Journal of
Clinical & Cellular Immunology

Liu and Issazadeh-Navikas, J Clin Cell Immunol 2012, 3:1
DOI: 10.4172/2155-9899.1000117

Research Article Open Access

NKT Cell Subsets and Proinflammatory Cytokine Changes in Mice as a Result of a Natural *Pasteurella pneumotropica* Infection

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Abstract

CD1d-dependent natural killer T (NKT) cells play an important protective role in a variety of autoimmune, allergic, tumor, and infectious diseases, but there are several controversial reports. The balance in subsets of NKT cells and their activation status prior to induction of such diseases could play a critical role in the clinical outcome. *Pasteurella pneumotropica* is a commonly found bacterium in laboratory rodents. Because this infection is often asymptomatic and hence can go undetected, and given different levels of acceptable health status in different animal facilities, this infection could impact the outcome of reported scientific studies. We report that subclinical natural infection with *P. pneumotropica* significantly affects the balance among different subsets of NKT cells. While the major CD1d-dependent population, i.e., $CD4^+$ NKT cells, was not significantly affected by infection in wild-type (WT) or *CD1d*$^{-/-}$ mice, $CD8^+$ NKT cells significantly increased in infected WT mice. Interestingly, double negative NKT cells, while significantly suppressed in WT mice due to infection, were unaffected in *CD1d*$^{-/-}$ mice. The pattern of proinflammatory cytokines IFN-γ and IL-17A considerably changed as a result of infection. The differential effects were even more pronounced when investigating different T-cell subsets and their cytokine profiles.

While the effect of infection on shaping immune cell balance could be expected, mice lacking CD1d-dependent NKT cells differed entirely in their response to a subclinical infection. This subsequently indicated influences of NKT cells on other T-cell subsets and their cytokine milieu. Collectively, these data confirm the importance of controlling environmental factors which could have strong impacts on immune balance, and consequently dictate the outcome of immune-mediated diseases.

Keywords: CD1 knockout mice; IFN-γ; IL-17; Autoimmunity

Introduction

Natural killer T (NKT) cells, characterized by expression of both NK1.1 and a T-cell receptor, play an important role in the response to a variety of autoimmune [1], allergic [2], viral and bacterial insults [3]. Their effects are in part mediated by the rapid release of large amounts of cytokines, including interferon (IFN)-γ [2] and IL-17, a proinflammatory cytokine [4], and action on a variety of cell types involved in the immune response [5]. Notably, they have been shown to have a suppressive function, such as on cytotoxic T lymphocytes [6]. Through cell-cell contact, they can impair differentiation of T cells into Th1 effector cells [7] and kill T cells through a Fas-FasL–mediated mechanism [8,9]. NKT cells' effects on bacteria are mediated by TCR recognition of bacterial antigens or self antigens, or an antigen-independent pathway involving cytokines [10]. The majority of NKT cells are CD1d-restricted, though some populations of $NK1.1^+TCR^+$ (NKT) cells remain in *CD1d*$^{-/-}$ mice [11], the functionality of which is not well characterized. In general *CD1d*$^{-/-}$ mice or other mice deficient in CD1d-restricted subsets of NKT cells have been shown to have reduced survival and immune responses due to infection with different species of bacteria [3,12-14]. However, controversy exists regarding the protective role of NKT cells in bacterial infection in mice [15-17]. For some bacterial infections, such as with *Chlamydia trachomatis*, NKT cells have even been reported to exert a detrimental effect involving promotion of a Th2-type response; *CD1d*$^{-/-}$ mice showed reduced morbidity, lower growth of *Chlamydia* in vivo, and less pathological changes in the lung [18].

Controversy over the role of NKT cells is not limited to studies on bacteria, but extends to the realm of autoimmunity, with different accounts offered for their role in such diseases as experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA), two disease models known to be influenced by IFN-γ, IL-17 and NKT cells [8,11,19-22]. Controversial results have been also reported in allergic asthma conditions [23]. These discrepant results may point to different modes of action of NKT cells depending upon the bacterial species, disease-induction protocols, and host factors such as genetic background. In addition, the varying and sometimes opposing accounts of the effects of NKT cells in disease may also reflect an underlying, asymptomatic infection with a benign bacterial species that could nonetheless affect NKT cells and other immune parameters. If this is the case then immunological studies on mice harboring this bacterium could inadvertently be introducing bias into the results. We thus sought to address this question by looking at the effects of *Pasteurella pneumotropica*, a non-pathogenic but frequently occurring subclinical bacterial infection in animal facilities world-wide, on NKT cells and other immune responses in healthy mice.

P. pneumotropica is a Gram-negative bacterium commonly found in laboratory rodents, including mice. *P. pneumotropica*, although included in the Federation of European Laboratory Animal Science

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Received December 15, 2011; **Accepted** January 24, 2012; **Published** January 28, 2012

Citation: Liu Y, Issazadeh-Navikas S (2012) NKT Cell Subsets and Proinflammatory Cytokine Changes in Mice as a Result of a Natural Pasteurella pneumotropica Infection. J Clin Cell Immunol 3:117. doi:10.4172/2155-9899.1000117

Associations (FELASA)–issued set of guidelines for health monitoring in breeding colonies of rodents [24], is among frequently reoccurring asymptomatic infections. The prevalence in mice in a large commercial rodent diagnostic laboratory was shown to be close to 13% [25], though as high as 46% in another study [26]. Although mice shipped from commercial laboratories are claimed to be free of *P. pneumotropica*, it is possible for mice to acquire the disease once in an infected research facility. Furthermore, it is possible that many research animal facilities do not screen for this organism. Given the absence of clinical signs of the infection in immunocompetent hosts, infection with *P. pneumotropica* is considered opportunistic [27,28]. Consequently, little attention may be given to excluding this organism from populations used in biological research. This is especially important given that the influence of this infection on immunological parameters in immunocompetent mice is largely unknown. However, as shown by Patten et al. [28] who examined changes in local and regional cytokine gene expression in C57BL/6 mice infected with high or low doses of *P. pneumotropica*, a slight, transient perturbation in the transcription of inflammatory cytokines, including IL-1β and TNF-α, was found to exist. Similarly, Kawai et al. [29] found that induction of an adaptive immune response to orally colonizing nonpathogenic *P. pneumotropica* resulted in RANKL-dependent periodontal bone loss in mice. A study by Chapes et al. tested the susceptibility of various gene-deleted mice (MHCII, *Tlr4*, *NrampI*) to *P. pneumotropica* infection and found a hierarchy of importance for these genes in staving off lung infection, with deletion of all three genes leading to a rapidly progressing pneumonia [30]. Another important study found that reconstitution of $TLR4^{Lps\text{-}del}$ mice with TLR4-positive macrophages provided protection against *P. pneumotropica* infection [31]. Nonetheless, data on the possible differential influence of *P. pneumotropica* infection on immunocompromised mice, especially mice lacking CD1d-dependent NKT cells important for regulation of immunity in infectious, allergic and autoimmune diseases, are missing. This prompted us to study how a discovered subclinical, natural *P. pneumotropica* infection affected CD1d-dependent NKT cells, their proinflammatory cytokine response and consequently other immune cells. For this purpose mice genetically lacking CD1d-dependent NKT cells ($CD1d^{-/-}$ mice) or depleted of $NK1.1^{+}$ cells in adult wild-type (WT) during this natural infection were compared with non-infected mice. The previous controversial studies on the importance of CD1d-dependent NKT cells for controlling inflammatory and autoimmune diseases raise concerns about use of mice infected with this bacterium for immunological studies, which the results of our current study strongly support given the tremendous effect such an infection could have on outcomes of immune responses. This is particularly true for studies comparing immunocompetent mice with mice lacking CD1d-dependent NKT cells. This also points towards the importance of the awareness of such environmental factors and the necessity for active exclusion of this infection.

Materials and Methods

Animals

Mice were bred and maintained with SPF standards at an animal facility at the University of Copenhagen, Denmark. After weaning mice were routinely transferred to another SPF standard experimental room. Each group of mice was housed in individualized ventilated cages during the entire period until mice were sacrificed. By a routine FELASA screen some groups of mice were determined to have subclinical *P. pneumotropica* infection. This finding prompted the study design, which by its nature is retrospective as mice were not intentionally infected with the bacteria.

All animal experiments were done in accordance with the ethical committees in Denmark, approval number 2007/561-1364.

Mice were screened and found to be negative for the following organisms: mouse hepatitis virus, mouse rotavirus (EDIM), parvoviruses, minute virus of mice, mouse parvovirus, pneumonia virus of mice, Sendai virus, Theiler's murine encephalomyelitis virus, ectromelia virus (yearly), lymphocytic choriomeningitis virus (yearly), mouse adenovirus type 1 (FL) (yearly), mouse adenovirus type 2 (K87) (yearly), mouse cytomegalovirus (yearly), reovirus type 3 (yearly), murine norovirus, *Citrobacter rodentium, Clostridium piliforme* (Tyzzer's disease), *Corynebacterium kutscheri, Mycoplasma* spp., *Salmonella* spp., Streptococci β-hemolytic (not group D), *Streptococcus pneumoniae, Helicobacter bilis* (yearly), *Helicobacter hepaticus* (yearly), other *Helicobacter* spp. (yearly), *Streptobacillus moniliformis* (yearly), ecto- and endo-parasites.

$CD1d^{-/-}$ mice are homozygous for the $Cd1^{tm1Gru}$ targeted mutation and are deficient in both the *Cd1d1* and *Cd1d2* genes (CD1.1, CD1.2) [54]. Mice on C57BL/6J (B6) background (10-generation backcross) were obtained and further backcrossed to B6 in our animal facility for an additional 10 generations. $CD1d^{-/-}$ and WT (homozygote) littermates were sex and age matched (10–14 weeks) in all experiments.

For identification of *P. pneumotropica*, a swab was taken from the pharynx. Bacteria were plated directly onto 5% horse blood agar plates and incubated aerobically at 37°C for 48 hr. After examining for colony morphology up to 10 colonies were selected and further cultured on plates containing agar and MacConkey agar. Isolates on blood agar which did not grow on MacConkey agar were tested for cytochromoxidase. Oxidase-positive colonies were propagated in brain heart infusion broth (Difco Laboratories) supplemented with 5% horse serum for a motility test and an indole test. Non-motile indole-positive colonies were inoculated in Hugh & Leifson medium. Further identification of *P. pneumotropica* was performed by 16s RNA gene sequencing for selected fermentative isolates.

In vivo NK1.1 cell depletion

After discovery of the *P. pneumotropica* infection, to deplete $NK1.1^{+}$ cells in vivo, mice were injected i.p. with 500 μg anti-NK1.1 (PK136, eBioscience) or isotype-matched control Ab (L243, BD Pharmingen) in 500 μl PBS. Mice were then sacrificed after 10 days.

Single-cell preparation from splenocytes and FACS staining

Spleens were collected for FACS analysis. Single-cell suspensions were made, erythrocytes were lysed with RBC-lysis buffer (#00-4333-57, eBioscience), washed in PBS and re-suspended in FACS buffer (2% BSA, 0.01% sodium azide in PBS).

Single cells prepared from spleens were incubated with anti-Fc receptor Ab (2.4G2) for 20 min on ice. Thereafter, splenocytes were incubated with biotinylated, fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled Abs. The following antibodies were used for FACS staining: PE-Cy5-anti-CD4 (GK1.5), FITC-anti-CD3 (145-2C11), APC-anti-CD8a (53-6.7), PE-anti-IFN-γ (XMG1.1, eBioscience), PE-anti-IL-17a (TC11-18H10), PE-anti-CD49b (DX5), FITC and PE-anti-NK1.1 (PK136).

For intracellular cytokine staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences). All Abs were purchased from BD Pharmingen and were used at 1–5 μg/ml unless stated otherwise. Antibodies were allowed to bind for 20 min on ice. Dead cells were discriminated in all stainings using the LIVE/DEAD® Fixable Dead Cell Stain Kit for 405 nm excitation (L34955, Invitrogen). Cells were acquired with a FACSAria (BD) using the FACSDiVa software for acquisition after exclusion of duplets. FlowJo 8.8.6 was used for further analysis. The experiment was repeated at least twice with similar results and hence the shown data are the summary of these experiments.

Statistical analysis

For multiple-group analyses of FACS data, ANOVA with Newman-Keuls multiple comparison test was utilized. Student's *t*-test was used for comparisons between two groups. Values of $p < 0.05$ were considered significant.

Results

P. pneumotropica infection induces significant differential effects on NKT cell subsets in CD1d$^{-/-}$ mice compared with WT mice

FACS analysis of gated NKT cells were based on exclusion of duplets and then gated on CD3$^+$ T cells. Next they were gated on NK1.1$^+$ and other markers for triple positive cells; hence the percentages discussed were calculated in the gated T cells and not total splenocytes. This expectedly revealed significantly lower percentages of all subsets of NK1.1$^+$ T (NKT) cells, including CD4$^+$CD3$^+$NK1.1$^+$ (hereby referred to as CD4$^+$ NKT cells), CD8$^+$CD3$^+$ NKT (CD8$^+$ NKT) cells, and double negative CD3$^+$ NKT (DN NKT) cells in *CD1d*$^{-/-}$ mice. Surprisingly, *P. pneumotropica* infection led to no major changes in the percentage of CD4$^+$ NKT cells (Figure 1A,1B), while the percentage of CD8$^+$ NKT cells was significantly increased (Figure 1C) with those of DN NKT cells significantly reduced in the WT mice upon infection (Figure 1D). These results reveal that *P. pneumotropica* infection not only induces greater differences in CD8$^+$ NKT cells but also changes the balance of DN NKT cells (i.e., to a much more elevated percentage) in *CD1d*$^{-/-}$ mice compared with WT mice, after infection.

P. pneumotropica infection increases CD4$^+$ T cells and DN T cells, but reduces NK cells in WT mice

Although genetic deletion of CD1d-dependent NKT cells did not result in major changes in the percentage of classical T-cell subsets in total splenocytes (gated only on CD3$^+$ T cells), such as CD4, CD8 T cells, their ratio, or DN T cells and/or NK cells as earlier published [11], *P. pneumotropica* infection resulted in reduced CD4$^+$ T, CD4/CD8 T cell ratio, and NK cells (gated on CD4$^-$CD8$^-$NK1.1$^+$) in both groups. It caused significant increases in DN T cells in WT and *CD1d*$^{-/-}$ mice, but with such a large increase in the WT group as to result in the major differences among the groups after infection (Figures 2A–2E).

While *P. pneumotropica* infection significantly increases DN T cells in WT mice, it differentially affects elevation of CD8$^+$ T cells in CD1d$^{-/-}$ mice

Notably, FACS analysis of splenocytes revealed that although the percentage of CD8$^+$ T cells (gated again for double positivity, i.e.,

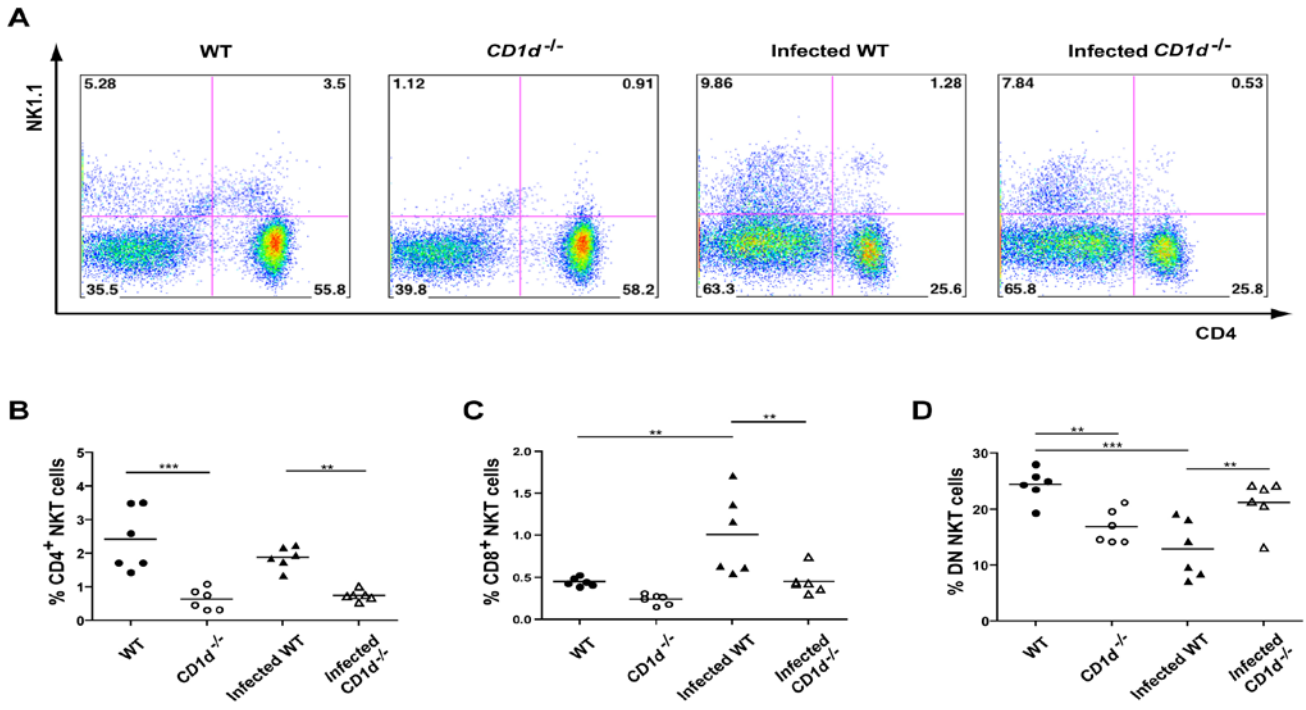

Figure 1: FACS analysis of NKT cell populations with or without *P. pneumotropica* infection. (A) Representative FACS plot showing the percentage of NK1.1$^+$ and CD4$^+$ NKT cells in the different groups as shown in (B). Percentage of (B) CD4$^+$ NKT cells and (C) CD8$^+$ NKT cells in gated CD3$^+$ T cells in analyzed splenocytes and (D) DN NKT (CD3$^+$CD4$^-$CD8$^-$NK1.1$^+$) cells for all groups of mice (non-infected WT and CD1d$^{-/-}$ mice; and infected WT and CD1d$^{-/-}$). Dot plots (B–D) show individual values and the line represents the mean. One-way ANOVA test with Newman-Keuls multiple comparisons correction was used to evaluate significant differences among the groups; n = 6 mice/group, **P ≤0.01, ***P ≤0.001.

$CD8^+CD3^+$) was stable among the different groups and remained stable after infection in the WT group, $CD1d^{-/-}$ mice showed significantly elevated percentages of cytotoxic $CD8^+$ T cells upon P. pneumotropica infection (Figure 2B) indicating that CD1d-dependent NKT cells are important for prevention of cytotoxic $CD8^+$ T-cell proliferation.

In summary, these results indicate that *P. pneumotropica* infection leads to significant differential effects in shaping immune cell balance in WT mice compared to mice lacking genetically CD1d-dependent NKT cells.

Presence of CD1d-dependent NKT cells is critical to suppress IFN-γ and IL-17A production upon *P. pneumotropica* infection

Proinflammatory cytokines IFN-γ and IL-17 play crucial roles in promoting many autoimmune diseases and are important in infectious diseases as well. NKT cells can produce these cytokines promptly upon activation [2]. However, other immune cells can also produce these cytokines and usually cytokine production by effector T cells, such as Th1 and Th17 cells, plays a critical role in shaping autoimmune and anti-inflammatory responses [32]. Interestingly, while *P. pneumotropica* infection significantly induced increases in the total percentage of cells producing both IFN-γ and IL-17A in WT and $CD1d^{-/-}$ mice, the elevation was significantly higher in the infected $CD1d^{-/-}$ mice (Figure 3B,4B). These data indicate that CD1d-dependent NKT cells are important in the regulation of other immune cells that produce proinflammatory cytokines.

P. pneumotropica infection induces a general elevation of IFN-γ and IL-17A production but this effect is entirely different with regard to NKT cells

In accordance with increased levels of total IFN-γ-producing cells (Figure 3A,3B), $CD4^+$ and $CD8^+$ T cells also upregulated IFN-γ production upon infection (Figure 3C and 3D, respectively). However, no major differences were observed in IFN-γ production by NK cells (gated on $CD4^-CD8^-NK1.1^+$) (Figure 3E). While no significant changes were observed in regard to IFN-γ positivity in the gated $CD4^+$ NKT cells (Figure 3F), $CD8^+$ NKT cells' (mostly CD1d-independent NKT cell populations in mice) [11] production of IFN-γ was significantly increased in both WT and $CD1d^{-/-}$ mice when infected (Figure 3G).

As depicted in Figure 4A–B, the total percentage of cells producing IL-17A was significantly increased in both infected groups. More interestingly, $CD1d^{-/-}$ mice mounted a significantly higher IL-17A response, indicating a suppressive effect of CD1d-dependent NKT cells in the modulation of IL-17A responses. Moreover, the increased IL-17A response was not correlated with Th17 cells as the percentage of $IL\text{-}17A^+CD4^+$ T cells, although not significantly changed, was slightly reduced in both infected groups (Figure 4C). In contrast, the main source of IL-17A positivity was in the $CD8^+$ T-cell population since $IL\text{-}17A^+CD8^+$ T cells were significantly increased in both groups as a consequence of infection (Figure 4D). No differences in the $IL\text{-}17A^+$ NK cells (gated on $CD4^-CD8^-NK1.1^+$) were detected (Figure 4E).

Additional differential effects of *P. pneumotropica* infection were

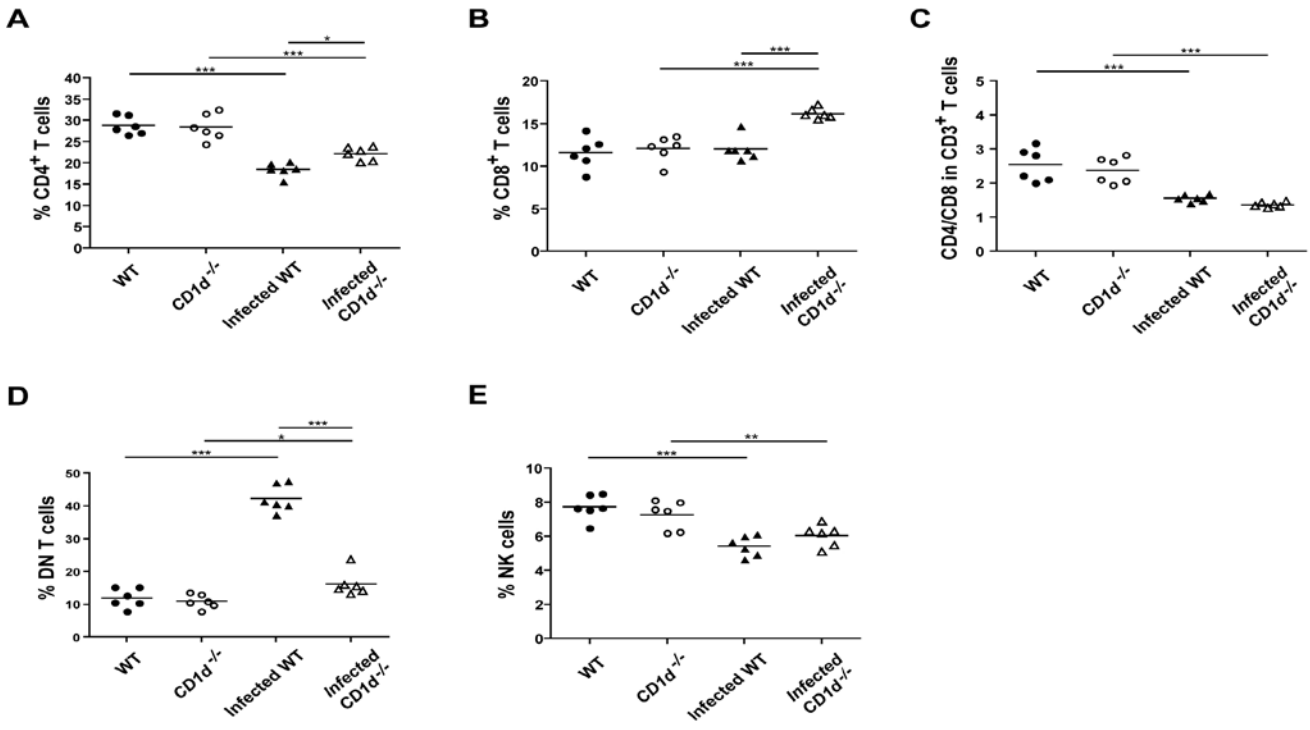

Figure 2: FACS analysis of T cell and NK cell populations with and without *P. pneumotropica* infection. Percentage of (**A**) $CD4^+$ T cells, (**B**) $CD8^+$ T cells, (**C**) the CD4/CD8 ratio in $CD3^+$ T cells, (**D**) double negative (DN) in $CD3^+$ T cells, and (**E**) NK cells (gated on $CD3^-NK1.1^+DX5^+$ cells), for all four groups of mice (non-infected WT and $CD1d^{-/-}$ mice; and infected WT and $CD1d^{-/-}$). Dot plots show individual values and the mean. One-way ANOVA test with Newman-Keuls multiple comparisons correction was used to evaluate significant differences among the groups, n = 6 mice/group, *P ≤0.05, **P ≤0.01, ***P ≤0.001.

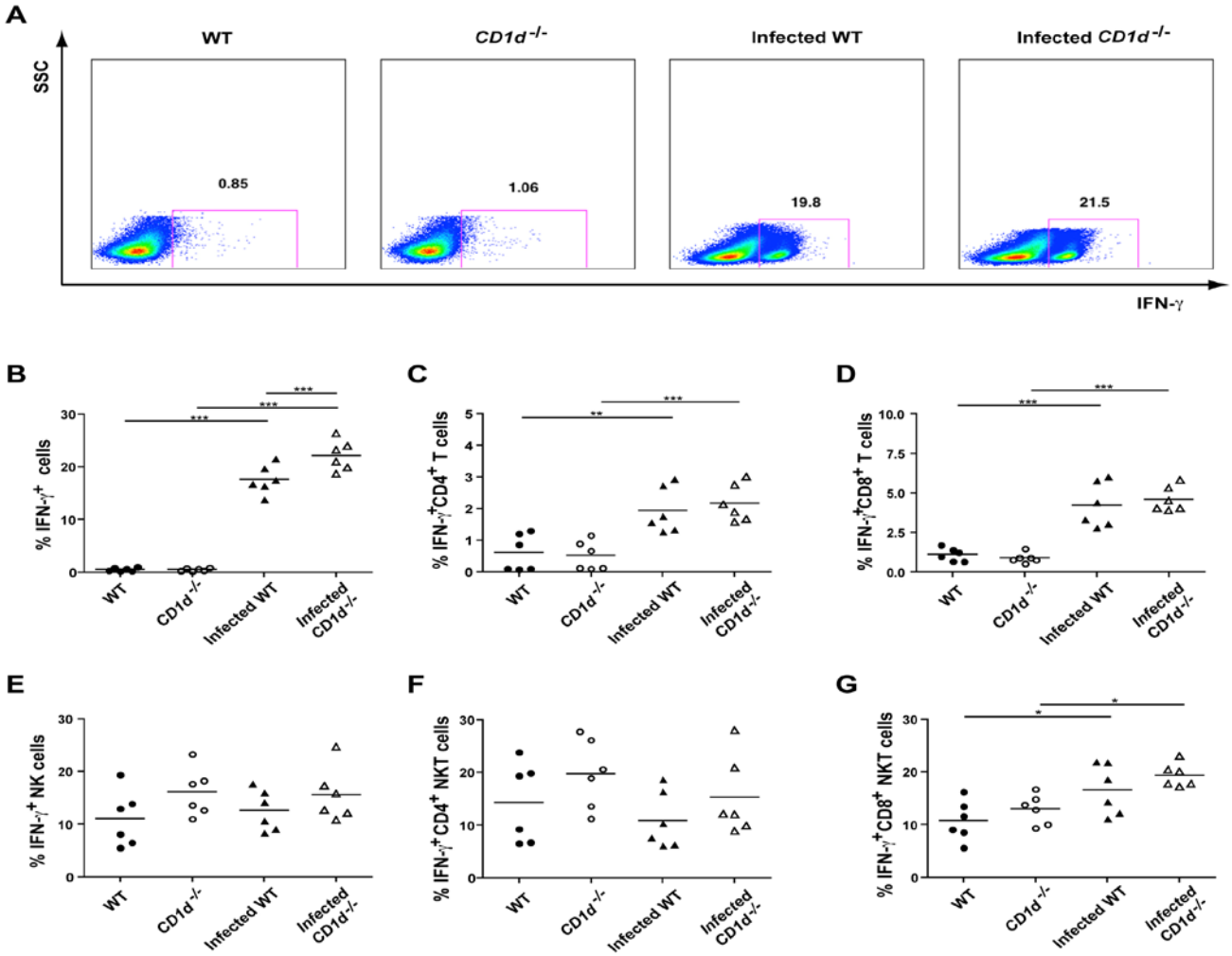

Figure 3: IFN-γ⁺ splenocytes elevated significantly after *P. pneumotropica* infection. (**A**) Representative FACS plot showing the percentage of IFN-γ⁺ cells in the different groups as shown in (B). **(B–G)** Total percentage of IFN-γ⁺ cells or in different subsets of T cells and NK cells analyzed by FACS. (**B**) Total percentage of IFN-γ⁺ cells (**C**) IFN-γ⁺CD4⁺ T cells, (**D**) IFN-γ⁺CD8⁺ T cells, (**E**) IFN-γ⁺ NK cells (CD3⁻NK1.1⁺), (**F**) IFN-γ⁺CD4⁺ NKT cells (CD3⁺CD4⁺NK1.1⁺ cells), and (**G**) IFN-γ⁺CD8⁺ NKT cells (CD3⁺CD8⁺NK1.1⁺ cells) – for all four groups of mice (non-infected WT and *CD1d*$^{-/-}$ mice; and infected WT and *CD1d*$^{-/-}$). Dot plots show individual values and the mean. One-way ANOVA test with Newman-Keuls multiple comparisons correction was used to evaluate significant differences among the groups; n = 6 mice/group, *P ≤0.05, **P ≤0.01, ***P ≤0.001.

detected in the regulation of IL-17A⁺ NKT cells in contrast to IFN-γ⁺ NKT cells. Although IL-17A⁺ cells were significantly increased in the infected groups, IL-17A⁺CD4⁺ NKT and IL-17A⁺CD8⁺ NKT cells were both reduced upon infection in the WT and *CD1d*$^{-/-}$ groups (Figure 4F and 4G, respectively).

In summary, results indicate that the main source of IL-17A-producing cells is non–T cells even if a small percentage of CD8⁺ T cells contribute to IL-17A production upon *P. pneumotropica* infection. Moreover, the presence of CD1d-dependent NKT cells in the WT group contributes to a significant reduction of total IL-17A⁺ cells.

NK1.1⁺-depleted infected WT mice have significantly reduced CD4⁺ T cells, increased DN T cells, CD4⁺ NKT and NK cells compared to non-infected WT mice

The role of NK1.1⁺ cells at an adult age during *P. pneumotropica* infection was investigated using the PK136 antibody which results in depletion of NK1.1⁺ cells—NK cells along with NKT cells. Although this treatment has been used mainly to address the role of NKT cells at an adult age compared to genetically depleting them, the treatment leads to depletion of all NK1.1⁺ cells and hence could affect the balance of cell subsets differently under pathogen-free conditions versus during infection. Therefore, infected WT and non-infected WT mice were treated with a single dose of PK136 and their T, NK and NKT cell changes were studied. The in vivo antibody treatment led to sufficient depletion of all NK1.1⁺ cells in the investigated groups, as reported earlier by us [11].

Depletion of NK1.1⁺ cells in vivo is known to not influence conventional T-cell subsets [33,34], although some similar results were achieved in comparison to *CD1d*$^{-/-}$ conditions (Figures 1–2) where as a result of infection some major differences were also observed. A significant reduction of the CD4⁺ T-cell populations were detected as a result of infection (Figure 5A), but no changes in CD8⁺ T cells were

detected (Figure 5B), while the CD4/CD8 T cell ratio was similarly reduced (Figure 5C). DN T cells were tremendously expanded in the infected WT group depleted of NK1.1$^+$ cells (Figure 5D). Such significant changes were not detected among non-infected *CD1d*$^{-/-}$ mice compared with infected *CD1d*$^{-/-}$ mice (Figure 2D). Moreover, the percentage of the remaining NK cells after in vivo treatment with PK136 was significantly higher in the infected versus non-infected group (Figure 5E). This is also in contrast to the NK-cell changes shown in Figure 2E.

The in vivo depletion of NK1.1$^+$ cells in adulthood before and after *P. pneumotropica* infection resulted in very different outcomes for NKT cell subsets compared to genetic depletion of CD1d-dependent NKT cells in *CD1d*$^{-/-}$ mice. While CD4$^+$ NKT cells were significantly elevated during infection (Figure 5F), no differences in the CD8$^+$ NKT or DN NKT cells were detected (Figure 5G and 5H, respectively).

Depletion of NK1.1$^+$ cells differentially affects cytokine balance and NKT cell cytokine production before and after *P. pneumotropica* infection

The total percentage of IFN-γ-producing cells was again increased in the infected group (Figure 6A) as was the case in genetically depleted CD1d-dependent NKT cells (Figure 3B). However, the changes in the relative percentages of IFN-γ-producing T and NKT cells were different from *CD1d*$^{-/-}$ mice (Figure 3). No differences in IFN-γ$^+$CD4$^+$ T cells could be detected in the PK136-treated WT groups before and after infection (Figure 6B). IFN-γ$^+$CD8$^+$ T cells were, however, significantly increased in the infected group (Figure 6C). No differences were observed in IFN-γ$^+$ NK cells (Figure 6D). Furthermore, while the percentage of IFN-γ$^+$CD4$^+$ NKT cells were significantly decreased in

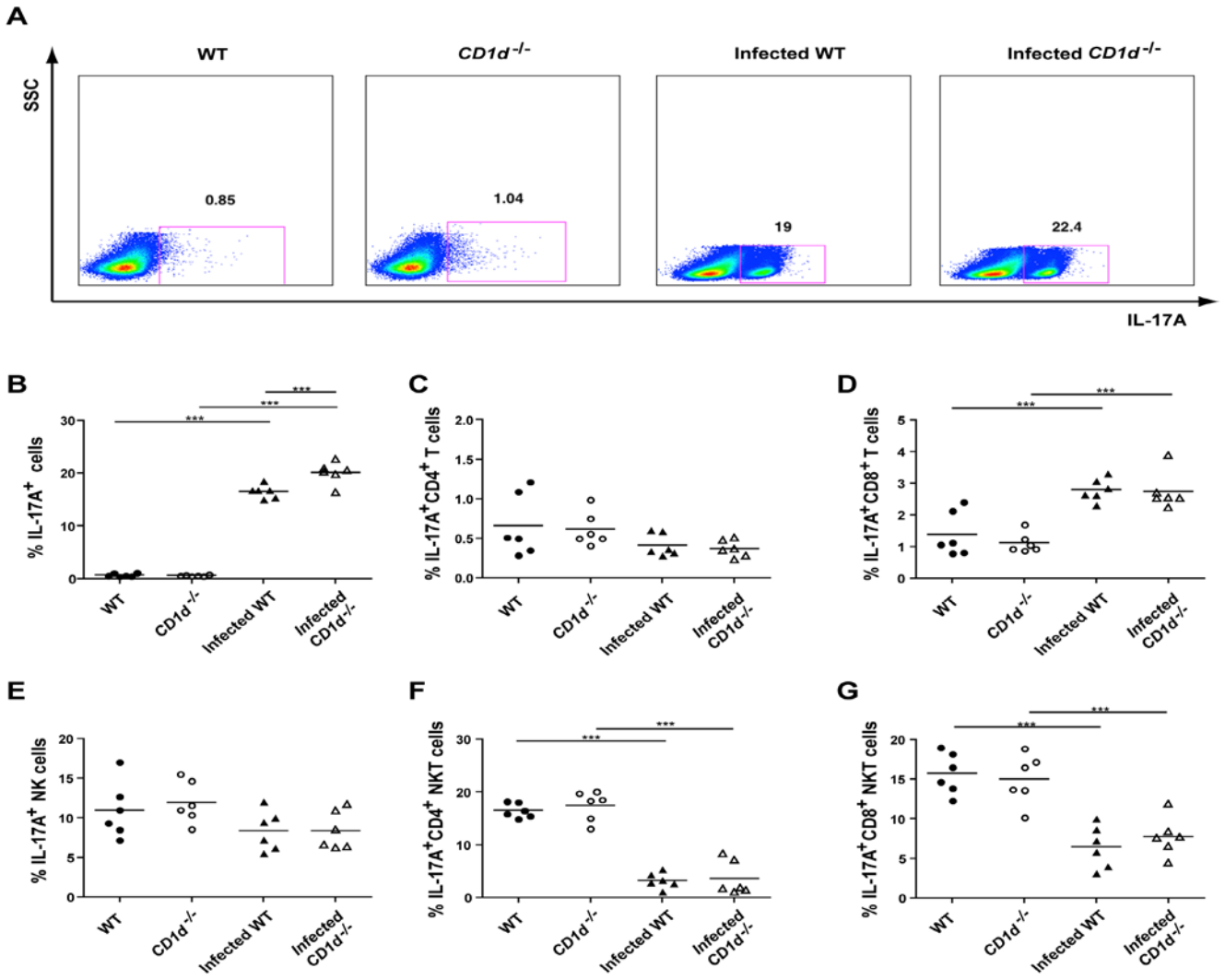

Figure 4: Elevated IL-17A$^+$ splenocytes particularly in CD8$^+$ T cells while IL-17A$^+$ NKT cells decreased in *P. pneumotropica*–infected groups. (**A**) Representative FACS plot showing the percentage of IL-17A$^+$ cells in the different groups as shown in (B). **(B–G)** Percentage of total IL-17A$^+$ cells and in different subsets of T and NK cells analyzed using FACS. (**B**) Total percentage of IL-17A$^+$ cells, (**C**) IL-17A$^+$CD4$^+$ T cells, (**D**) IL-17A$^+$CD8$^+$ T cells, (**E**) IL-17A$^+$NK cells (CD3$^-$ NK1.1$^+$), (**F**) IL-17A$^+$CD4$^+$NKT cells, and (**G**) IL-17A$^+$CD8$^+$ NKT cells – for all four groups of mice (non-infected WT and *CD1d*$^{-/-}$ mice; and infected WT and *CD1d*$^{-/-}$). Dot plots individual values and the mean. One-way ANOVA test with Newman-Keuls multiple comparisons correction was used to evaluate significant differences among the groups; n = 6 mice/group, *P ≤0.05, **P ≤0.01, ***P ≤0.001.

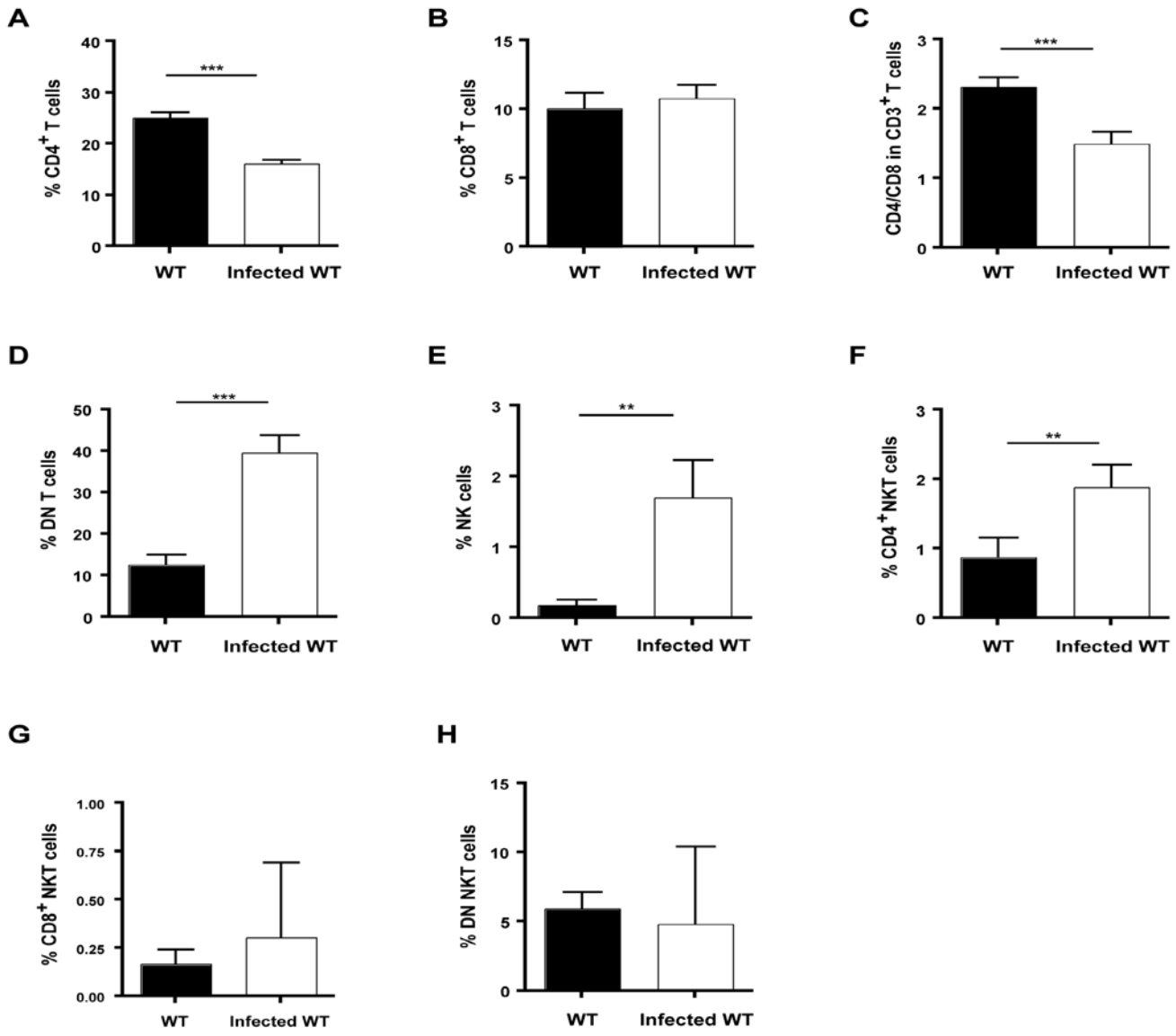

Figure 5: Depletion of NK1.1⁺ cells using PK136 at an adult age has differential effects on splenocyte populations in infected WT mice. FACS analysis of percentage of (**A**) CD4⁺ T cells, (**B**) CD8⁺ T cells, (**C**) the CD4/CD8 ratio in CD3⁺ T cells, (**D**) DN cells in CD3⁺ T cells, (**E**) NK cells (gated on CD3⁻NK1.1⁺DX5⁺ cells) and (**F**) CD4⁺ NKT cells, (**G**) CD8⁺ NKT cells, (**H**) or DN NKT cells for WT mice treated with the NK1.1-depleting antibody PK136 with and without *P. pneumotropica* infection. Data show mean ± SD. Student's *t*-test was used to evaluate significant differences between the groups; n = 3–6 mice/group, **P ≤0.01, ***P ≤0.001.

the infected group (Figure 6E), there were no significant changes in IFN-γ⁺CD8⁺ NKT cells (Figure 6F).

As depicted in Figure 7, similar tendencies regarding IL-17A⁺ cells were detected in adult WT groups depleted of NK1.1⁺ cells with or without *P. pneumotropica* infection. The total percentage of IL-17A⁺ cells was significantly increased in the infected WT group versus the non-infected WT group both lacking NK1.1⁺ cells (Figure 7A) indicating that NK1.1⁺ T cells could have a critical IL-17A-suppressive role during infection. No increased IL-17A positivity could be observed in any of T cells, NK cells, or NKT-cell subsets (Figure 7B–F). Although a significant reduction of IL-17A positivity was detected in both CD4⁺ T and CD4⁺ NKT cell subsets (Figure 7B and 7E, respectively), these cells comprised only a very small population of total IL-17A⁺ cells. Hence, these data also indicate that the main source of IL-17A-producing cells could be antigen-presenting cells (APCs) or other innate immune cells rather than different T-cell subpopulations, NK or NKT cells.

Discussion

A variety of NKT cell types exist, including type I "invariant" NKT cells and type II NKT cells. Type I NKT cells possess an invariant TCR α chain with a bias towards certain types of Vβ chains, whereas type II NKT cells possess diverse α and β chains [35]. *Cd1d*$^{-/-}$ mice lack type I and II NKT cells, though they may possess other NKT-like cells that do not rely on CD1d but that express NK receptors such as DX5 and NK1.1 and T cell receptors [11,36] although the function of these cells are not well explored. NKT cells have received vast support as important regulatory cells in autoimmune, infectious and cancer diseases [2] in

Citation: Liu Y, Issazadeh-Navikas S (2012) NKT Cell Subsets and Proinflammatory Cytokine Changes in Mice as a Result of a Natural Pasteurella pneumotropica Infection. J Clin Cell Immunol 3:117. doi:10.4172/2155-9899.1000117

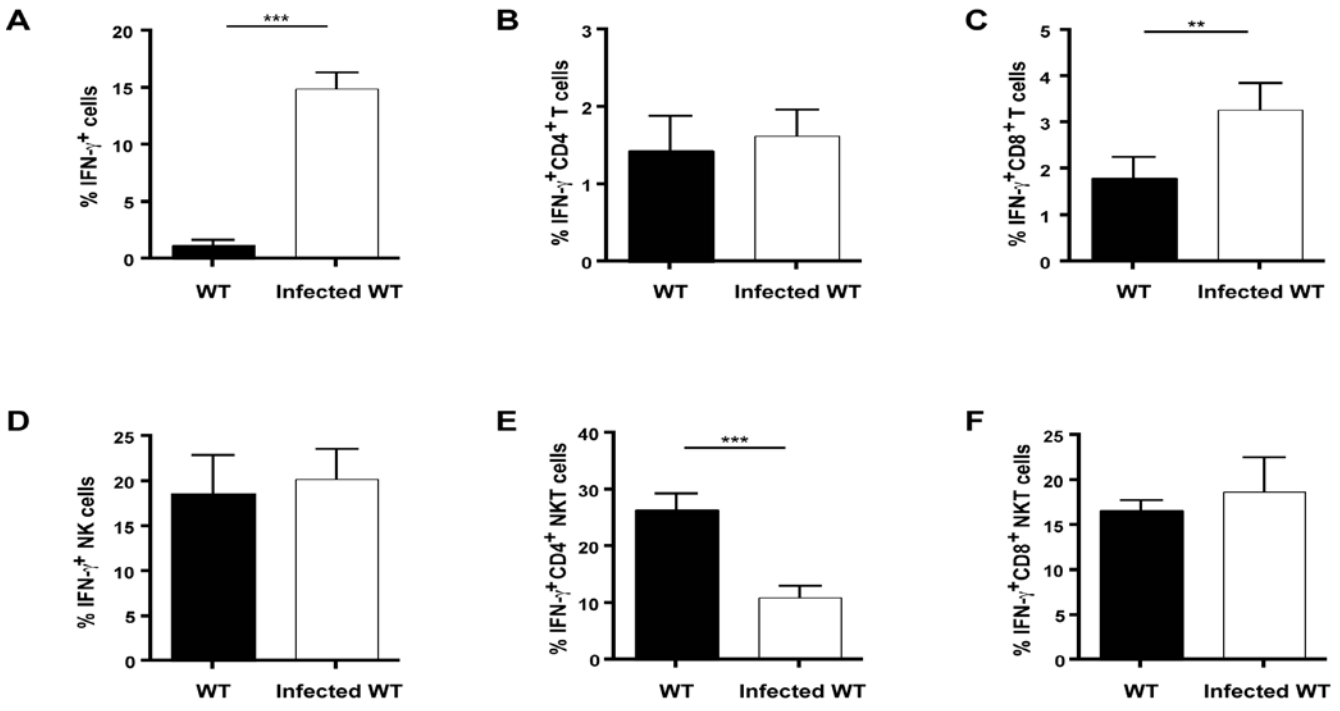

Figure 6: FACS analysis of IFN-γ+ splenocytes with and without *P. pneumotropica* infection in WT mice depleted of NK1.1⁺ cells. (A–F) Total percentage of IFN-γ⁺ cells and in different subsets – CD4⁺ T cells, CD8⁺ T cells, NK cells (CD3⁻NK1.1⁺), CD4⁺ NKT cells, and CD8⁺ NKT cells – for WT mice treated with the NK1.1-depleting antibody PK136. Data show mean ± SD. Student's *t*-test was used to evaluate significant differences between the groups; n = 3–6 mice/group, **P ≤0.01, ***P ≤0.001.

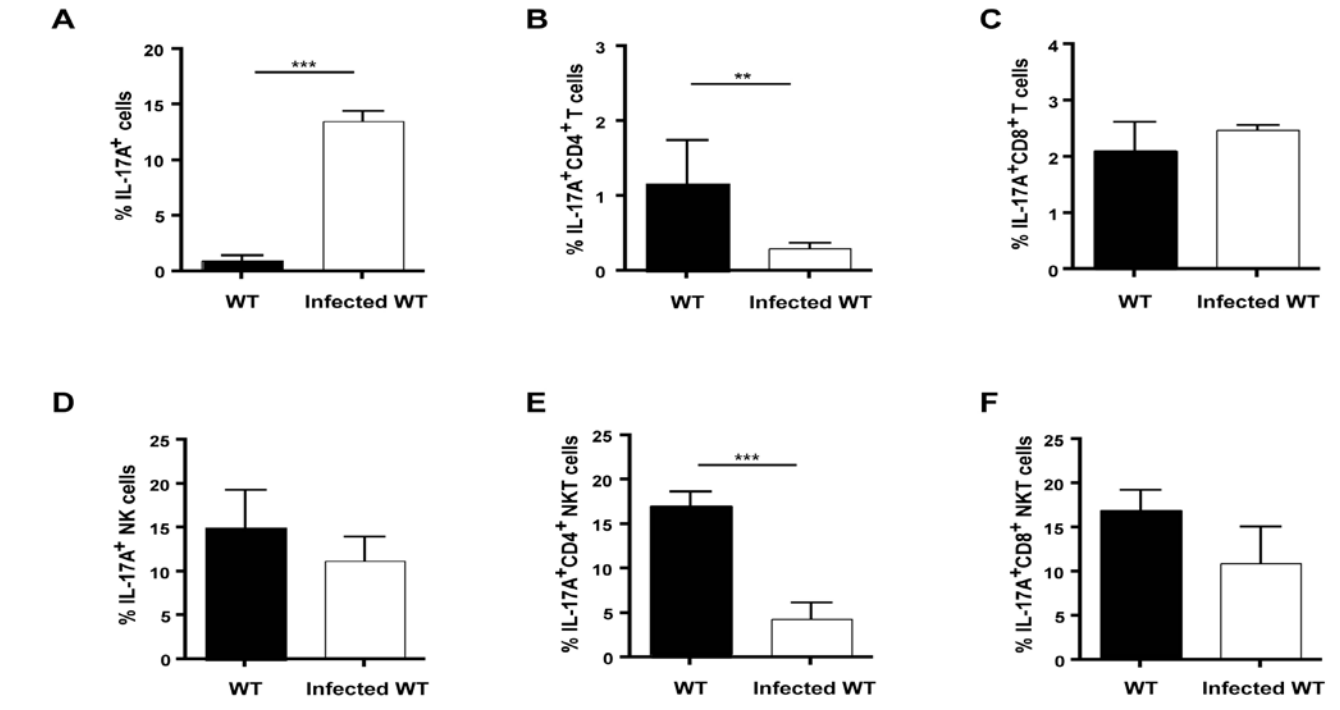

Figure 7: FACS analysis of IL-17A⁺ splenocytes with and without *P. pneumotropica* infection in WT mice depleted of NK1.1⁺ cells. (A–F) Total percentage of IL-17A⁺ cells and IL-17A⁺ in different subsets – CD4⁺ T cells, CD8⁺ T cells, NK cells (CD3⁻NK1.1⁺), CD4⁺ NKT cells, and CD8⁺ NKT cells – for WT mice treated with the NK1.1-depleting antibody PK136. Data show mean ± SD. Student's *t*-test was used to evaluate significant differences between the groups; n = 3–6 mice/group, **P ≤0.01, ***P ≤0.001.

spite of the fact that they only comprise small percentages of the total immune cells. Their immunoregulatory functions in counteracting inflammatory conditions have been attributed partly to the fact that they rapidly release large amount of cytokines such as IL-4, IFN-γ and IL-17 [2,4]. Cytokine production by NKT cells could have important roles in immune-mediated diseases directly [4], or indirectly by inhibiting cytokine production by effector T helper cells such as Th1 and Th17 responses [37]. The large diversity in types of cytokines produced by NKT cells could at least in part underlie the wide range of effects NKT cells have on a variety of disease states. As recently emphasized, this could also be due to the presence of different subsets of NKT cells [4].

NKT cells are widely believed to have a protective role in autoimmune, inflammatory and infectious diseases [1,3]. However, such a function has also been challenged, showing that NKT cells can worsen inflammatory and autoimmune conditions [38]. One of the most important factors which could have bearing on the outcome of immune-mediated diseases and the result of such immunological studies, and the reason for controversies in the literature, could be the fact that some laboratories might use mice infected with the commonly occurring bacteria, *P. pneumotropica*, which could have a significant impact on immune cells. As infection with *P. pneumotropica* is usually asymptomatic, and given the high prevalence of the infection, the results of such investigations could have been influenced. Additionally, it is often accepted that if the genetically modified mice have been backcrossed more than 10 generations they can be compared to WT mice. Quite often, though WT mice from commercial sources are purchased instead of producing littermates, which could thus introduce variance in studies. In addition to possible differences in the genetic background, transferring mice from different facilities could introduce unaccounted for differences, such as under conditions where mice either from the conventional animal house or from the commercial source are infected. Comparisons among these mice, if either group were infected, could thus generate bias.

Given the strong involvement of NKT cells in regulation of bacterial infections, as well as the high prevalence of subclinical *P. pneumotropica* infection in laboratory mice, we were motivated to investigate how a naturally occurring *P. pneumotropica* infection could impact the outcome of immune cell balance and their cytokine profile. Therefore we investigated the influence of presence and absence of CD1d-dependent NKT cells upon subclinical natural infection with *P. pneumotropica*. We utilized *CD1d*$^{-/-}$ mice, which genetically lack CD1d-dpendent NKT cells, and compared them to their WT littermates. As the *P. pneumotropica* infection was discovered upon routine laboratory FELASA screens that also excluded the presence of other pathogens, any differences in effects in WT and knockout mice could be attributed to the presence of this organism. We report here that mice naturally infected with *P. pneumotropica* display altered levels and subsets of NKT cells, conventional T cells and NK cells and an altered cytokine profile, in regard to IFN-γ- and IL-17-producing cells. Interestingly, changes in the percentage of CD4^{+}, CD8^{+} and DN T cells were also observed in the infected mice regardless of the existence of CD1d. But more importantly we found that mice genetically lacking CD1d-dependent NKT cells reacted to the subclinical *P. pneumotropica* infection very differently compared to their WT littermates, both with regard to the shift in the T cell, NK cell and NKT cell subsets as well as the proinflammatory cytokine profile of the mice.

Data revealed that *P. pneumotropica* infection in WT mice resulted in significant expansion of CD8^{+} NKT cells but significant reduction of DN NKT cells. In contrast, *CD1d*$^{-/-}$ mice did not show major changes in CD8^{+} NKT cell populations while responding to *P. pneumotropica* infection by expanding the DN NKT cell population. When CD1d-dependent NKT cells along with other NK1.1^{+} cells were depleted in vivo, at an adult age, this led to a very different influence on NKT cell homeostasis. Under such conditions, CD4^{+} NKT cells were significantly increased even if both groups were treated with PK136. However, no differences were observed in other NKT cell populations. Such differences in the CD1d-independent NKT cell subsets could indeed influence the outcome of immune-mediated diseases.

P. pneumotropica infection affected the balance among conventional T cell subsets and NK cells in infected WT and *CD1d*$^{-/-}$ mice compared to non-infected groups. Moreover, the NK1.1^{+} cell depletion led to similar changes in these cells. However, a major difference was observed in the percentage of NK cells. While NK cells were completely diminished in non-infected WT mice as a result of PK136 treatment, a substantial percentage of NK cells remained in the infected group. Since NK cells have also been suggested to have a modifying role in autoimmune [39], allergic [40], tumor [41] and infectious diseases [42], such impacts of a non-pathological infection such as *P. pneumotropica* could have bearings on the ensuing disease.

Of note, we found that the total levels and the pattern of intracellularly IFN-γ- and IL-17A-producing cells were tremendously affected as a result of this subclinical infection. NKT cells producing IFN-γ differed entirely from NKT cells producing IL-17A and hence how they might regulate the cytokine production by other T cells as well as APCs. Surprisingly, while both these proinflammatory cytokines were significantly elevated upon infection, IFN-γ elevations were also seen in subsets of NKT cells. This is in agreement with a recent report [43]. This finding could be the result of IL-12 production by professional APCs like dendritic cells that could produce large amounts of IL-12. Since NKT cells have been shown to express the IL-12 receptor [43], this could consequently lead to rapid production of intracellular IFN-γ production. Such a cytokine milieu could also trigger production of IFN-γ by conventional T cells, as seen in the current report.

The major IL-17A-producing cells among infected splenocytes were found to be non–T and non–NK1.1^{+} cells. This strongly points towards other innate immune cells such as APCs as the main source of IL-17A production upon subclinical *P. pneumotropica* infection. It is also important to point out that *CD1d*$^{-/-}$ mice and NK1.1-depleted WT mice mounted significantly higher IL-17A responses. Although the pathogenic role of IL-17 production in autoimmune diseases has received serious challenges [11,44-46], it is widely reported both in autoimmune conditions [47-49] and especially in allergic asthma conditions [50] that IL-17 plays a pathologic role. Thus, factors that change the IL-17 levels could have a serious impact on how the diseases under investigation will be affected. Because of this, close monitoring of laboratory mice for the presence of *P. pneumotropica* even in the absence of clinical signs is warranted to eliminate skewed results in immunological studies. This is particularly of concern as very few immunological studies [51,52] explicitly state that mice were free of *P. pneumotropica*, and one even indicated that two thirds of mice were infected [53]. Though it is plausible that many immunological studies do use *P. pneumotropica*–free mice but do not state so, given the high

prevalence of the infection and lack of symptoms, there is a likelihood that such subclinical infection could have contributed to the existing controversies. This study lends further emphasis to the importance even subclinical infections have in the regulation of immune cell homeostasis and the inflammatory cytokine milieu.

Funding Acknowledgements

This work has been supported by grants from The Lundbeck Foundation, Denmark; The Novo Nordisk Foundation, The Danish Research Council – Medicine; and The Danish Cancer Society.

Conflict of Interest Statement

The authors have declared that no conflict of interest exists.

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