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Evidence of Invariant Natural Killer T (iNKT) Cell Exhaustion in Sarcoidosis

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Summary

Invariant natural killer T (iNKT) cells are integral components of immune responses during many chronic diseases, yet their surface phenotypes, subset distribution, and polyfunctional capacity in this environment are largely unknown. Therefore, using flow cytometry, we determined iNKT phenotypic and functional characteristics in subjects with the chronic inflammatory disease sarcoidosis and matched controls. We found that sarcoidosis subjects displayed lower iNKT frequencies, which correlated with lung fibrosis, C-reactive protein levels, and other measures of clinical disease. The CD4− CD8− (DN) iNKT cell population was selectively lower in diseased individuals and the remaining DN iNKT cells exhibited higher frequencies of the activation markers CD69 and CD56. Functionally, both total IFN-gamma+ and the dual-functional IFN-gamma+ TNF-alpha+ iNKT cells were decreased in sarcoidosis subjects and these functional defects correlated with total iNKT circulating frequencies. As the loss of polyfunctionality can reflect functional exhaustion, we measured the surface antigens PD-1 and CD57 and found that levels inversely correlated with dual-functional iNKT cell percentages. These findings reveal that, similar to traditional T cells, iNKT cells may also undergo functional exhaustion, and that circulating iNKT frequencies reflect these defects. PD-1 antagonists may therefore be attractive therapeutic candidates for sarcoidosis and other iNKT-mediated chronic diseases.

Keywords

invariant natural killer T cells; sarcoidosis; flow cytometry; inflammation; interferon-gamma

Introduction

Invariant natural killer T (iNKT) cells are a unique population of T cells that possess qualities of both innate and adaptive immune cells. iNKT cells recognize glycolipid antigens bound to the non-classical MHC CD1d [1–3] and are also stimulated in response to cytokines and TLR signaling [4–5]. Possibly due in part to the multiple ways iNKT cells can be activated, this unique T cell subset is likely a key determinant in the course of autoimmune [6] and infectious diseases [7–8] as well as malignancy [6, 9–13].

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Conflict of interest
The authors have no conflicts of interest.

In many chronic diseases, prolonged inflammation leads to dysfunctional T cell populations with ablated effector function and/or proliferative capability; these T cells are referred to as "exhausted" [14]. In the early-mid stages of exhaustion, antigen-specific T cells lose the capacity to produce multiple cytokines (e.g. polyfunctionality) [15–17]. Over time, chronic inflammation can lead to complete ablation of T cell effector function capacity, loss of proliferative potential, and ultimate depletion of antigen-specific T cells [14]. Programmed Death Receptor-1 (PD-1) expression is associated with T cell exhaustion [14]; *in vivo*PD-1 up-regulation during chronic lymphocytic choriomeningitis virus (LCMV) in mice or SIV infection in non-human primates leads to suppression of T cell function with concomitant reduced pathogen clearance [18–19]. In humans with advanced solid organ malignancies, treatment with antibodies against PD-1 or PD-1 ligand produced durable anti-tumor responses [20–21]. These examples illustrate the robust inhibitory role associated with PD-1 expression on T cell function in chronic disease states.

Similar to antigen-specific CD8+ T cells, iNKT cells express higher levels of PD-1 in HIV [22] and *Mycobacterium tuberculosis* [23] infections. However, given the challenges of studying low frequency cell populations in humans, it is unknown whether increased PD-1 expression on circulating iNKT cells is associated with polyfunctional impairment. Understanding the functional role of iNKT cells in states of chronic inflammation will help identify whether novel therapies, such as PD-1 antibody antagonists (against either PD-1 receptor [21], or a PD-1 receptor Ligand, PD-L1 [20]), should be considered possible molecular targets for treatment.

Here, we used flow cytometry to determine the phenotype and functional status of iNKT cells in the chronic inflammatory disease sarcoidosis. This condition is associated with persistent antigenic stimulation by insoluble microbial proteins [24–26] and low numbers of iNKT cells have been reported [27–28]. Little is known about the correlation of iNKT cell numbers to phenotypic and functional properties and whether markers of immune exhaustion are associated with impaired cytokine production in these cells. Our goal was to improve the understanding of iNKT cell defects in a chronic human inflammatory disease and therefore elucidate the potential for novel iNKT biomarkers and therapeutic targets.

Results

Study Subject Characteristics

Twenty-nine sarcoidosis subjects and 33 healthy controls were included in the iNKT cell phenotype study (Table I). Twenty-seven subjects with sarcoidosis had controls matched for gender, race, and age (within 10 years). Pulmonary function and additional clinical characteristics are presented in Table I. Since pulmonary disease is the most common manifestation in sarcoidosis, Table II provides clinical characteristics of these subjects by chest radiographic stage. Stage IV subjects tended to have higher dyspnea scores (i.e., worse dyspnea) and lower pulmonary function compared to other stages (trend in statistical significance for % FVC and % DLCO in Stage IV compared to other groups; P = 0.06 and 0.08 respectively by one-way ANOVA). A subgroup of these subjects had samples available for iNKT cell functional and PD-1/CD57 analysis (see Supplemental Table 1).

Sarcoidosis subjects have low numbers of circulating iNKT cells, with the lowest counts found in subjects with high CRP levels and irreversible fibrotic lung disease

Circulating iNKT cell frequencies and absolute counts have been reported to be abnormally low in sarcoidosis subjects in the UK and Japan [27–28]. For our US cohort, we used a tetramer-based gating strategy (Figure 1A) and found a trend toward lower frequencies (p=0.099, Figure 1B) and significantly lower absolute numbers of iNKT cells (p=0.0133,

Figure 1C) in the PBMC of sarcoidosis subjects compared to controls. As the sarcoidosis subjects demonstrated a range of pulmonary function abnormalities and chest radiographic disease, we also compared total iNKT cell frequencies and absolute counts between subjects in each of the defined disease stages (Stages I-IV, as described in Materials and Methods). Stage IV disease is defined by evidence of lung fibrosis and is considered to be irreversible compared to Stage I disease, which has been reported to resolve spontaneously in up to 70% of subjects [29]. Stage IV subjects had significantly lower iNKT cell frequencies compared to both controls and subjects with Stage I disease (Figure 1D) and significantly lower iNKT cell absolute counts compared with controls (Figure 1E). Concurrent use of systemic corticosteroids or alternative immunosuppression (as used by 10/29 subjects, Figure 1) had no significant effect on iNKT frequencies or numbers (data not shown), consistent with a previous report [27].

We next compared iNKT cell frequencies with quantitative and qualitative measures of lung disease and inflammation and found significantly lower frequencies of iNKT cells in subjects with chest CT scan evidence of fibrosis, nodules, and the symptom of cough (Figure 1F). There were also trends toward lower iNKT cell percentages for subjects with CT scan evidence of bronchiectasis and the symptom of wheezing (Figure 1F). In addition, the continuous variables of shortness of breath (dyspnea score) and forced vital capacity (FVC) (a measure of lung restriction) were significantly correlated with iNKT cell frequencies (Figures 1G & 1H, respectively). Overall, subjects presenting with more severe lung disease had significantly lower frequencies of circulating iNKT cells. Similar results were found when we analyzed iNKT cell frequencies as a percentage of CD3+ cells (data not shown), indicating the differences noted are iNKT cell-specific and not reflective of general lymphopenia. Also, serum levels of C reactive protein (CRP), a marker of general inflammation, were measured in the plasma of sarcoidosis subjects. Lower iNKT frequencies were significantly associated with higher CRP levels (Figure 1I). Taken together, these data strongly implicate a link between circulating iNKT frequencies and both inflammation and measures of disease severity.

To assess whether iNKT frequencies changed over time during the course of disease, levels were assessed at two visits over a span of 36 months in six subjects. Between these two visits, four subjects remained clinically stable with no significant change in iNKT cell frequency (Figure 1J, open triangles). One subject developed progressive lung disease (from stage II to fibrotic stage IV disease), and had a 53% decrease in iNKT frequency (Figure 1J, closed squares), while one subject demonstrated improved lung function (11% increase in FVC) with a concomitant increase in iNKT cell level (Figure 1J, closed circles). These preliminary results, taken together with our cross-sectional data stratified by disease stage (Figure 1E) suggest that iNKT cell numbers may decline as disease progresses. These changes were documented prior to any change in therapy.

Lower numbers and distinct surface phenotype of the CD4−CD8− (Double Negative) iNKT subset in sarcoidosis

We next determined whether any alterations in iNKT cell subset distributions are present in PBMC from sarcoidosis subjects. iNKT cells can express CD4 and/or CD8 to comprise up to four distinct subsets in humans. The two most frequent subsets in PBMC are CD4−CD8− (DN) and CD4+CD8− (CD4 SP) and differences in *ex vivo* functional profiles of these subsets have been reported [30–31]. Therefore, we explored the frequencies and phenotypes of these subsets (Figure 2A demonstrates the gating strategy). We found significantly lower absolute numbers of circulating DN iNKT cells compared to healthy controls (Figure 2B). A similar distribution was found when analyzing the percentages of CD4, CD8 and DN iNKT cell subsets while there was no measurable difference in these percentages for CD3+ T cells

between sarcoidosis and controls (Supplemental Figure 1 & 2). The cell counts of all other iNKT cell subsets that differ in CD4 and CD8 expression, including CD4 SP, CD8 SP, and CD4+ and CD8+ (DP) fractions, were not significantly different between groups (Figure 2B). We next compared DN iNKT cell numbers between subjects divided by disease stage and found the lowest numbers of DN iNKT cells in subjects with fibrotic disease (Stage IV) compared to all other subject groups (Figure 2C).

To assess whether the remaining DN iNKT cells in diseased subjects were phenotypically distinct, we measured the activation markers CD69 and CD56, as well as CD161, an antigen associated with iNKT cell maturation [32–33] and iNKT cell function in chronic infection [34]. We found that DN iNKT cells exhibited higher percentages of CD69 and CD56 in sarcoidosis subjects versus controls (Figure 2D). Analysis of the other major iNKT cell subset, CD4+ CD8− (CD4 SP), yielded no significant differences in these surface markers between the healthy and sarcoidosis cohorts (Figure 2E).

Functional analysis reveals a defect of total IFNγ+ and IFNγ+ TNFα+ dual functional iNKT cells in sarcoidosis; these functional alterations correlate with total circulating iNKT cell frequencies

Given the association of Th1 cytokines in the pathogenesis of sarcoidosis [25, 35–36], we determined the functional status of the circulating iNKT cell compartment in sarcoidosis and controls by measuring IFNγ and TNFα production directly *ex vivo* (gating strategy shown in Figure 3A). We found an overall defect in total IFNγ production by iNKT cells from sarcoidosis subjects compared to controls following PMA and Ionomycin stimulation, while total production of TNFα was similar between groups (Figure 3B). All sarcoidosis subjects taking immunosuppression demonstrated intact iNKT IFNγ production. We did not find a similar defect in IFNγ production by total CD3+ T cells (Figure 3C). Taken together, these data reveal an iNKT cell-specific defect in IFNγ production in sarcoidosis.

To examine for defects of an iNKT cell's ability to secrete multiple cytokines simultaneously, as has been described for early-to-mid stages of traditional T cell exhaustion [14, 37], we compared the percentages of iNKT cells that produce all combinations of IFNγ and TNFα after stimulation between healthy and sarcoidosis groups. There were significantly lower frequencies of IFNγ+ TNFα+ (dual functional) iNKT cells in the sarcoidosis subjects compared with controls (Figure 3D) yet the percentages of iNKT cells secreting only IFNγ or only TNFα were similar between groups. We also compared the frequencies of iNKT cells that did not secrete either IFNγ or TNFα after stimulation, as T cells without effector function are found in chronic diseases and are believed to represent late stage exhaustion [14, 38]. The IFNγ− TNFα− iNKT cell percentages were significantly higher in sarcoidosis subjects compared with controls (Figure 3D).

Lower numbers of circulating iNKT cells have been described in several chronic diseases and infections [39–44], however the significance of this finding is largely unknown. We directly compared iNKT frequency and functionality and found a significant positive correlation between circulating iNKT frequencies and percentages of both total IFNγ+ cells (Figure 3E), as well as IFNγ+ TNFα+ dual functional cells (Figure 3F). Taken together, we identified significant, selective defects in the ability of iNKT cells from sarcoidosis subjects to produce IFNγ generally, as well as exhibit specific IFNγ and TNFα dual functionality. Within individual sarcoidosis subjects, these functional abnormalities correlated with iNKT cell circulating frequencies, a parameter that associated with both general inflammation (CRP) and the severity of lung disease (Figure 1).

Dual functional defects of iNKT cells in sarcoidosis correlate with expression of PD-1 and CD57

We next investigated whether the iNKT cell dual functional defect we found in sarcoidosis was associated with increased expression of PD-1 on iNKT cells. We compared the percentage (Figure 4A) and the Mean Fluorescence Intensity (MFI) (Figure 4B) of PD-1 on iNKT cells from healthy individuals and sarcoidosis subjects stratified by iNKT dual functionality. Sarcoidosis subjects with impaired dual functional iNKT cells demonstrated significantly higher expression of PD-1 compared with both healthy controls and sarcoidosis subjects with normal iNKT function (Figures 4A, 4B). Also, a significant inverse correlation was found between PD-1 expression on iNKT cells and iNKT dual functionality within individual sarcoidosis subjects (Figure 4C). A similar finding was found when PD-1 was assessed by MFI (data not shown). These results implicate immune exhaustion as one possible mechanism accounting for the lack of *ex vivo* iNKT cytokine production observed.

As a second measure of immune exhaustion, we determined CD57 levels on iNKT cells, as CD57 has been linked to chronic immune stimulation [45]. There was a significant positive correlation between the percentages of CD57+ and PD-1+ iNKT cells within our sarcoidosis cohort (Figure 5A). Also, similar to PD-1, we found a significant inverse relationship between the percentages of CD57+ and IFNγ+TNFα+ dual functional iNKT cells (Figure 5B). No differences in CD57 expression were found on total CD3+ T cells between healthy and sarcoidosis subject groups (data not shown). Together, these data support the concept that selective immune exhaustion of iNKT cells may be contributing to the iNKT functional defects observed in sarcoidosis subjects. Representative histogram plots from a sarcoidosis subject are displayed in Supplemental Figure 3.

Discussion

Shortly after an immune response begins, iNKT cells become activated and have been ascribed to shift the strength and character of the composite immune response through cross-talk with other immune cells [46–49]. This characteristic is likely to account for the integral role of iNKT cells in autoimmunity, infection and malignancy. Here, we determined the phenotypic and functional attributes of circulating iNKT cells in sarcoidosis, linking iNKT cell deficiencies and skewed iNKT subsets to disease severity. iNKT cells from sarcoidosis subjects also had functional defects in both total IFNγ and IFNγ+ TNFα+ dual functionality. These impairments were associated with higher PD-1 and CD57 expression. Collectively, these data indicate that iNKT cells become phenotypically skewed and functionally exhausted in sarcoidosis. These alterations/impairments might limit the ability to resolve inflammation and result in persistent or progressive disease.

One of the challenges in the field of human iNKT cell biology is not only understanding the implications of why humans exhibit such a large range of circulating iNKT cell frequencies, but why the frequency of iNKT cells has been reported to be lower than control populations in many different types of infectious and inflammatory diseases [39–44]. Our data have shed new light on the biological implications of these observations, as we found iNKT numbers linked to clinical markers of disease severity (Figure 1) as well as iNKT functional capacity (Figure 3). Future studies will need to address whether the loss of circulating iNKT cells in sarcoidosis is due to apoptosis and death from persistent activation or selective homing to organs affected by sarcoidosis inflammation. Finally, the more challenging possibility that will take future longitudinal studies to determine is whether individuals who possess lower iNKT cell frequencies are more prone to develop “a” particular disease itself or more severe forms of the disease. Answering these questions will be important steps toward harnessing the full potential of iNKT-targeted therapeutics.

iNKT cell subsets may have different immunological roles in disease processes. Human iNKT cell populations are comprised of CD4+ CD8− (CD4 SP), CD8+ CD4− (CD8 SP), CD4− CD8− (DN) [31, 50–51], and the CD4+ CD8+ (DP) population reported here (Figure 2) and previously by our group [51]. Few data exist about the distribution of these iNKT subsets in the context of a human disease, likely due to the extremely low frequencies in PBMC and the number of simultaneous markers required for accurate visualization by flow cytometry. Here, we found lower absolute numbers of CD4− CD8− DN iNKTs in PBMC in subjects with the most progressive disease as well as higher percentages of CD56 and CD69 on these cells in the sarcoidosis subjects compared with controls (Figure 2). As CD4+ and CD4− iNKTs can have distinct functional profiles [31] and homing markers [50] and given our results, we propose that the CD4− CD8− DN population is the predominant iNKT subset involved in the sarcoidosis immune response. Furthermore, as all CD4 and CD8 iNKT cell subsets possess the same T cell receptor specificity, we predict that in sarcoidosis, iNKT cell subsets are differentially activated by combinations of cytokines and/or other inflammatory signals. A further understanding of the activation requirements and functional capacities of different iNKT subsets may help elucidate the reported contrasting roles of iNKT cells in disease pathogenesis.

In chronic viral infections, persistent antigenic stimulation leads to loss of polyfunctionality and up-regulation of PD-1 on virus-specific CD8+ T cells. In sarcoidosis we found iNKT cells exhibited similar characteristics to that found in exhausted antigen-specific T cells in chronic viral infections; this raises the question as to whether iNKT cells undergo a similar type of functional exhaustion. Although a possible alternative explanation for the loss of Th-1 type effector function by iNKT cell is that they are undergoing polarization to a Th2 phenotype, we do not think the data support this interpretation. First, impairment of TNFα and IFNγ dual-functional iNKT cells correlated highly significantly with the marker most strongly associated with T cell exhaustion, PD-1 ($p<0.0001$, Figure 4A). Second, the expression of PD-1 and another marker of exhaustion, CD57, were significantly inter-correlated in iNKT cell populations within individuals sarcoidosis subjects (Pearson $r=0.7$, $p=0.002$, Figure 5A).

Immune exhaustion in traditional antigen-specific memory T cells is thought to be due to persistent stimulation of the TCR by antigen. In the case of iNKT cells, the TCR is, by definition, invariant, but theoretically remains susceptible to chronic stimulation by cognate ligands such as glycolipids. Stimulation of iNKT cells with the glycolipid α–galactosylceramide leads to up-regulation of PD-1, resulting in anergic responses after subsequent attempts of TCR stimulation [52–53]. Whether long-term *in vivo* stimulation of iNKT cells by specific cognate ligands leads to a slow loss of polyfunctionality and eventual exhaustion has not been demonstrated experimentally. We identified defects in iNKT cell cytokine production that were not due to anergy as we stimulated with PMA and ionomycin which bypasses the proximal steps in TCR signaling [54]. Unlike traditional T cells, we propose that functional exhaustion of iNKT cells may also occur through TCR-independent mechanisms such as chronic cytokine stimulation. This may explain preferential activation of specific iNKT cell subsets (e.g. CD4− CD8− DN iNKTs) despite expression of the same invariant TCR. Elucidation of the precise mechanisms of iNKT cell exhaustion may reveal how to restore and/or harness iNKT functions to reverse the course of a variety of chronic illnesses.

Materials and Methods

Subjects

Sarcoidosis subjects were recruited prospectively through the Interstitial Lung Disease Clinic at UCSF (San Francisco, CA, USA). Control subjects were recruited through

advertisements. All subjects were ≥21 years of age and excluded for known pulmonary or systemic illness. Subjects with sarcoidosis met the diagnostic criteria defined by the American Thoracic Society and the European Respiratory Society [55–56] which is detailed in the Online Supplemental methods section. Clinical information was collected from sarcoidosis subjects on the blood draw date and included: complete medical history, clinical questionnaires with validated shortness of breath scale (Dyspnea Score) [57] and physical exam. Pulmonary function data, CXR and high resolution CT scanning of the chest was performed within two months prior to the blood draw. Clinical Stages I-IV of sarcoidosis disease was defined as previously described [58–59], where Stage I disease displays hilar adenopathy without parenchymal change; Stage II/III disease shows parenchymal change with or without hilar adenopathy; and Stage IV disease shows parenchymal disease with fibrosis. Control subjects had a normal standardized respiratory health and medical questionnaire on the data of the blood draw. Before initiating this study, sample size calculations were performed using preliminary data from a separate group of disease and control subjects. This study was approved by the Committee of Human Research at UCSF and informed consent was obtained on all participants.

Human lymphocyte preparation/thawing

Standard procedures were followed for PBMC isolation and the thawing protocol is detailed in the Supplemental methods section.

Staining, flow cytometry acquisition and gating of iNKT cells

Additional details of fluorescent antibodies, staining methods, enumeration and gating of iNKT cells are found in Supplemental methods section. In brief, for surface staining, PBMCs were incubated with fluorescent antibodies and CD1d-tetramer-PBS57 for 30 minutes at 4°C, washed and incubated with streptavidin-Qdot 655 for 20–30 minutes at 4°C. After washing and fixing, cells were analyzed on a LSR-II Flow Cytometer. A mean ± SD of 2.2 ± 1.5 and 3.0 ±1.9 million of acquisition events from each subject within our healthy and sarcoidosis groups, respectively, were collected (Supplemental Figure 4). For functional analysis, PBMC were incubated at 37°C, and 5% CO_2 ± 50ng/ml PMA, 500ng/ml ionomycin and 5μg/ml brefeldin for ~22 hr. Cells were then stained/washed for surface markers as described above and then fixed in 2% paraformaldehyde for 20 minutes followed by FACS Permeabilizing Solution 2 for 10 minutes. Intracellular staining with anti-TNFα and anti-IFNγ, anti-CD4 and anti-CD8 antibodies was performed for 30min at 4°C. Cells were washed and analyzed on a LSR-II Flow Cytometer. Titration of each reagent and "Fluorescence Minus One" controls [60] were used for optimization and placement of gates. iNKT cells were identified by gating on lymphocytes within forward and side scatter plots, exclusion of doublets, selection of live CD3+ cells and the iNKT cell-specific gating detailed in Supplemental methods.

Statistical analysis

The data are expressed as box plots with lines reflecting the medians for each group, boxes showing the interquartile range and whiskers showing the minimum to maximum values unless otherwise indicated. For two group comparisons we used Student's t test for normally distributed data or Mann Whitney U test for non-normally distributed data as appropriate. For more than two-group comparisons we used one-way analysis of variance for normally distributed data or Kruskal-Wallis test for non-normally distributed data as appropriate. Chi-square or Fisher's exact test was applied for comparison of groups with respect to categorical variables. Pearson correlation was applied for correlation of continuous variables after log-transformation for normality or Spearman correlation for non-normally distributed data where indicated. A p-value of < 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Non-standard abbreviations

iNKT	invariant Natural Killer T cell
DN	CD4− CD8− (Double Negative) T cell
CRP	C Reactive Protein
PD-1	Programmed Death-1 Receptor
FVC	Forced vital capacity

References

1. Bendelac A, Lantz O, Quimby ME, Yewdell JW, Bennink JR, Brutkiewicz RR. CD1 recognition by mouse NK1+ T lymphocytes. Science. 1995; 268:863–865. [PubMed: 7538697]
2. Bendelac A, Rivera MN, Park SH, Roark JH. Mouse CD1-specific NK1 T cells: development, specificity, and function. Annu Rev Immunol. 1997; 15:535–562. [PubMed: 9143699]
3. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. Science. 1997; 278:1626–1629. [PubMed: 9374463]
4. Tyznik AJ, Tupin E, Nagarajan NA, Her MJ, Benedict CA, Kronenberg M. Cutting edge: the mechanism of invariant NKT cell responses to viral danger signals. J Immunol. 2008; 181:4452–4456. [PubMed: 18802047]
5. Nagarajan NA, Kronenberg M. Invariant NKT cells amplify the innate immune response to lipopolysaccharide. J Immunol. 2007; 178:2706–2713. [PubMed: 17312112]
6. Subleski JJ, Jiang Q, Weiss JM, Wiltrout RH. The split personality of NKT cells in malignancy, autoimmune and allergic disorders. Immunotherapy. 2011; 3:1167–1184. [PubMed: 21995570]
7. Juno JA, Keynan Y, Fowke KR. Invariant NKT Cells: Regulation and Function during Viral Infection. PLoS Pathog. 2012; 8:e1002838. [PubMed: 22916008]
8. Reilly EC, Thompson EA, Aspeslagh S, Wands JR, Elewaut D, Brossay L. Activated iNKT cells promote memory CD8+ T cell differentiation during viral infection. PLoS One. 2012; 7:e37991. [PubMed: 22649570]
9. Berzofsky JA, Terabe M. The contrasting roles of NKT cells in tumor immunity. Curr Mol Med. 2009; 9:667–672. [PubMed: 19689293]
10. Casorati G, de Lalla C, Dellabona P. Invariant natural killer T cells reconstitution and the control of leukemia relapse in pediatric haploidentical hematopoietic stem cell transplantation. Oncoimmunology. 2012; 1:355–357. [PubMed: 22737613]
11. Najera Chuc AE, Cervantes LA, Retiguin FP, Ojeda JV, Maldonado ER. Low number of invariant NKT cells is associated with poor survival in acute myeloid leukemia. J Cancer Res Clin Oncol. 2012; 138:1427–1432. [PubMed: 22692855]
12. Smyth MJ, Thia KY, Street SE, Cretney E, Trapani JA, Taniguchi M, Kawano T, et al. Differential tumor surveillance by natural killer (NK) and NKT cells. J Exp Med. 2000; 191:661–668. [PubMed: 10684858]
13. Vivier E, Ugolini S, Blaise D, Chabannon C, Brossay L. Targeting natural killer cells and natural killer T cells in cancer. Nat Rev Immunol. 2012; 12:239–252. [PubMed: 22437937]
14. Wherry EJ. T cell exhaustion. Nat Immunol. 2011; 12:492–499. [PubMed: 21739672]

15. Fuller MJ, Zajac AJ. Ablation of CD8 and CD4 T cell responses by high viral loads. J Immunol. 2003; 170:477–486. [PubMed: 12496434]
16. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. J Virol. 2003; 77:4911–4927. [PubMed: 12663797]
17. Wherry EJ, Ahmed R. Memory CD8 T-cell differentiation during viral infection. J Virol. 2004; 78:5535–5545. [PubMed: 15140950]
18. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature. 2006; 439:682–687. [PubMed: 16382236]
19. Velu V, Titanji K, Zhu B, Husain S, Pladevega A, Lai L, Vanderford TH, et al. Enhancing SIV-specific immunity in vivo by PD-1 blockade. Nature. 2009; 458:206–210. [PubMed: 19078956]
20. Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med. 2012; 366:2455–2465. [PubMed: 22658128]
21. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med. 2012; 366:2443–2454. [PubMed: 22658127]
22. Moll M, Kuylenstierna C, Gonzalez VD, Andersson SK, Bosnjak L, Sonnerborg A, Quigley MF, et al. Severe functional impairment and elevated PD-1 expression in CD1d-restricted NKT cells retained during chronic HIV-1 infection. Eur J Immunol. 2009; 39:902–911. [PubMed: 19197939]
23. Kee SJ, Kwon YS, Park YW, Cho YN, Lee SJ, Kim TJ, Lee SS, et al. Dysfunction of natural killer T cells in patients with active Mycobacterium tuberculosis infection. Infect Immun. 2012; 80:2100–2108. [PubMed: 22409933]
24. Song Z, Marzilli L, Greenlee BM, Chen ES, Silver RF, Askin FB, Teirstein AS, et al. Mycobacterial catalase-peroxidase is a tissue antigen and target of the adaptive immune response in systemic sarcoidosis. J Exp Med. 2005; 201:755–767. [PubMed: 15753209]
25. Chen ES, Moller DR. Sarcoidosis--scientific progress and clinical challenges. Nat Rev Rheumatol. 2011; 7:457–467. [PubMed: 21750528]
26. Iannuzzi MC, Rybicki BA, Teirstein AS. Sarcoidosis. N Engl J Med. 2007; 357:2153–2165. [PubMed: 18032765]
27. Ho LP, Urban BC, Thickett DR, Davies RJ, McMichael AJ. Deficiency of a subset of T-cells with immunoregulatory properties in sarcoidosis. Lancet. 2005; 365:1062–1072. [PubMed: 15781102]
28. Kobayashi S, Kaneko Y, Seino K, Yamada Y, Motohashi S, Koike J, Sugaya K, et al. Impaired IFN-gamma production of Valpha24 NKT cells in non-remitting sarcoidosis. Int Immunol. 2004; 16:215–222. [PubMed: 14734606]
29. Kirtland SH, Winterbauer RH. Pulmonary Sarcoidosis. Semin. Respir. Med. 1993; 14:344–352.
30. Lee PT, Benlagha K, Teyton L, Bendelac A. Distinct functional lineages of human V(alpha)24 natural killer T cells. J Exp Med. 2002; 195:637–641. [PubMed: 11877486]
31. Gumperz JE, Miyake S, Yamamura T, Brenner MB. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. J Exp Med. 2002; 195:625–636. [PubMed: 11877485]
32. Berzins SP, Cochrane AD, Pellicci DG, Smyth MJ, Godfrey DI. Limited correlation between human thymus and blood NKT cell content revealed by an ontogeny study of paired tissue samples. Eur J Immunol. 2005; 35:1399–1407. [PubMed: 15816002]
33. Baev DV, Peng XH, Song L, Barnhart JR, Crooks GM, Weinberg KI, Metelitsa LS. Distinct homeostatic requirements of CD4+ and CD4- subsets of Valpha24-invariant natural killer T cells in humans. Blood. 2004; 104:4150–4156. [PubMed: 15328159]
34. Snyder-Cappione JE, Loo CP, Carvalho KI, Kuylenstierna C, Deeks SG, Hecht FM, Rosenberg MG, et al. Lower cytokine secretion ex vivo by natural killer T cells in HIV-infected individuals is associated with higher CD161 expression. AIDS. 2009; 23:1965–1970. [PubMed: 19590406]
35. Terasaki F, Ukimura A, Tsukada B, Fujita S, Katashima T, Otsuka K, Kanzaki Y, et al. Enhanced expression of type 1 helper T-cell cytokines in the myocardium of active cardiac sarcoidosis. Circ J. 2008; 72:1303–1307. [PubMed: 18654018]

36. Antoniou KM, Tzouvelekis A, Alexandrakis MG, Tsiligianni I, Tzanakis N, Sfiridaki K, Rachiotis G, et al. Upregulation of Th1 cytokine profile (IL-12, IL-18) in bronchoalveolar lavage fluid in patients with pulmonary sarcoidosis. J Interferon Cytokine Res. 2006; 26:400–405. [PubMed: 16734560]
37. Day CL, Abrahams DA, Lerumo L, Janse van Rensburg E, Stone L, O'Rie T, Pienaar B, et al. Functional capacity of Mycobacterium tuberculosis-specific T cell responses in humans is associated with mycobacterial load. J Immunol. 2011; 187:2222–2232. [PubMed: 21775682]
38. Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, Ahmed R. Viral immune evasion due to persistence of activated T cells without effector function. J Exp Med. 1998; 188:2205–2213. [PubMed: 9858507]
39. Lucas M, Gadola S, Meier U, Young NT, Harcourt G, Karadimitris A, Coumi N, et al. Frequency and phenotype of circulating Valpha24/Vbeta11 double-positive natural killer T cells during hepatitis C virus infection. J Virol. 2003; 77:2251–2257. [PubMed: 12525661]
40. Azakami K, Sato T, Araya N, Utsunomiya A, Kubota R, Suzuki K, Hasegawa D, et al. Severe loss of invariant NKT cells exhibiting anti-HTLV-1 activity in patients with HTLV-1-associated disorders. Blood. 2009
41. Sandberg JK, Fast NM, Palacios EH, Fennelly G, Dobroszycki J, Palumbo P, Wiznia A, et al. Selective loss of innate CD4(+) V alpha 24 natural killer T cells in human immunodeficiency virus infection. J Virol. 2002; 76:7528–7534. [PubMed: 12097565]
42. Snyder-Cappione JE, Nixon DF, Loo CP, Chapman JM, Meiklejohn DA, Melo FF, Costa PR, et al. Individuals with pulmonary tuberculosis have lower levels of circulating CD1d-restricted NKT cells. J Infect Dis. 2007; 195:1361–1364. [PubMed: 17397008]
43. Tahir SM, Cheng O, Shaulov A, Koezuka Y, Bubley GJ, Wilson SB, Balk SP, et al. Loss of IFN-gamma production by invariant NK T cells in advanced cancer. J Immunol. 2001; 167:4046–4050. [PubMed: 11564825]
44. van der Vliet HJ, von Blomberg BM, Hazenberg MD, Nishi N, Otto SA, van Benthem BH, Prins M, et al. Selective decrease in circulating V alpha 24+V beta 11+ NKT cells during HIV type 1 infection. J Immunol. 2002; 168:1490–1495. [PubMed: 11801694]
45. Focosi D, Bestagno M, Burrone O, Petrini M. CD57+ T lymphocytes and functional immune deficiency. J Leukoc Biol. 2010; 87:107–116. [PubMed: 19880576]
46. Bendelac A, Savage PB, Teyton L. The biology of NKT cells. Annu Rev Immunol. 2007; 25:297–336. [PubMed: 17150027]
47. Matsuda JL, Mallevaey T, Scott-Browne J, Gapin L. CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system. Curr Opin Immunol. 2008
48. De Santo C, Salio M, Masri SH, Lee LY, Dong T, Speak AO, Porubsky S, et al. Invariant NKT cells reduce the immunosuppressive activity of influenza A virus-induced myeloid-derived suppressor cells in mice and humans. J Clin Invest. 2008; 118:4036–4048. [PubMed: 19033672]
49. De Santo C, Arscott R, Booth S, Karydis I, Jones M, Asher R, Salio M, et al. Invariant NKT cells modulate the suppressive activity of IL-10-secreting neutrophils differentiated with serum amyloid A. Nat Immunol. 2010; 11:1039–1046. [PubMed: 20890286]
50. Kim CH, Johnston B, Butcher EC. Trafficking machinery of NKT cells: shared and differential chemokine receptor expression among V alpha 24(+)V beta 11(+) NKT cell subsets with distinct cytokine-producing capacity. Blood. 2002; 100:11–16. [PubMed: 12070001]
51. Snyder-Cappione JE, Tincati C, Eccles-James IG, Cappione AJ, Ndhlovu LC, Koth LL, Nixon DF. A comprehensive ex vivo functional analysis of human NKT cells reveals production of MIP1-alpha and MIP1-beta, a lack of IL-17, and a Th1-bias in males. PLoS One. 2010; 5:e15412. [PubMed: 21082024]
52. Parekh VV, Lalani S, Kim S, Halder R, Azuma M, Yagita H, Kumar V, et al. PD-1/PD-L blockade prevents anergy induction and enhances the anti-tumor activities of glycolipid-activated invariant NKT cells. J Immunol. 2009; 182:2816–2826. [PubMed: 19234176]
53. Chang WS, Kim JY, Kim YJ, Kim YS, Lee JM, Azuma M, Yagita H, et al. Cutting edge: Programmed death-1/programmed death ligand 1 interaction regulates the induction and maintenance of invariant NKT cell anergy. J Immunol. 2008; 181:6707–6710. [PubMed: 18981087]

54. Sullivan BA, Kronenberg M. Activation or anergy: NKT cells are stunned by alpha-galactosylceramide. J Clin Invest. 2005; 115:2328–2329. [PubMed: 16138189]

55. Costabel U, Hunninghake GW. ATS/ERS/WASOG statement on sarcoidosis. Sarcoidosis Statement Committee. American Thoracic Society. European Respiratory Society. World Association for Sarcoidosis and Other Granulomatous Disorders. Eur Respir J. 1999; 14:735–737. [PubMed: 10573213]

56. Hunninghake GW, Costabel U, Ando M, Baughman R, Cordier JF, du Bois R, Eklund A, et al. ATS/ERS/WASOG statement on sarcoidosis. American Thoracic Society/European Respiratory Society/World Association of Sarcoidosis and other Granulomatous Disorders. Sarcoidosis Vasc Diffuse Lung Dis. 1999; 16:149–173. [PubMed: 10560120]

57. Watters LC, King TE, Schwarz MI, Waldron JA, Stanford RE, Cherniack RM. A clinical, radiographic, and physiologic scoring system for the longitudinal assessment of patients with idiopathic pulmonary fibrosis. Am Rev Respir Dis. 1986; 133:97–103. [PubMed: 3942381]

58. James DG, Thomson AD. The course of sarcoidosis and its modification by treatment. Lancet. 1959; 1:1057–1061. [PubMed: 13655669]

59. Scadding JG. Prognosis of intrathoracic sarcoidosis in England. A review of 136 cases after five years' observation. Br Med J. 1961; 2:1165–1172. [PubMed: 14497750]

60. Roederer M. Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. Cytometry. 2001; 45:194–205. [PubMed: 11746088]

61. Sandberg JK, Bhardwaj N, Nixon DF. Dominant effector memory characteristics, capacity for dynamic adaptive expansion, and sex bias in the innate Valpha24 NKT cell compartment. Eur J Immunol. 2003; 33:588–596. [PubMed: 12616479]

62. Lee PT, Putnam A, Benlagha K, Teyton L, Gottlieb PA, Bendelac A. Testing the NKT cell hypothesis of human IDDM pathogenesis. J Clin Invest. 2002; 110:793–800. [PubMed: 12235110]

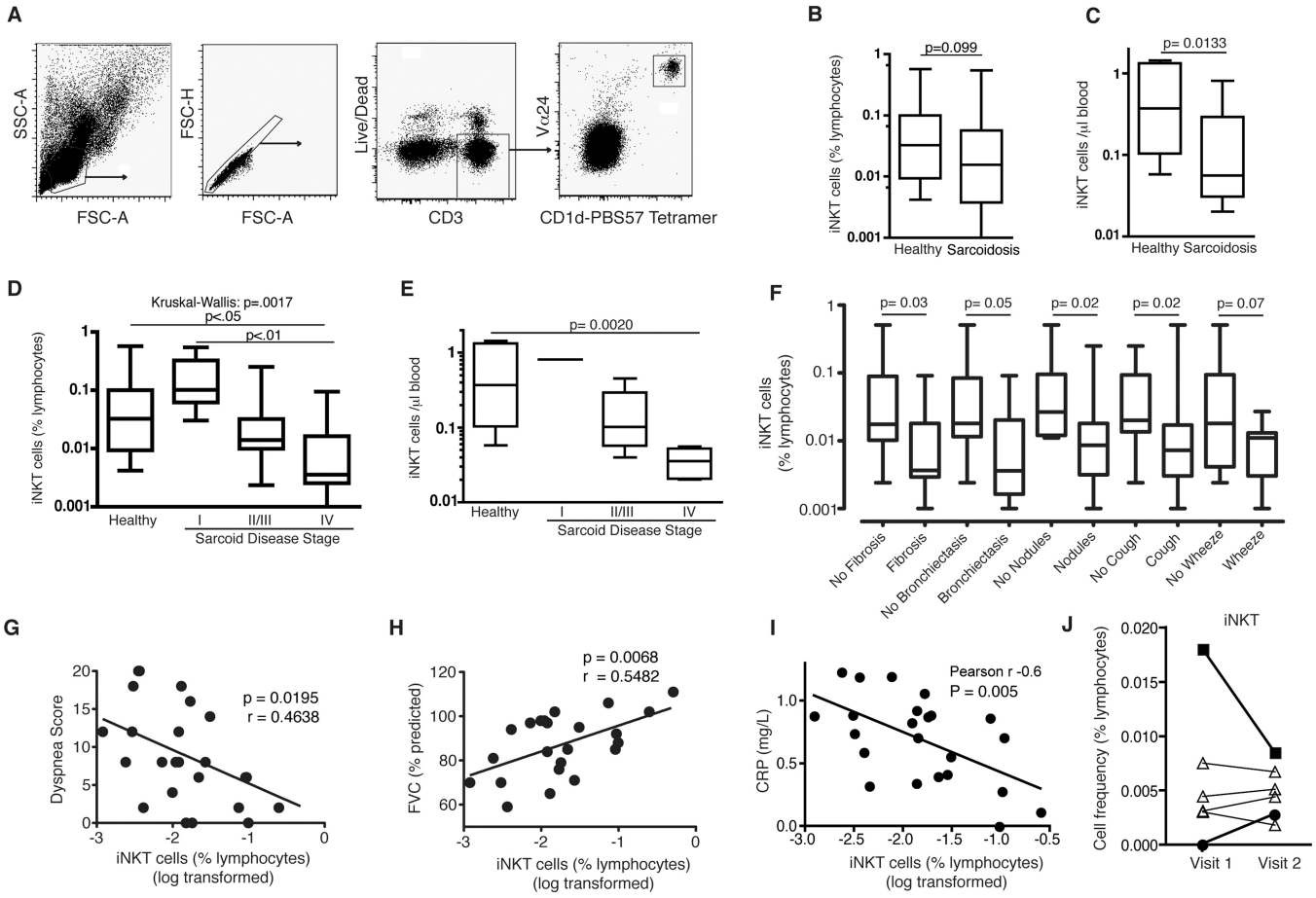

Figure 1. Correlation of sarcoidosis severity and clinical phenotype with numbers of circulating iNKT cells

PBMC's from sarcoidosis subjects and matched controls were stained with fluorescently labeled reagents (including a CD1d tetramer for iNKT cell specificity) and analyzed by flow cytometry. (A) Shown is the gating strategy for enumerating live iNKT cells. (B) Comparison of the frequencies of iNKT cells in PBMC from 29 sarcoidosis subjects and 33 healthy controls. (C) Comparison of the absolute numbers of iNKT cells in PBMC from ten of these sarcoidosis and control subjects who had CBC data available for analysis. (D & E) Comparison of the (D) frequencies and (E) absolute numbers of circulating iNKT cells from these same individuals stratified by radiographic Stage, where Stage I sarcoidosis has the highest spontaneous remission rates while Stage IV sarcoidosis is characterized by irreversible fibrosis (29 sarcoidosis subjects stratified by Stage as follows: Stage 1 [n=5], stage 2/3 [n=13] and stage 4 [n=11]). (F) The frequencies of circulating iNKT cells among sarcoidosis subjects were compared with qualitative and quantitative measures of lung disease, including chest CT scan evidence of fibrosis, bronchiectasis, or nodules, or symptoms of cough or wheeze (n ≥10 sarcoidosis subjects/group for each comparison). (G, H & I) Correlations of (G) Dyspnea Score, (H) FVC (percent predicted forced vital capacity, a quantitative measurement of lung restriction) and (I) CRP levels were compared with circulating iNKT cell frequencies in 24 sarcoidosis subjects who had complete clinical data available for analysis. (J) The iNKT frequencies of six subjects were followed longitudinally. iNKT cell frequencies are shown at two points in time separated by 36 months. Between visits, four subjects remained clinically stable with no significant change in iNKT cell frequency (open triangles). One subject developed progressive lung disease

(from stage II to fibrotic stage IV disease), and had a 53% decrease in iNKT frequency (closed squares), while one subject demonstrated improved lung function (11% increase in FVC) with a concomitant increase in iNKT cell level (closed circles). Statistical significance between groups is shown by horizontal bars displaying P values. Mann-Whitney test used for two group comparisons in B, C, and F; Kruskal-Wallis test for multiple comparisons used in D & E; Pearson correlations used for G-I. Data in (B-F) are displayed as box plots where boxes equal the 25th and 75th percentile range, the middle band represents the median, and the whiskers represent the minimum and maximum values.

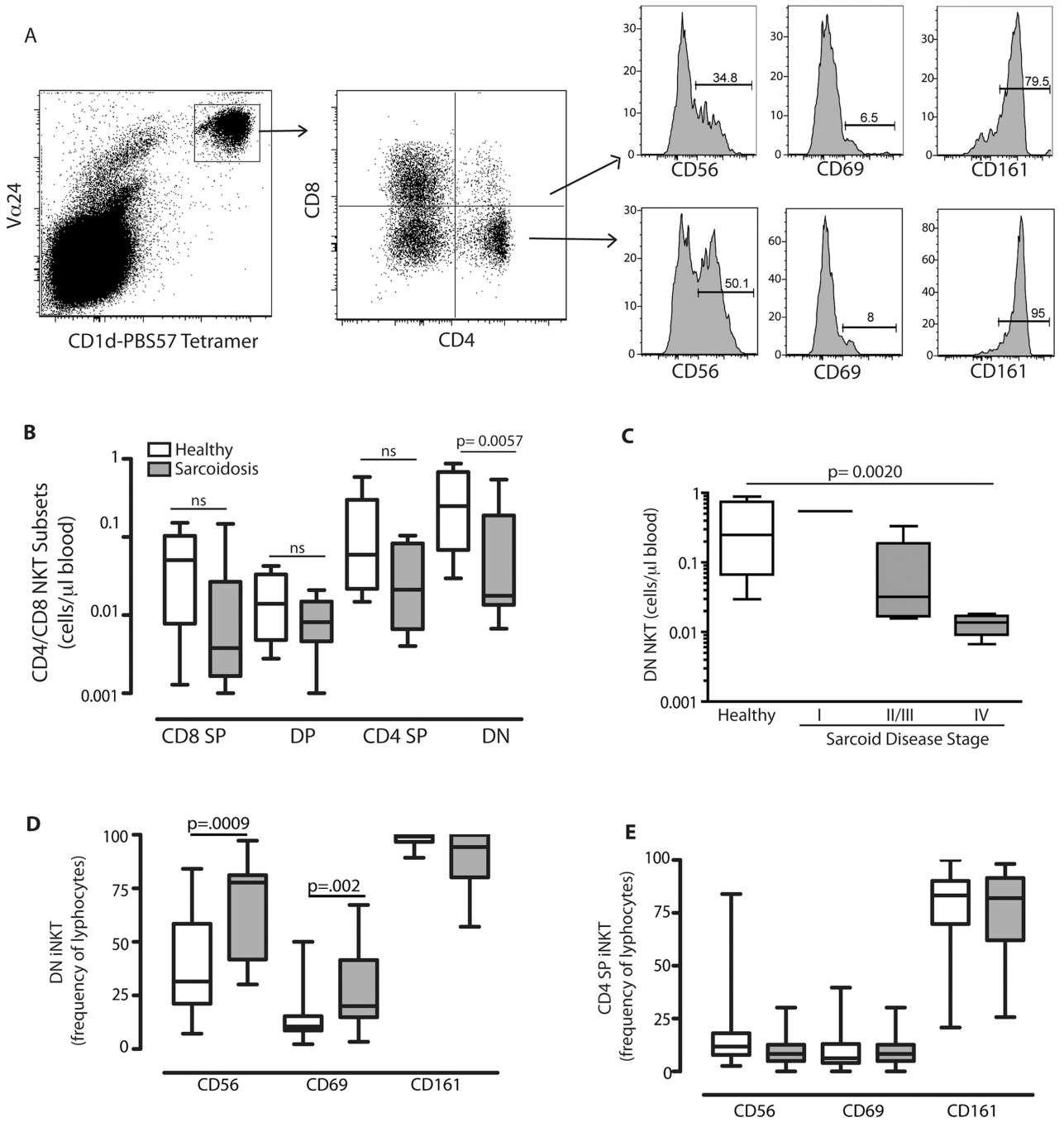

Figure 2. Measurement of absolute numbers and surface phenotype of circulating CD4− CD8− iNKT cells in sarcoidosis subjects

PBMC's from sarcoidosis subjects and matched controls were stained with fluorescently labeled reagents (as described in Figure 1) including surface antigen markers for CD56, CD69 and CD161 and analyzed by flow cytometry. (A) Shown is the gating strategy to enumerate these surface antigens on iNKT cells. (B) Measurement of the absolute numbers of CD4 and CD8 iNKT subsets between ten healthy and nine sarcoidosis subjects who had CBC data available for analysis (similar findings found for percentages of CD4 and CD8 iNKT subsets between 33 healthy and 29 sarcoidosis subjects shown in Supplemental Figure 2). In (C), the absolute number of CD4− CD8− double negative (DN) iNKT cells from these

same subjects were stratified by radiographic Stage, where Stage I sarcoidosis has the highest spontaneous remission rates while Stage IV sarcoidosis is characterized by irreversible fibrosis. (D & E) Percentage of (D) DN iNKT cells or (E) CD4 single positive (SP) iNKT cells expressing CD56, CD69 or CD161 amongst 33 healthy and 29 sarcoidosis subjects. Statistical significance between groups is shown by horizontal bars displaying P values. Mann-Whitney test used for two group comparisons in B, D, and E; Kruskal-Wallis test for multiple comparisons used in C. Data in (B–E) are displayed as box plots where boxes equal the 25th and 75th percentile range, the middle band represents the median, and the whiskers represent the minimum and maximum values.

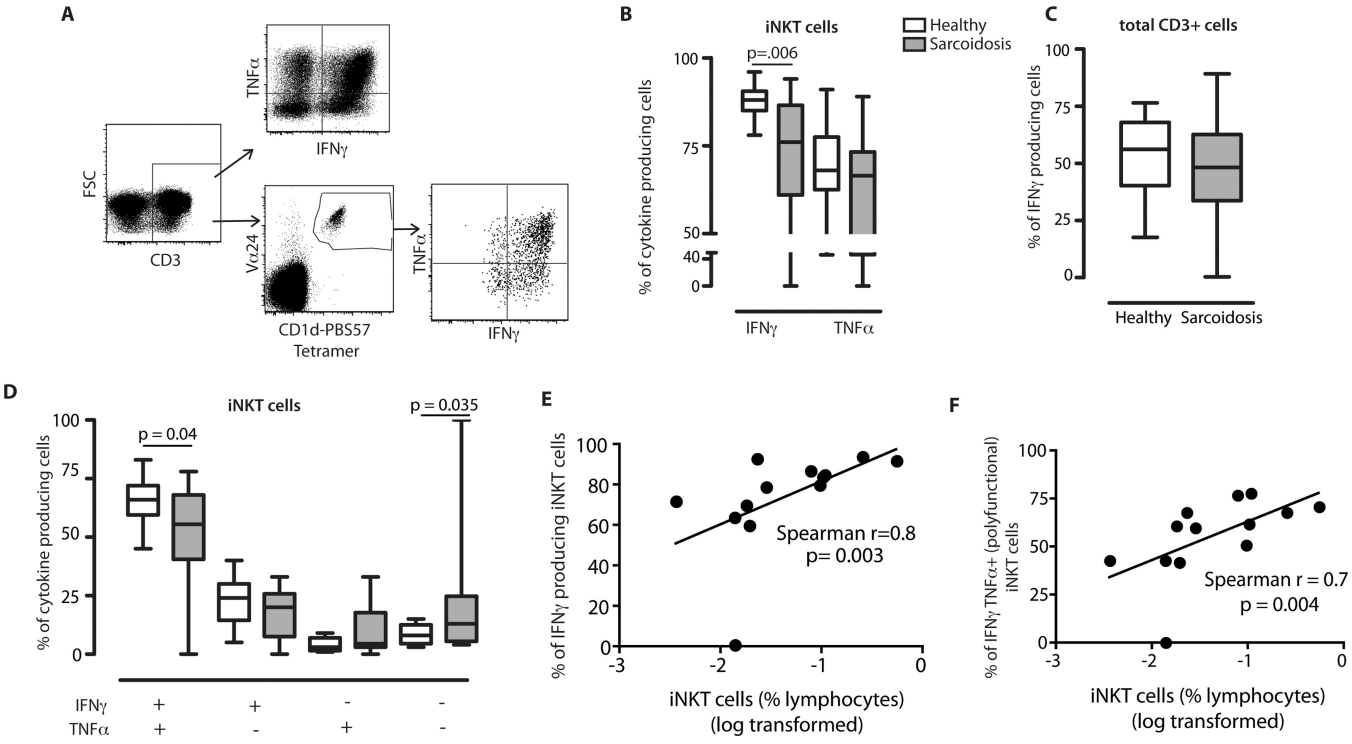

Figure 3. Functional analysis of iNKT cells in sarcoidosis

PBMC's from sarcoidosis subjects and matched controls were stimulated ex-vivo with PMA and ionomycin and subsequently stained with fluorescently labeled reagents to measure intracellular interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) in iNKT cells by flow cytometry. (A) Shown is the gating strategy to enumerate cytokine-producing iNKT cells. (B) Measurement of total IFNγ and TNFα production by iNKT cells in 16 sarcoidosis and 17 matched controls. (C) Total IFNγ production by CD3+ T cells in 16 sarcoidosis and 17 matched controls. (D) Measurement of the percentages of individual iNKT cells producing all combinations of IFNγ and TNFα in 16 sarcoidosis and 17 matched controls. (E & F) Correlation between total circulating iNKT frequencies and the percentages of (E) total IFNγ+ and (F) IFNγ+ TNFα+ dual functional iNKT cells in 13 sarcoidosis subjects. Statistical significance between groups is shown by horizontal bars displaying P values. Mann-Whitney test used for two group comparisons in B-D; Spearman correlations used for E & F. Data in B-D are displayed as box plots where boxes equal the 25th and 75th percentile range, the middle band represents the median, and the whiskers represent the minimum and maximum values.

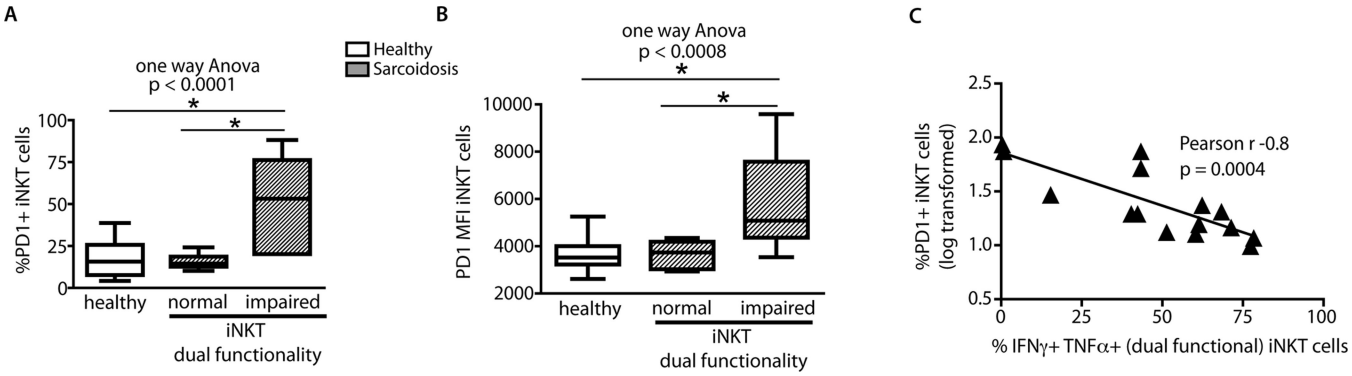

Figure 4. Relationship between PD-1 expression on iNKT cells and dual functionality in sarcoidosis subjects

PBMC's from sarcoidosis subjects and matched controls were stained with fluorescently labeled reagents (as described in Figure 1) including surface antigen markers for PD-1 and analyzed by flow cytometry. (A & B) The percentage of (A) PD-1+ iNKT cells or (B) PD-1 Mean Fluorescence Intensity (MFI) from 15 sarcoidosis subjects and 16 controls are displayed as box plots. Eight sarcoidosis subjects with IFNγ and TNFα dual functionality similar to that measured in controls samples are shown as one box plot compared to seven sarcoidosis subjects with defective dual functionality to display the difference in PD-1 staining between subjects with intact vs. defective dual functionality. Defective dual functionality was defined by the lowest percentage of dual functional iNKT cells in the control group (<45% of IFNγ and TNFα dual functional iNKT cells). (C) Linear regression plot correlating the percentage of PD-1+ iNKT cells versus the percentage of IFNγ+ TNFα+ dual functional iNKT cells in 15 sarcoidosis subjects. Statistical significance between groups is shown by horizontal bars displaying P values. One way Anova test used for three group comparisons in A & B; Pearson correlation used in C. Data in A & B are displayed as box plots where boxes equal the 25th and 75th percentile range, the middle band represents the median, and the whiskers represent the minimum and maximum values.

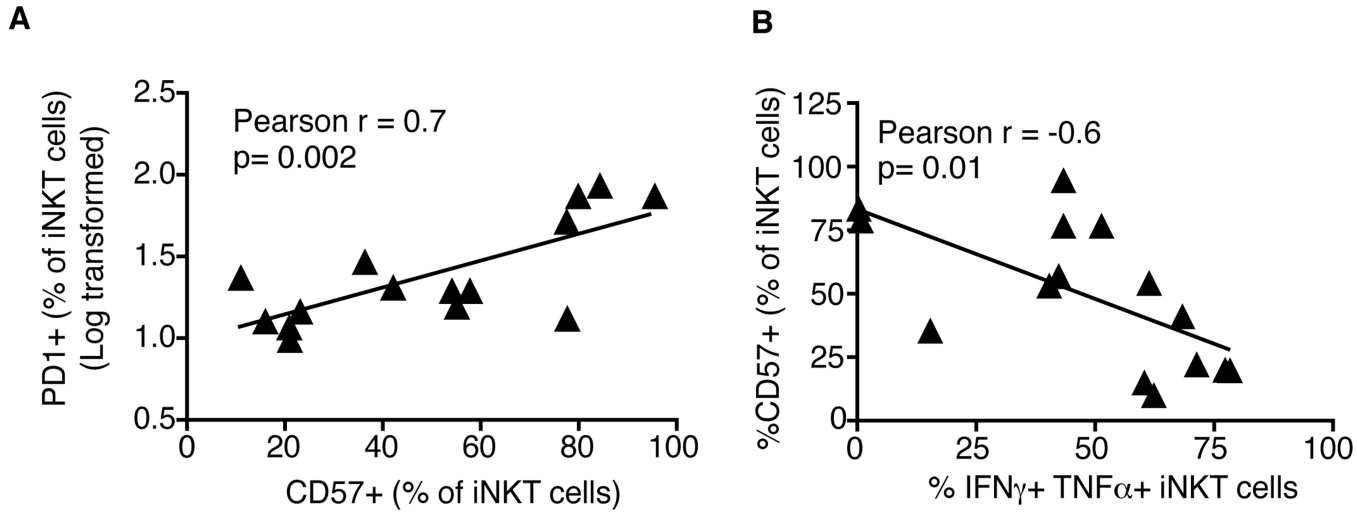

Figure 5. Correlation between CD57, PD-1, and IFNγ+ TNFα+ dual functional iNKT cells in sarcoidosis

PBMC's from sarcoidosis subjects were stimulated with PMA and ionomycin, and subsequently stained with fluorescently labeled reagents to enumerate iNKT cells including surface antigens for PD-1 and CD57 and analyzed by flow cytometry. (A) Linear regression plot correlating the percentage of PD-1+ iNKT cells and the percentage of CD57+ iNKT cells in 15 sarcoidosis subjects. (B) Linear regression plot correlating the percentage of CD57+ iNKT cells versus the percentage of IFNγ+ TNFα+ dual functional iNKT cells in 15 sarcoidosis subjects. Statistical significance of correlations is displayed in plots. Pearson correlation used to analyze data in A & B.

Table I

Clinical characteristics of control and sarcoidosis subjects

	Controls (N=33)	Sarcoidosis (N=29)
Age (± SD)	50 ± 13	52 ± 11
Gender (F/M)	23/10	21/8
Race		
Caucasian	26	22
African American	3	5
Other	4	2
Current immunosuppression use	0	10
Current smoker	0	2
Evidence of extrathoracic disease	N/A	14
Forced Expiratory Volume, 1 sec (FEV1)[a]	N/A	79% ± 19%
Forced Vital Capacity (FVC)[a]	N/A	89% ± 14%
FEV1/FVC ratio[a]	N/A	70% ± 12%
Total Lung Capacity (TLC)[a]	N/A	89% ± 12%
Diffusing Capacity (DLCO)[a]	N/A	75% ± 20%

[a]Data are presented as average values expressed as percent predicted ± Standard Deviation

Table II

Clinical characteristics by radiographic stage of disease

Total N = 29	**Stage I (N=5)**	**Stage II/III (N=13)**	**Stage IV (N=11)**
Age (± SD)	54 ± 15	49 ± 12	56 ± 10
Gender (F/M)	4/1	9/4	8/3
Race			
Caucasian	5	11	6
African American	0	1	4
Other	0	1	1
Current immunosuppression use	1	4	5
Current smoker	0	2	0
Evidence of extrathoracic disease	3	7	4
Dyspnea Score (± SD)	7 ± 5	7 ± 5	12 ± 7
Forced Expiratory Volume, 1 sec (FEV1)[a]	90% ± 16%	81% ± 20%	70% ±20%
Forced Vital Capacity (FVC)[a]	98% ± 9%	91% ± 12%	81% ± 16%
FEV1/FVC ratio[a]	73 ± 14	71 ± 12	67 ± 13
Total Lung Capacity (TLC)[a]	95% ± 14%	91% ± 11%	84% ± 14
Diffusing Capacity (DLCO)[a]	83% ± 27%	82% ± 20%	61% ± 10%

[a]Data are presented as average values expressed as percent predicted ± Standard Deviation