Reactive Oxygen Species Regulate the Inflammatory Function of NKT Cells through Promyelocytic Leukemia Zinc Finger

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Running title: ROS regulate NKT cell function through PLZF

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Abstract

Reactive oxygen species (ROS) contribute as second messengers in immune cells but little is known about their role in NKT cell function. We found that ROS levels in NKT cells dramatically increased compared to T cells in the spleen and liver but not in thymus or adipose tissues. Freshly isolated NKT cells produced ROS using primarily NADPH oxidases not mitochondria. Accordingly, NKT cells were susceptible to oxidative stress. ROS levels regulate the inflammatory function of NKT cells. ROS-high NKT cells had more NKT1 and NKT17 subsets but fewer NKT2 cells than ROS-low cells. Antioxidant treatment or activation of NKT cells resulted in reduced ROS, IFN-γ^+ and IL-17^+ cells. These characteristics are regulated by PLZF as evidenced by low ROS in NKT cells from PLZF haplo-deficient mice and highly elevated ROS in CD4 T cells from mice ectopically-expressing PLZF. Together, our study reveals that ROS regulate NKT cell function through PLZF.

Introduction

Invariant Natural Killer T (NKT) cells express highly restricted T cell receptor repertoire and share characteristics of both T cells and natural killer (NK) cells (1). In the thymus, they undergo progressive maturation after positive selection to develop into functional subsets, NKT1, NKT2 and NKT17, which are defined based on their distinct expression of transcription factors T-bet, GATA3 and RORγt together with promyelocytic leukemia zinc finger (PLZF), respectively (2). NKT cells express PLZF that is a master transcription factor in determining the innate T cell fate and indispensable for NKT cell development (3, 4). In addition, PLZF has been implicated to play a role in cell proliferation in myeloid cells (5, 6). A recent study showed that PLZF promotes hepatic gluconeogenesis (7), suggesting a plausible role of PLZF in cell metabolism.

NKT cells play a unique and important role in many immune diseases including metabolic diseases (8-11). Currently, the role of cell metabolism in controlling NKT cell functions is not well understood although metabolic factors like Vps34 (a class III PI3 kinase) (12), Fnip1 (13), autophagy (14, 15), and mTOR pathway (16, 17) have been shown to play a role in NKT cell development and function. Unlike conventional T cells that undergo antigen dependent differentiation in the periphery to become effector cells, each effector NKT cell type is generated as they develop in the thymus (18). With studies showing that the metabolic profile of T cells is responsible for defining the T cell subsets (19, 20), it remains to be investigated if NKT cell functions are also controlled by cell metabolism.

Reactive oxygen species (ROS) are byproducts of aerobic metabolism which include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH) (21). ROS mediate redox

biology and oxidative stress, and contribute to both physiological and pathological conditions (21). ROS are essential second messengers in innate and adaptive immune cells (22). In T cells, ROS levels increase upon activation and in the absence of ROS produced by mitochondria, antigen specific responses of T cells are compromised (23). However, NADPH oxidase (NOX) can be invoked in response to mitochondria produced ROS to further sustain ROS levels so as to maintain T cell activation (24).

In the current study, we investigated the role of ROS in NKT cell functions and found that the high levels of ROS in NKT cells from peripheral tissues are important for their inflammatory function. Our study also revealed that ROS levels and ROS-mediated changes of NKT cells are controlled by PLZF.

Materials and Methods

Mice

PLZF transgenic mice driven by the *cd4* promoter (4), the *lck* promoter (3) PLZF deficient mice (25), and Vα14 TCR transgenic mice (26) have been previously described. All the mice were bred and maintained under specific pathogen-free conditions at the University of Michigan animal facility and used at 8-12 weeks of age. All animal experiments were performed under protocols approved by the University of Michigan Institutional Animal Care and Use Committee.

Cell preparation and purification

Single cell suspensions were prepared from thymi, spleens and livers of mice as described earlier (17). To sort NKT and CD4 T cells, spleen cells were stained with PBS57-loaded CD1d

tetramers (NIH), and anti-TCR-β and anti-CD4 antibodies. NKT and CD4 T cells were then sorted using FACS Aria-III (BD). To sort ROS-low and ROS-high NKT cells, splenocytes were stained with DCFDA prior to surface staining.

Flow cytometry assay

The following anti-mouse antibodies from eBioscience were used: anti-TCR-β (H57-597), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-NK1.1 (PK-136), anti-CD44 (IM7, anti-CD69 (H1-2F3), anti-CD62L (MEL-14), anti-IFN-γ (XMG1.2), anti-IL-4 (11B11), anti-IL-17 (TC11-18H10), anti-T-bet (eBio4B10), anti-RORγt (AFKJS-9), anti-GATA3 (L50-823), and anti-PLZF (Mags-21F7). Dead cells were excluded by staining with 1 μg/ml propidium iodide (Sigma-Aldrich). Intracellular cytokines were measured as described earlier after stimulating the cells with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml, Sigma-Aldrich) and ionomycin (1.5 μM, Sigma-Aldrich) (17). Transcription factors were stained was using the Foxp3/transcription factor staining kit (eBioscience) Data were acquired on a FACS Canto II (BD) and analyzed using FlowJo (TreeStar software ver. 9.9).

ROS detection

To measure total ROS, cells (1 x 10^6/ml FACS tube) were incubated in RPMI media containing 10% fetal bovine serum (FBS) with 1 μM DCFDA (Invitrogen) for 30 minutes at 37˚C before staining with surface antibodies for flow cytometry. To measure ROS produced by mitochondria, 2.5 μM MitoSox (Invitrogen) was used.

Apoptosis assay

1×10^6 surface-stained splenocytes or hepatocytes were analyzed for apoptosis after Annexin V staining according to the manufacturer's instructions (BD).

T cell activation

3×10^5 sorted NKT or CD4 T cells were cultured with soluble α-Galcer (100 ng/ml) or plate bound anti-CD3 antibody (5 μg/ml) and soluble anti-CD28 antibody (2 μg/ml), respectively, per well of a 96-well tissue culture plate in RPMI medium supplemented with 10% FBS with for 3 days on 37˚C with 5% CO_2.

qRT-PCR

Total RNA was isolated from sorted NKT and CD4 T cells from C57BL/6 spleens using RNeasy Plus Mini Kit (QIAGEN) following the manufacturer's instructions. cDNA was synthesized using the RT2 First Strand Kit (QIAGEN). Predesigned primer sets for genes Nox1, Nox2, Nox3, Nox4, Duox1 and Duox2 were purchased from IDT. Either β-actin or GAPDH were used as internal controls. The inverse log of the delta delta-CT was calculated.

Statistical analysis

Data for all experiments were analyzed with Prism software (GraphPad Prism ver. 6, San Diego, CA). Unpaired Student's t-test was used for comparison of experimental groups. Correlation coefficient (r^2) was calculated by fitting the data to a linear regression curve.

Results and Discussion

Peripheral NKT cells have greatly elevated ROS

To examine if NKT cells show different metabolism, we measured ROS levels as a parameter of cell metabolism using 2',7'–dichlorofluorescin diacetate (DCFDA), a fluorogenic dye that detects hydroxyl, peroxyl and other ROS activity within live cells (27). ROS levels of NKT cells were similar to CD4 or CD8 single positive cells in the thymus but greatly increased in the spleen and liver with a mixture of ROS high and low cells (Fig. 1A). We further characterized the NKT cell population using CD44 and CD62L that are markers of NKT cell maturity and activation (4). CD44-high or CD62L-low splenic NKT cells had higher levels of ROS, while NK1.1 expression did not correlate with ROS levels (Fig. 1B). Neither $CD44^+$ effector CD4 T cells nor NK cells from the spleen and liver showed higher ROS indicating that ROS in NKT do not associate with the effector phenotype or being innate type cells (Fig. 1C). Hepatic NKT cells showed the same pattern as splenic NKT cells (Supplemental Fig. 1A). Therefore, highly elevated ROS are a unique property of NKT cells.

ROS are mainly produced by mitochondria and NADPH oxidases (NOX) (28). We first measured ROS produced by mitochondria (mtROS) using MitoSox (14). Unlike high ROS measured by DCFDA, only a small fraction of NKT cells produced mtROS (Fig. 1D). Both the frequency of mtROS-producing NKT cells and the amount of mtROS as measured by MFI were lower than CD4 T cells indicating that ROS in NKT cells are not produced by mitochondria in the steady state. To determine if ROS were produced by NOX, we compared the gene expression of members of the NOX family - Nox1, Nox2, Nox3, Nox4, dual oxidases 1 and 2 (Duox1 and Duox2) - in NKT and CD4 T cells using qPCR. CD4 T cells predominantly expressed Nox2,

whereas NKT cells expressed both Nox1 and Nox2 (Fig. 1E). Nox3, Nox4 and DUOXs mRNA levels were below detection or very low (data not shown). Together, ROS in NKT cells are likely generated by NOX1 and NOX2.

ROS high NKT cells are susceptible to oxidative stress but not ER stress

Because excessive ROS can cause damage and pathology, we asked whether NKT cells are susceptible to oxidative stress by treating the cells with hydrogen peroxide (H_2O_2). We treated total splenocytes with a range of H_2O_2 concentrations (10 μM to 150 μM) and measured apoptosis to compare responses. NKT cells showed an increase in apoptotic cell death at 30 μM concentration, whereas a higher dose of H_2O_2 was required to induce cell death of CD4 T cells (Supplemental Fig. 1B, Fig. 2*A*). The data were consistent with the reduced NKT cell frequencies and lower ROS in remaining live cells (Fig. 2B, Supplemental Fig. 1C). Hepatic cells showed the same pattern (Supplemental Fig. 1D). To probe if cell death can be prevented by reducing ROS, we pre-treated cells with antioxidants N-acetyl-cysteine (NAC) or diphenylene-iodonium (DPI) prior to the addition of H_2O_2. Both NAC and DPI pre-treatments reduced NKT cell death caused by H_2O_2 (Fig. 2C, Supplemental Fig. 1E). However, MitoQ, a mitochondria-targeted antioxidant that inhibits mitochondrial ROS (29), failed to protect the oxidative damage caused by H_2O_2 (Fig. 2C, Supplemental Fig. 1E), indicating that mtROS do not induce oxidative stress in NKT cells. Next, we treated NKT and CD4 T cells with different concentrations of thapsigargin that inhibits endoplasmic reticulum (ER) Ca_2^+ ATPase and thus induces stress (30). In contrast to H_2O_2 treatment, NKT cells were highly tolerant to thapsigargin-induced ER stress, whereas CD4 T cells showed cell death at high concentrations (Fig. 2D).

Amount of ROS correlates with the function of NKT cells

Because effector functions of T cells are regulated by ROS (23, 31), we asked if ROS also control the function of NKT cells. The ROS levels decreased upon pre-treatment with 20 μM DPI in NKT cells but not in CD4 T cells from the spleen (Supplemental Fig. 1F). Upon stimulation with PMA and Ionomycin, DPI-treated NKT cells had more IL-4$^+$ cells but fewer IFN-γ$^+$ and IL-17$^+$ cells than without DPI treatment (Fig. 3A, top panel). CD4 T cells did not show a measurable change with DPI treatment except an increase in IFN-γ$^+$ cells (Fig. 3A, bottom panel). To rule out that DPI treatment might have caused a selective loss or gain of an effector cell type, we sorted ROS-high and ROS-low NKT cells and examined for the expression of T-bet, GATA3 and RORγt, the transcription factors responsible for NKT1, NKT2, and NKT17 cells, respectively. ROS-high NKT cells had more NKT1 but fewer NKT2 than ROS-low cells (Fig. 3B). NKT17 cells showed a similar trend as the cytokine data, albeit statistically insignificant. Together, both the cytokine data and the functional subset analyses suggest that the amount of ROS correlates with the NKT cell function.

It is reported that CD4 T cells increase mtROS production upon activation (23) and therefore we asked if NKT cells also produce mtROS upon activation. NKT cells from Vα14 transgenic (Vα14tg) mice were used to obtain a sufficient number of NKT cells for these assays. ROS levels of Vα14tg NKT cells were comparable to those from C57BL/6 mice (Supplemental Fig. 1G). Splenic Vα14 NKT and CD4 T cells from C57BL/6 mice were sorted and stimulated with α-Galcer or anti-CD3 plus anti-CD28, respectively for 3 days. As expected, CD4 T cells increased both total ROS and mtROS upon activation (Supplemental Fig. 1H, Fig. 3C). In NKT

cells however total ROS levels were reduced despite an increase of mtROS (Supplemental Fig. 1H, Fig. 3C). Increase of CD69 expression indicates that both cell populations were activated (Supplemental Fig. 1H). CD62L-low and -high NKT cells exhibited decreased and increased ROS, respectively, after stimulation (Supplemental Fig. 1I). NKT cells showed similar response when stimulated with α-Galcer or with anti-CD3 and anti-CD28 antibodies (data not shown). CD62L-high but not CD62L-low CD4 T cells also showed an increase in ROS levels. We showed that low ROS by DPI pretreatment changed cytokine expression (Fig. 3A). Therefore, we tested if cytokine expression in activated NKT cells would be similar to that of DPI pretreated NKT cells due to lower ROS. As shown in Figure 3D, activated NKT cells showed lower IFN-γ^+ and IL-17^+ but more IL-4^+ cells than before activation. Activated CD4 T cells also showed a similar response to those treated with DPI (Supplemental Fig. 1J).

PLZF regulates the ROS levels

Next, we asked if PLZF, expressed by NKT cells but not CD4 T cells, is involved in controlling the ROS levels. To answer this, we examined γδ T cells that are known to express PLZF (32, 33). We found that splenic and hepatic γδ T cells had low and high ROS respectively (Fig 4A). To further investigate the role of PLZF in ROS, we compared the amounts of ROS with PLZF expression levels using CD4, NKT and γδ T cells from C57BL/6 mice. As shown in Figure 4B, PLZF and ROS showed a positive correlation. To further confirm the direct role of PLZF in ROS regulation, we employed two complementary approaches. First, we examined NKT cells from PLZF haplo-deficient ($PLZF^{+/-}$) mice which express less PLZF. Total deficiency of PLZF is not informative because PLZF is required for NKT cell development (3, 4). The ROS levels were greatly decreased in NKT but not CD4 T cells from $PLZF^{+/-}$ mice compared to the wild type

(WT) cells (Supplemental Fig. 2A, Fig. 4C). Consequently, PLZF$^{+/-}$ NKT cells were more tolerant to oxidative stress than WT NKT cells (Supplemental Fig. 2B, Fig. 4D). Next, we examined CD4 T cells from PLZF transgenic (PLZF^{Tg}) mice and found that ROS levels were greatly increased in CD4 T cells from PLZF^{Tg} mice compared to WT CD4 T cells, from both spleen and liver (Supplemental Fig. 2C, Fig. 4E) and these cells became sensitive to a low concentration of H_2O_2 treatment (Supplemental Fig. 2D, Fig. 4F). NKT cells were comparable between WT and PLZF^{Tg} mice (Supplemental Fig. 2C, Fig. 4E). The induction of ROS in PLZF^{Tg} CD4 T cells could also be seen from another PLZF transgenic mouse line (Supplemental Fig. 2E). Similar to NKT cells, activation of PLZF^{Tg} CD4 T cells resulted in the reduction of ROS (Supplemental Fig. 2F, Fig. 4G) and decreased IFN-γ and IL-17 expressing cells but not IL-4^{+} cells (Fig. 4H). Studies reported that visceral adipose tissues (VAT) NKT cells do not express PLZF and exhibit the regulatory function (34, 35). In line with this, we observed lower levels of ROS in VAT NKT cells compared to those from spleen or liver (Supplemental Fig. 2G). Lastly, we compared PLZF expression in sorted ROS-high and ROS-low NKT cells from C57BL/6 that were used in Figure 3B. ROS-high NKT cells expressed higher PLZF than ROS-low cells (Supplemental Fig. 2H). Taken all together, PLZF regulates ROS levels, which in turn controls inflammatory or regulatory function of NKT cells.

Compared to CD4 T cells, the increase of ROS in the peripheral NKT cells, but not in thymic NKT cells does not seem to be due to TCR-mediated signaling because NKT cells receive very weak, if any, TCR stimulation in the steady-state (36). In addition, we show that α–GalCer stimulation reduces ROS. Therefore, it is unlikely that NKT cells are continuously undergoing TCR-mediated activation leading to the increase of ROS. The primary source of ROS in NKT

cells seems to be NADPH oxidases and NKT cells may have a higher capacity to produce ROS using the NOX system. However, upon activation via TCR signaling, in contrast to CD4 T cells, NKT cells down-regulate total ROS despite increased mtROS. Perhaps ROS produced by the NOX system in the steady state of NKT cells is reduced and switched to mitochondria production of ROS. Although NKT cells are effector cells, the high level of ROS is not simply due to the effector phenotype either, because CD44-high CD4 T effector cells did not have as high ROS as NKT cells. It is not clear why NKT cells make more ROS in the steady state because the consequence of high ROS is likely detrimental to NKT cell survival. To prevent spontaneous cell death *in vivo*, NKT cells may have a built-in mechanism to cope with high ROS.

Reducing ROS by either TCR-mediated activation or antioxidant treatment *in vitro* decreased frequencies of NKT cells expressing IFN-γ or IL-17 but increased IL-4^+ cells. The role of ROS in NKT cell function is further strengthened by the results that more inflammatory (NKT1 and NKT17) cells were found among ROS-high cells, whereas NKT2 cells are more abundant in ROS-low cells. It is possible that the expression of T-bet, GATA3 and RORγt is regulated by ROS-mediated signaling. Alternatively, the functional difference may reflect the heterogeneity of glycolytic potential of NKT cells. It is known that both Th1 and Th17 are highly glycolytic, which induces ROS generation (37).

The current study provided several evidence demonstrating PLZF as a regulator of ROS and that the level of ROS correlates with the inflammatory and regulatory function of NKT cells. PLZF might act by regulating the expression of genes that are involved in antioxidant pathways or by directly regulating the metabolic pathways. In depth studies of mechanisms for PLZF-mediated

ROS regulation and NKT cell metabolism are warranted to have a better understanding of NKT cell-mediated inflammatory and regulatory immune responses under a different local environment *in vivo*. However, these studies require an appropriate tool that allows deleting PLZF after NKT cell development in a tissue type specific manner. In addition, it is challenging to explore the role of ROS in NKT function *in vivo*. Use of pro-oxidants is toxic and administration of antioxidants would affect multiple cell types complicating the interpretation of the results. It should be noted that although clinical studies showed the beneficial effects of antioxidants to prevent and treat metabolic liver diseases, the therapeutic effect of antioxidants on immune cells has not yet been established (38-40). Without long-term longitudinal studies, it is difficult to assess the clinical effect of antioxidant.

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Competing financial interests

The authors declare no competing financial interests.

References

1. Bendelac, A., M. N. Rivera, S. H. Park, and J. H. Roark. 1997. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annual review of immunology* 15: 535-562.
2. Lee, Y. J., K. L. Holzapfel, J. Zhu, S. C. Jameson, and K. A. Hogquist. 2013. Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells. *Nature Immunology* 14: 1146-1154.
3. Kovalovsky, D., O. U. Uche, S. Eladad, R. M. Hobbs, W. Yi, E. Alonzo, K. Chua, M. Eidson, H. J. Kim, J. S. Im, P. P. Pandolfi, and D. B. Sant'Angelo. 2008. The BTB-zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nature Immunology* 9: 1055-1064.
4. Savage, A. K., M. G. Constantinides, J. Han, D. Picard, E. Martin, B. Li, O. Lantz, and A. Bendelac. 2008. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity* 29: 391-403.
5. Yeyati, P. L., R. Shaknovich, S. Boterashvili, J. Li, H. J. Ball, S. Waxman, K. Nason-Burchenal, E. Dmitrovsky, A. Zelent, and J. D. Licht. 1999. Leukemia translocation protein PLZF inhibits cell growth and expression of cyclin A. *Oncogene* 18: 925-934.
6. Shaknovich, R., P. L. Yeyati, S. Ivins, A. Melnick, C. Lempert, S. Waxman, A. Zelent, and J. D. Licht. 1998. The promyelocytic leukemia zinc finger protein affects myeloid cell growth, differentiation, and apoptosis. *Molecular and cellular biology* 18: 5533-5545.
7. Chen, S., J. Qian, X. Shi, T. Gao, T. Liang, and C. Liu. 2014. Control of hepatic gluconeogenesis by the promyelocytic leukemia zinc finger protein. *Mol Endocrinol* 28: 1987-1998.
8. Takeda, K., Y. Hayakawa, L. Van Kaer, H. Matsuda, H. Yagita, and K. Okumura. 2000. Critical contribution of liver natural killer T cells to a murine model of hepatitis. *Proceedings of the National Academy of Sciences of the United States of America* 97: 5498-5503.
9. Lynch, L., M. Nowak, B. Varghese, J. Clark, A. E. Hogan, V. Toxavidis, S. P. Balk, D. O'Shea, C. O'Farrelly, and M. A. Exley. 2012. Adipose tissue invariant NKT cells protect against diet-induced obesity and metabolic disorder through regulatory cytokine production. *Immunity* 37: 574-587.
10. Schipper, H. S., M. Rakhshandehroo, S. F. van de Graaf, K. Venken, A. Koppen, R. Stienstra, S. Prop, J. Meerding, N. Hamers, G. Besra, L. Boon, E. E. Nieuwenhuis, D. Elewaut, B. Prakken, S. Kersten, M. Boes, and E. Kalkhoven. 2012. Natural killer T cells in adipose tissue prevent insulin resistance. *The Journal of clinical investigation* 122: 3343-3354.
11. Wu, L., V. V. Parekh, C. L. Gabriel, D. P. Bracy, P. A. Marks-Shulman, R. A. Tamboli, S. Kim, Y. V. Mendez-Fernandez, G. S. Besra, J. P. Lomenick, B. Williams, D. H. Wasserman, and L. Van Kaer. 2012. Activation of invariant natural killer T cells by lipid excess promotes tissue inflammation, insulin resistance, and hepatic steatosis in obese mice. *Proceedings of the National Academy of Sciences of the United States of America* 109: E1143-1152.
12. Parekh, V. V., L. Wu, K. L. Boyd, J. A. Williams, J. A. Gaddy, D. Olivares-Villagomez, T. L. Cover, W. X. Zong, J. Zhang, and L. Van Kaer. 2013. Impaired autophagy, defective T cell homeostasis, and a wasting syndrome in mice with a T cell-specific deletion of Vps34. *Journal of Immunology* 190: 5086-5101.

13. Park, H., M. Tsang, B. M. Iritani, and M. J. Bevan. 2014. Metabolic regulator Fnip1 is crucial for iNKT lymphocyte development. *Proceedings of the National Academy of Sciences of the United States of America* 111: 7066-7071.
14. Salio, M., D. J. Puleston, T. S. Mathan, D. Shepherd, A. J. Stranks, E. Adamopoulou, N. Veerapen, G. S. Besra, G. A. Hollander, A. K. Simon, and V. Cerundolo. 2014. Essential role for autophagy during invariant NKT cell development. *Proceedings of the National Academy of Sciences of the United States of America* 111: E5678-5687.
15. Pei, B., M. Zhao, B. C. Miller, J. L. Vela, M. W. Bruinsma, H. W. Virgin, and M. Kronenberg. 2015. Invariant NKT cells require autophagy to coordinate proliferation and survival signals during differentiation. *J Immunol* 194: 5872-5884.
16. Zhang, L., B. O. Tschumi, S. Corgnac, M. A. Ruegg, M. N. Hall, J. P. Mach, P. Romero, and A. Donda. 2014. Mammalian target of rapamycin complex 1 orchestrates invariant NKT cell differentiation and effector function. *Journal of Immunology* 193: 1759-1765.
17. Prevot, N., K. Pyaram, E. Bischoff, J. M. Sen, J. D. Powell, and C. H. Chang. 2015. Mammalian Target of Rapamycin Complex 2 Regulates Invariant NKT Cell Development and Function Independent of Promyelocytic Leukemia Zinc-Finger. *Journal of Immunology* 194: 223-230.
18. Bendelac, A., P. B. Savage, and L. Teyton. 2007. The biology of NKT cells. *Annual review of immunology* 25: 297-336.
19. Chang, C. H., J. D. Curtis, L. B. Maggi, Jr., B. Faubert, A. V. Villarino, D. O'Sullivan, S. C. Huang, G. J. van der Windt, J. Blagih, J. Qiu, J. D. Weber, E. J. Pearce, R. G. Jones, and E. L. Pearce. 2013. Posttranscriptional control of T cell effector function by aerobic glycolysis. *Cell* 153: 1239-1251.
20. MacIver, N. J., R. D. Michalek, and J. C. Rathmell. 2013. Metabolic regulation of T lymphocytes. *Annual review of immunology* 31: 259-283.
21. Schieber, M., and N. S. Chandel. 2014. ROS function in redox signaling and oxidative stress. *Current biology : CB* 24: R453-462.
22. West, A. P., G. S. Shadel, and S. Ghosh. 2011. Mitochondria in innate immune responses. *Nature reviews. Immunology* 11: 389-402.
23. Sena, L. A., S. Li, A. Jairaman, M. Prakriya, T. Ezponda, D. A. Hildeman, C. R. Wang, P. T. Schumacker, J. D. Licht, H. Perlman, P. J. Bryce, and N. S. Chandel. 2013. Mitochondria are required for antigen-specific T cell activation through reactive oxygen species signaling. *Immunity* 38: 225-236.
24. Kaminski, M., M. Kiessling, D. Suss, P. H. Krammer, and K. Gulow. 2007. Novel role for mitochondria: protein kinase Ctheta-dependent oxidative signaling organelles in activation-induced T-cell death. *Molecular and cellular biology* 27: 3625-3639.
25. Barna, M., N. Hawe, L. Niswander, and P. P. Pandolfi. 2000. Plzf regulates limb and axial skeletal patterning. *Nature genetics* 25: 166-172.
26. Taniguchi, M., H. Koseki, T. Tokuhisa, K. Masuda, H. Sato, E. Kondo, T. Kawano, J. Cui, A. Perkes, S. Koyasu, and Y. Makino. 1996. Essential requirement of an invariant V alpha 14 T cell antigen receptor expression in the development of natural killer T cells. *Proceedings of the National Academy of Sciences of the United States of America* 93: 11025-11028.
27. LeBel, C. P., H. Ischiropoulos, and S. C. Bondy. 1992. Evaluation of the probe 2',7'-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. *Chemical research in toxicology* 5: 227-231.
28. Panday, A., M. K. Sahoo, D. Osorio, and S. Batra. 2015. NADPH oxidases: an overview from structure to innate immunity-associated pathologies. *Cellular & molecular immunology* 12: 5-23.
29. Kelso, G. F., C. M. Porteous, C. V. Coulter, G. Hughes, W. K. Porteous, E. C. Ledgerwood, R. A. Smith, and M. P. Murphy. 2001. Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and antiapoptotic properties. *J Biol Chem* 276: 4588-4596.

30. Lytton, J., M. Westlin, and M. R. Hanley. 1991. Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. *The Journal of biological chemistry* 266: 17067-17071.
31. Kaminski, M. M., S. W. Sauer, C. D. Klemke, D. Suss, J. G. Okun, P. H. Krammer, and K. Gulow. 2010. Mitochondrial reactive oxygen species control T cell activation by regulating IL-2 and IL-4 expression: mechanism of ciprofloxacin-mediated immunosuppression. *J Immunol* 184: 4827-4841.
32. Alonzo, E. S., and D. B. Sant'Angelot. 2011. Development of PLZF-expressing innate T cells. *Current Opinion in Immunology* 23: 220-227.
33. Kreslavsky, T., A. K. Savage, R. Hobbs, F. Gounari, R. Bronson, P. Pereira, P. P. Pandolfi, A. Bendelac, and H. von Boehmer. 2009. TCR-inducible PLZF transcription factor required for innate phenotype of a subset of gamma delta T cells with restricted TCR diversity. *Proceedings of the National Academy of Sciences of the United States of America* 106: 12453-12458.
34. Lynch, L., X. Michelet, S. Zhang, P. J. Brennan, A. Moseman, C. Lester, G. Besra, E. E. Vomhof-Dekrey, M. Tighe, H. F. Koay, D. I. Godfrey, E. A. Leadbetter, D. B. Sant'Angelo, U. von Andrian, and M. B. Brenner. 2015. Regulatory iNKT cells lack expression of the transcription factor PLZF and control the homeostasis of T(reg) cells and macrophages in adipose tissue. *Nature Immunology* 16: 85-95.
35. Vieth, J. A., J. Das, F. M. Ranaivoson, D. Comoletti, L. K. Denzin, and D. B. Sant'Angelo. 2017. TCRalpha-TCRbeta pairing controls recognition of CD1d and directs the development of adipose NKT cells. *Nat Immunol* 18: 36-44.
36. Moran, A. E., K. L. Holzapfel, Y. Xing, N. R. Cunningham, J. S. Maltzman, J. Punt, and K. A. Hogquist. 2011. T cell receptor signal strength in T-reg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *Journal of Experimental Medicine* 208: 1279-1289.
37. Michalek, R. D., V. A. Gerriets, S. R. Jacobs, A. N. Macintyre, N. J. MacIver, E. F. Mason, S. A. Sullivan, A. G. Nichols, and J. C. Rathmell. 2011. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. *Journal of Immunology* 186: 3299-3303.
38. Sanyal, A. J., N. Chalasani, K. V. Kowdley, A. McCullough, A. M. Diehl, N. M. Bass, B. A. Neuschwander-Tetri, J. E. Lavine, J. Tonascia, A. Unalp, M. Van Natta, J. Clark, E. M. Brunt, D. E. Kleiner, J. H. Hoofnagle, and P. R. Robuck. 2010. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *The New England journal of medicine* 362: 1675-1685.
39. Melhem, A., M. Stern, O. Shibolet, E. Israeli, Z. Ackerman, O. Pappo, N. Hemed, M. Rowe, H. Ohana, G. Zabrecky, R. Cohen, and Y. Ilan. 2005. Treatment of chronic hepatitis C virus infection via antioxidants: results of a phase I clinical trial. *J Clin Gastroenterol* 39: 737-742.
40. Phillips, M., H. Curtis, B. Portmann, N. Donaldson, A. Bomford, and J. O'Grady. 2006. Antioxidants versus corticosteroids in the treatment of severe alcoholic hepatitis--a randomised clinical trial. *J Hepatol* 44: 784-790.

Figure legends

FIGURE 1. Study of ROS levels in NKT and CD4 T cells. (**A**) Freshly isolated thymocytes, splenocytes and liver lymphocytes were compared for the amount of ROS using 2',7'-dichlorofluorescin diacetate (DCFDA) as described in the Methods. N=8 mice per group. (**B**) Expression level of CD44, CD62L, and NK1.1 was used to compare the amount of ROS in two subsets of splenic NKT cells. N=4 mice per group. (**C**) NKT, $CD44^+$ and $CD44^-$ CD4 T cells, and NK cells from spleen were compared for the levels of ROS. N=6 mice per group. (**D**) NKT and CD4 T cells from total splenocytes were compared for mitochondria-produced ROS using MitoSox. N=3 mice per group. (**E**) Gene expression of the members of the Nox gene family was assessed from sorted splenic NKT and CD4 T cells using qPCR. N=4 mice per group. Gene expression was normalized to either β–actin or GAPDH. Error bars represent the mean ± SEM. **p <0.01; ***p<0.001; ****p<0.0001.

FIGURE 2. NKT cells are susceptible to oxidative stress. (**A**) Total splenocytes were treated with H_2O_2 for 30 minutes at indicated concentrations and then measured for apoptosis. The graph illustrates the summary of % of Annexin V^+ cells from 6 mice per group. (**B**) Total splenocytes were treated without or with 30 μM H_2O_2 for 30 minutes and examined for the % of NKT cells. N=8 mice per group. (**C**) Total lymphocytes from spleen and liver were pre-treated with 15 mM

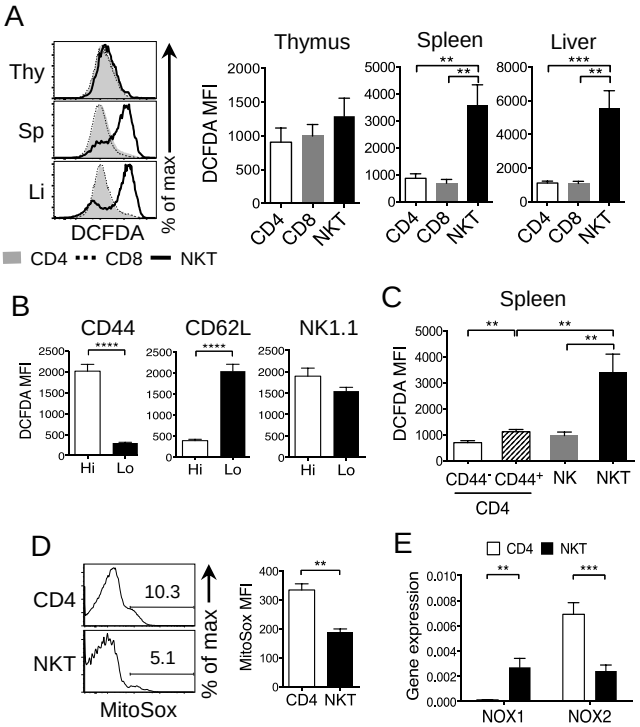
NAC or 20 μM DPI for 30 minutes, or 100 nM MitoQ before treating with H_2O_2 as in (B). N=6 mice per group of NAC and DPI and N=3 mice per group of MitoQ treatment. (**D**) Total splenocytes were treated with Thapsigargin for 30 minutes at indicated concentrations before measuring apoptosis. N=4 mice per group. Error bars represent the mean ± SEM. *p<0.05; **p <0.01; ****p<0.0001.

FIGURE 3. Regulation of cytokine expression by ROS. (**A**) Total splenocytes from C57BL/6 mice were pre-treated with 20 μM DPI for 30 minutes and then stimulated with PMA and Ionomycin to measure cytokine expression. N=6 mice per group. (**B**) ROS-low and ROS-high NKT cells were sorted from C57BL/6 mice spleens and analyzed for the expression of indicated transcription factors. N=4 mice per group. (**C**) NKT and CD4 T cells were sorted from Vα14 and C57BL/6 mice spleens, respectively, followed by stimulation for 3 days as mentioned in Materials and Methods section. Cells at day 0 and 3 were analyzed to measure total ROS (DCFDA) and mtROS (MitoSox). N=6 mice per group. (**D**). The NKT cells from (C) were re-stimulated with PMA and Ionomycin to measure cytokine expression. N=5 mice per group. Error bars represent the mean ± SEM. *p<0.05; **p <0.01; ***p<0.001; ****p<0.0001.

FIGURE 4. PLZF regulates the level of ROS. (**A**) Total lymphocytes from spleen and liver of C57BL/6 mice were used to measure ROS and PLZF levels of CD4 T, NKT and γδ T cells. (**B**) The graph showing the correlation between DCFDA and PLZF levels of CD4 T, NKT and γδ T cells. N=4 (spleen) or N=3 (liver) mice per group (**C**) NKT and CD4 T cells from spleen and liver of $PLZF^{+/-}$ mice and their WT litter mates were examined for ROS. N=5 mice per group. (**D**) Splenic NKT cells from $PLZF^{+/-}$ and $PLZF^{+/+}$ were measured for apoptosis after 30 μM H_2O_2

treatment. N=5 mice per group. (**E**) CD4 T cells from spleen and liver of PLZF^{Tg} (Tg) mice and their litter mates (WT) were compared for ROS. N=5 mice per group. (**F**) Splenic CD4 T cells from Tg and WT mice were measured for apoptosis as in (C). N=5 mice per group. (**G, H**) Enriched splenic PLZF^{Tg} CD4 T cells were stimulated for 3 days as in Figure 3C to measure ROS (G) and cytokine expression (H). N=4 mice per group. Error bars represent the mean ± SEM. *$p<0.05$; **$p<0.01$; ****$p<0.0001$.

FIGURE 1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Thy
Sp
Li
DCFDA
% of max
CD4 CD8 NKT
Thymus
Spleen
Liver
DCFDA MFI
CD4
CD8
NKT
B
CD44
CD62L
NK1.1
DCFDA MFI
Hi
Lo
C
Spleen
DCFDA MFI
CD44⁻ CD44⁺
NK
NKT
CD4
D
CD4
NKT
10.3
5.1
MitoSox
% of max
MitoSox MFI
CD4 NKT
E
CD4 NKT
Gene expression
NOX1
NOX2

FIGURE 2

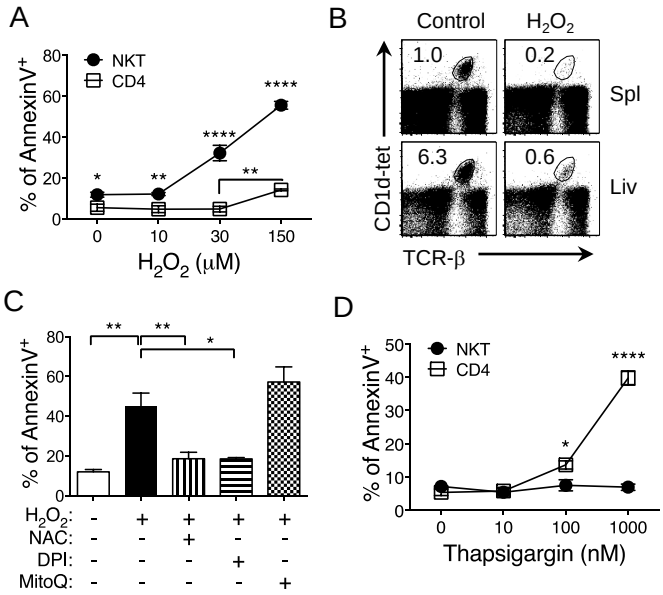

A
NKT
CD4
% of AnnexinV+
80
60
40
20
0
*
**
****
**
****
0
10
30
150
H2O2 (μM)
B
Control
H2O2
1.0
0.2
Spl
6.3
0.6
Liv
CD1d-tet
TCR-β
C
% of AnnexinV+
80
60
40
20
0
**
**
*
H2O2: - + + + +
NAC: - - + - -
DPI: - - - + -
MitoQ: - - - - +
D
NKT
CD4
% of AnnexinV+
50
40
30
20
10
0
*
****
0
10
100
1000
Thapsigargin (nM)

FIGURE 3

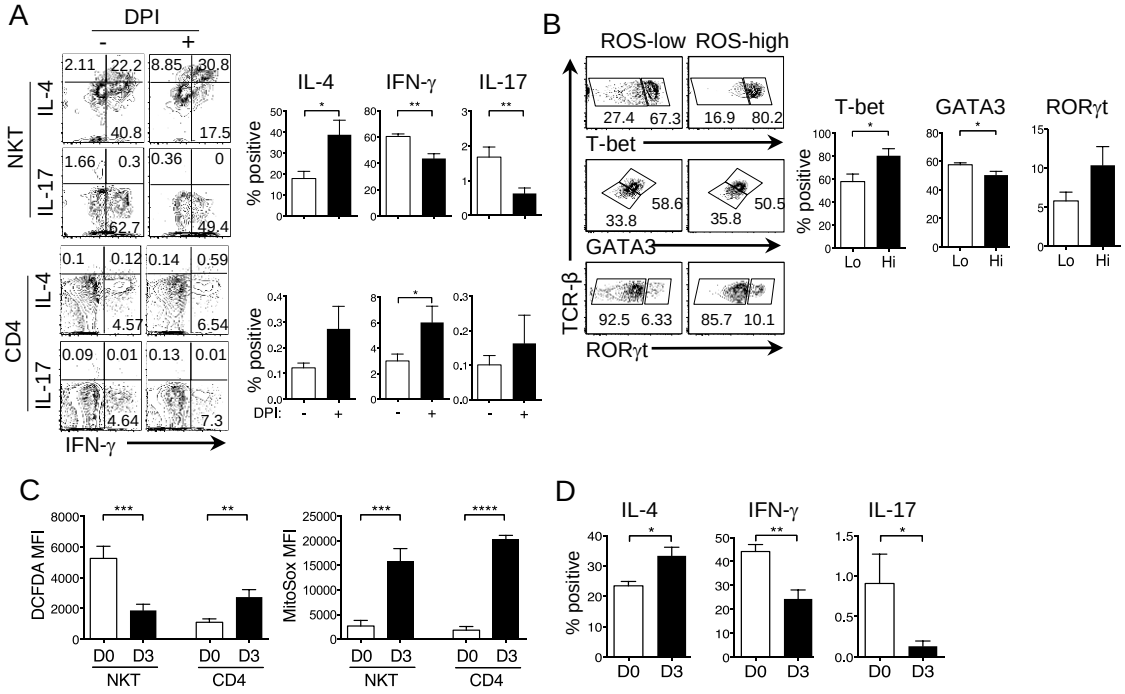
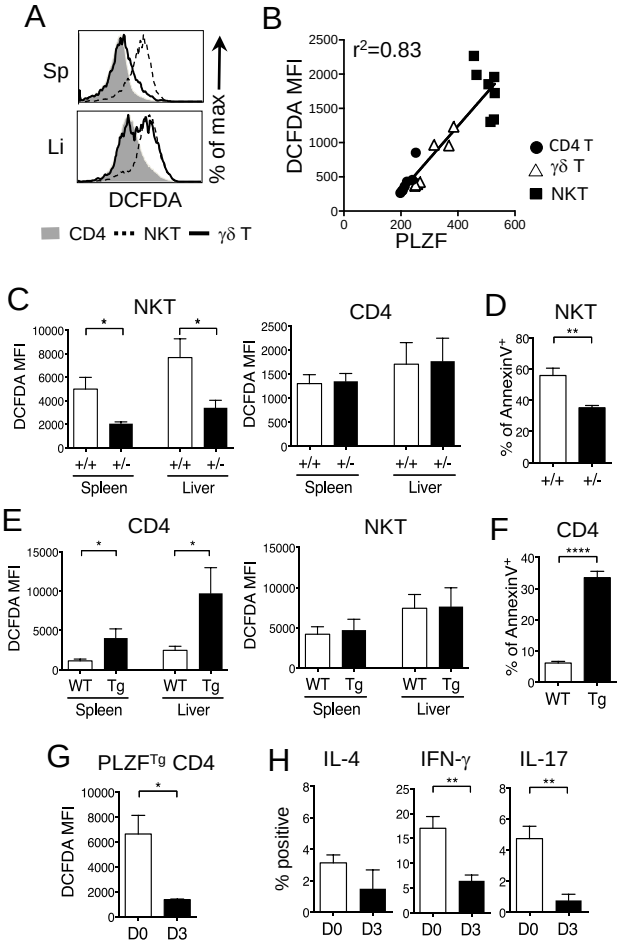

A
DPI
-
+
NKT
IL-4
IL-17
CD4
IFN-γ
% positive
DPI:
B
ROS-low
ROS-high
T-bet
GATA3
TCR-β
RORγt
Lo
Hi
C
DCFDA MFI
MitoSox MFI
D0
D3
NKT
CD4
D
IL-4
IFN-γ
IL-17

FIGURE 4


A
Sp
Li
% of max
DCFDA
CD4 NKT γδ T
B
r2=0.83
DCFDA MFI
PLZF
CD4 T
γδ T
NKT
C
NKT
CD4
DCFDA MFI
+/+ +/-
Spleen
Liver
D
NKT
% of AnnexinV+
E
CD4
NKT
WT Tg
F
CD4
G
PLZFTg CD4
D0 D3
H
IL-4
IFN-γ
IL-17
% positive