examined each had a mixed expression of CD4, CD8 and CD4-CD8-subsets (data not shown).

3.4. Effect of CSE on iNKT phenotype

Upon iNKT cell activation, the cell up regulates functional surface molecules. We aimed to investigate the effect of CSE

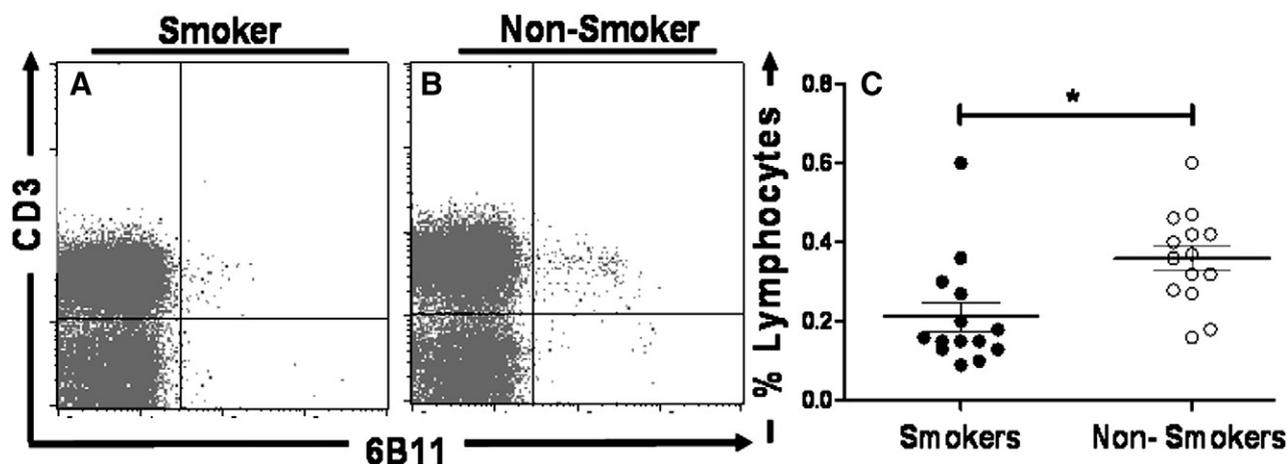


Figure 1 Enumeration of iNKT cells in cigarette smoking individuals. Representative flow cytometry dot plots showing peripheral blood mononuclear cells from (A) a cigarette smoking subject and (B) a non-smoking subject stained with the mAbs 6B11 PE and CD3 PE-Cy5. (C), scatter plot of iNKT frequencies in cigarette smoking cohort (black circles) and non-smoking cohort (unfilled circles), the black line denotes the mean frequency (n=12). * = $p < 0.05$.

on the expression levels of iNKT activation markers (CD56, CD69) and the killing associated molecule CD107. Invariant NKT cells were co-cultured with CD1d-transfected C1R cells loaded or unloaded with 100 ng/ml of α GalCer for 24 h in the presence or absence of CSE. The expression of surface molecules was examined by flow cytometry. The expression of CD56, CD69 and CD107 was increased with the addition of 100 ng/ml of α GalCer. The presence of CSE only significantly inhibited the up regulation of CD107 ($p < 0.05$) (Fig. 2C). No significant difference was observed in the surface expression of CD56 or CD69 on the iNKT cells exposed to CSE and controls (Fig. 2A–B).

3.5. Effect of CSE on iNKT cytokine production

Invariant NKT cells were co-cultured with CD1d-transfected C1R cells loaded or unloaded with 100 ng/ml of α GalCer for 24 and 72 h in the presence or absence of CSE. After 24 h supernatants were assessed by Th1/Th2 flow cytometry assay and ELISA (Fig. 3A and B). There were significant increases in IFN- γ , TNF- α , IL-4 and IL-5 ($p < 0.005$) secretion by iNKT cells in the presence of α GalCer compared to the iNKT and CD1d-transfected C1R cells in the absence of α GalCer (Fig. 3A and B). The addition of CSE resulted in the significant inhibition of IFN- γ secretion as detected by flow cytometry assay ($p < 0.005$) and ELISA ($p < 0.05$) (Fig. 3A and B). CSE also inhibited the secretion of IL-4 by iNKT cells compared to the co-cultures in the presence of α GalCer when assessed by flow cytometry assay ($p < 0.05$) and ELISA ($p < 0.05$) (Fig. 3A and B). TNF- α and IL-5 secretion by iNKT cells was examined by the flow cytometry assay and both were significantly increased in the presence of α GalCer. The addition of CSE resulted in a significant reduction in both TNF- α ($p < 0.005$) and IL-5 ($p < 0.005$) secretion after 24 h (Fig. 3A). After the 72 h culture, supernatants were assessed by ELISA for IFN- γ and IL-4 production. CSE had no effect on IFN- γ levels, with comparable levels detected by ELISA between α GalCer with

and without CSE. Invariant NKT cells produced significantly more IL-4 in the presence of CSE ($p < 0.05$) than those cultured without CSE (Fig. 3B).

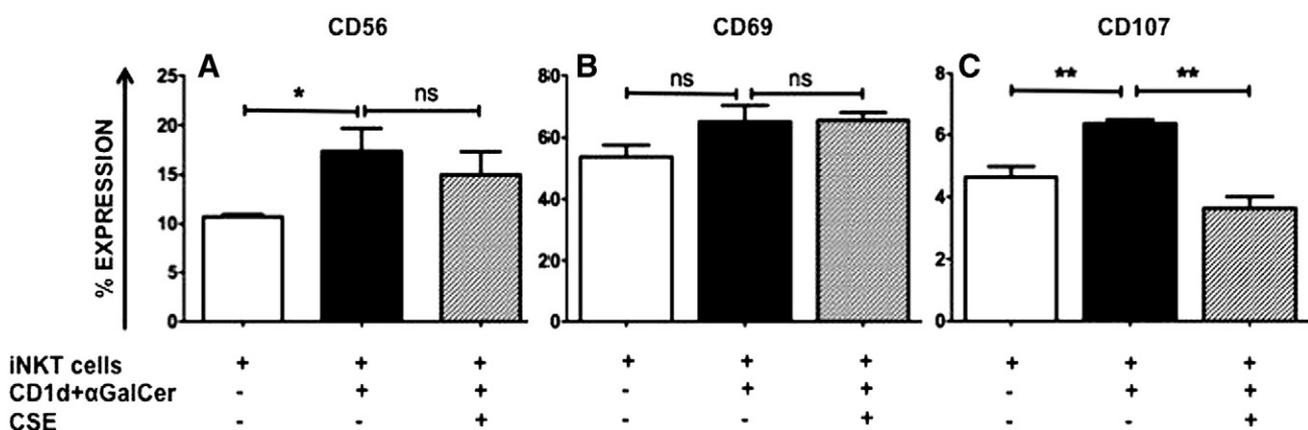
3.6. Effect of CSE on iNKT cytotoxicity

Invariant NKT cells were co-cultured with CFSE-labeled CD1d-transfected HeLa cells unloaded or loaded with 100 ng/ml of α GalCer for 4 h in the presence or absence of CSE. After 4 h cell death was measured. Invariant NKT cells lysed 28% of HeLa target cells. The addition of CSE resulted in a reduction in the lysis to a mean of 17% (Fig. 4A). In the presence of 100 ng/ml α GalCer, iNKT cells lysed 31% of HeLa target cells. The addition of CSE resulted in a reduction in the lysis to a mean of 21% (Fig. 4B), illustrating that CSE reduces iNKT cell cytotoxicity.

4. Discussion

Cigarette smoke has strong associations with several cancers and has previously been shown to inhibit important anti-tumor innate immune responses by NK cells [3,9]. Invariant NKT cells are important regulatory innate effector cells, with the potential to elicit specific immune responses through cytokine production [15,29]. This ability has highlighted iNKT cells as an attractive target for cancer immunotherapy [31]. The effect of tumor environment on iNKT cell frequency has been previously reported [18,19], however the effect of other environmental factors such as CSE has not been investigated. We aimed to determine if CSE altered the iNKT cell anti-tumor activity. Our study suggests the effect of CSE on iNKT cells is specific and reduces the population number and function.

We investigated the frequency of circulating iNKT cells in the peripheral blood of healthy cigarette-smoking subjects and found a significant reduction in the iNKT cell number compared to a non-smoking age-matched cohort (Fig. 1).



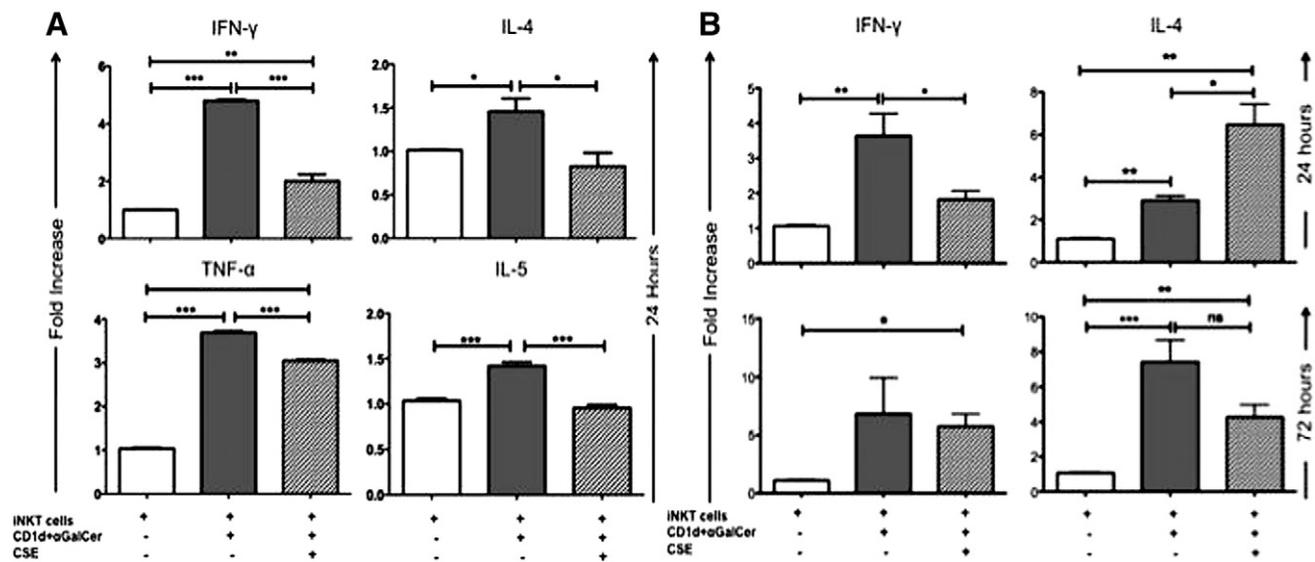


Figure 3 Investigation of CSE inhibition of iNKT cell derived cytokine secretion. Bar graphs showing IFN- γ , TNF- α , IL-5 and IL-4 secretion by iNKT cell lines after 24 h measured by Flow cytometry multiplex assay (A). Bar graphs showing IFN- γ , and IL-4 secretion by iNKT cell lines after 24 and 72 h measured by ELISA (B). The iNKT cells were cultured with either CD1d transfected C1R cell in the absence or presence of 100 ng/ml α GalCer, with or without CSE for 24 or 72 h. The values are expressed as fold increases in cytokine production over the iNKT cell cultured with CD1d transfected C1R cells. Graphs are representative of 5 individual experiments. * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 0.001$.

Tahir et al. previously showed that tumor environment depletes iNKT cell number [18]. Our findings add cigarette smoke as another environmental factor that leads to depletion of iNKT cells. The finding that iNKT cell numbers are reduced in cigarette-smoking subjects is novel. Reduced iNKT cell number has also been reported in conditions affected by smoking such as diabetes and head and neck squamous cell carcinoma (HNSCC). In HNSCC low levels of circulating iNKT cells predicted poor clinical outcomes in a group of 47 patients compared to individuals with intermediate or high levels of circulating iNKT cells [30].

In addition to a reduction in iNKT cell number our study also shows significant alteration in iNKT cell function. We examined iNKT cell cytokine secretion in response to α GalCer stimulation after 24 h in the absence or presence of CSE. As previously described, iNKT cells secrete significant amounts of IFN- γ and IL-4 simultaneously when stimulated [13,14]. The addition of CSE resulted in a significant reduction in the production of both cytokines after 24 h (Fig. 3). We have previously reported that CSE inhibited intracellular IFN- γ and increased IL-10 production by NK cells [9]. This would bias the immune system towards a T helper type 2 (Th2) response that does not have a significant anti-tumor activity. Cigarette smoke therefore appears to have multiple negative effects on the innate immune cells that are important in tumor surveillance and protection against infections.

CSE was also found to significantly inhibit the production of TNF- α and IL-5 by iNKT cells (Fig. 3). Cytokine production was examined after 72 h to determine if the inhibitory effect observed persisted without the further addition of CSE. Invariant NKT cells stimulated with α GalCer produced

comparable levels of IFN- γ when co-cultured for 72 h in the absence or presence of CSE. The addition of CSE resulted in significantly increased levels of IL-4 after 72 h compared to iNKT cells incubated with CD1d-transfected C1R cells loaded with α GalCer (Fig. 3B). This finding demonstrates that iNKT cell cytokine production is inhibited in the presence of CSE and subsequently altered in a Th2 manner after 72 h. The production of a Th2-biased cytokine milieu by iNKT cells has previously been reported in subjects with cancer [18].

The iNKT cell lines expressed the activation-associated surface molecules CD56 and CD69. These molecules were increased following stimulation with α GalCer. The addition of CSE to the cultures had no significant effect on the expression of either activation markers suggesting the iNKT cells were still activated by α GalCer (Fig. 2). Invariant NKT cells can lyse target cells by perforin CD107 [32,33]. We examined the expression of CD107 and observed that it was upregulated in the presence of α GalCer-loaded CD1d cells. This may be due to the specific lysing of CD1d-positive cells by iNKT cells as previously described [32,33]. With the addition of CSE, the upregulation of CD107 was inhibited (Fig. 2). We have shown that CSE also inhibited CD107 expression in NK cells without affecting other NK cell markers [9]. As a result of the inhibition of CD107-upregulation we examined the effect of CSE on the iNKT cell cytotoxic ability. We demonstrated the significant inhibition of iNKT cell lysis of HeLa target cells both in the absence (Fig. 4C) and presence (Fig. 4D) of α GalCer (Fig. 4). This suggests further impairment of iNKT cell anti-tumor activity due to exposure to CSE (IFN- γ production and tumor lysis).

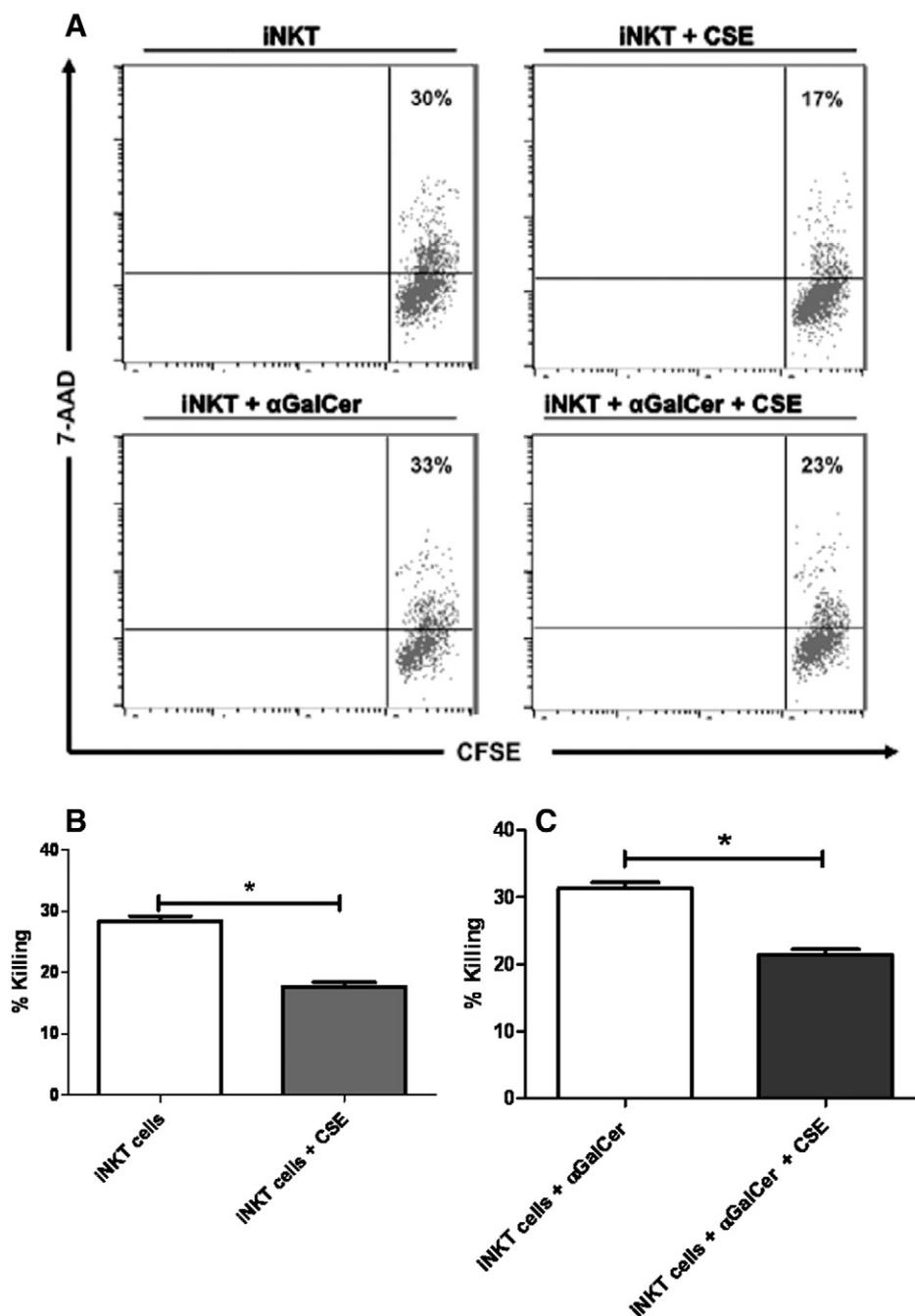


Figure 4 Investigation of CSE inhibition of iNKT cell cytotoxicity. Representative dot plots (A) showing the lysis of target cells by iNKT cells and (B and C) bar graphs displaying the mean percentage lysis. The iNKT cells were cultured with CFSE labeled CD1d transfected C1R cells in the absence or presence of 100 ng/ml α GalCer, with or without CSE for 4 h. Graphs are representative of 3 individual experiments. * = $p < 0.05$.

These findings demonstrate that cigarette smokers have a reduction in the number of circulating iNKT cells and that CSE directly alters iNKT cells anti-tumor activity.

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