(O)

2020　2023

Elucidation of Natural Killer T cell development in a mice model of rheumatoid arthritis

Diez, Diego

3,400,000

BALB/c WT　　SKG　　　　　　　　　　　NKT
27
WT　　NKT

NKT　　2

%

NKT cells are important regulators of immune responses that differentiate into subtypes with potential opposing effects in health. Understanding the mechanisms regulating NKT differentiation and their relationship to disease can help develop improved therapies.

We have performed single cell sequencing of the transcriptome, immune repertoire, and proteome for twenty-seven selected proteins of developing NKT cells from the thymus of BALB/c WT and SKG mice. Bioinformatics analysis including clustering and trajectory analysis identified cell states compatible with current knowledge of NKT development in WT mice. Identification of genes and proteins changing along differentiation trajectories found a smooth transition in expression profiles. Analysis of the immune repertoire identified 2% of NKT harbor a non-canonical alpha chain, but identical beta chain gene usage. This suggests that NKT phenotype is determined during beta chain recombination during thymocyte differentiation.

Computational Biology

Immunology　Computational Biology　Bioinformatics

１．研究開始当初の背景

Natural Killer T (NKT) cell are innate-like T cells that develop in the thymus and can secrete large amounts of cytokines after TCR binding. NKT cells can also secrete cytokines in a TCR independent manner and so they have important immunoregulatory functions. NKTs have protective and pathogenic roles in several human diseases, including asthma, insulin resistance and hepatitis. NKT cells develop in the thymus following a developmental pathway shared with other T cells, and fully differentiate before migrating to the periphery. Differentiation is known to start from a progenitor state (named NKT0), and continues through different stages leading at least three NKT subtypes defined based on transcription factors and secreted cytokines profiles: NKT1 (T-bet and interferon gamma), NKT2 (IL4) and NKT17 (RORγ and IL17). In wild type BALB/c mice, NKT subtypes are produced with predominance of NKT2, followed by NKT17 and finally with NKT1 being at the lowest numbers. BALB/c SKG mice have a mutation in the ZAP70 molecule that causes a weakening of the signal transduced upon TCR activation. SKG mice show altered development of T cells and develop arthritis when exposed to the adjuvant mannan. SKG mice are used as a model of autoimmunity, in particular Rheumatoid Arthritis (RA). NKT cells are expanded in the spleen and other tissues of SKG mice compared to wild type (WT), and NKT1 is the most abundant subtype. This suggests that ZAP70 mutation affects the development of NKT cells. Zao et al. (2018) have shown that NKT1 cells have protective effects, but NKT17 cells accumulate in the synovial fluid of the joints and are associated with disease progression. TCR strength has been shown to influence NKT development and lineage differentiation. However, how changes in TCR signaling due to mutation in the downstream molecule ZAP70 influence the transcriptional program during NKT development is not fully understood.

２．研究の目的

The purpose of this project is to get insight into how TCR binding strength, weakened in the SKG mice due to a mutation in the downstream molecule ZAP70, alters the development of NKT cells. To this aim we used single cell transcriptomics, protein expression and TCR repertoire. Single cell RNA-seq will measure the transcriptome, and the expression levels of thousands of genes will enable us to characterize NKT cell subpopulations. Because NKT subtypes are defined based on the expression of protein markers and the correlation between transcription and protein levels can be poor, canonical protein markers have been selected to accurately assess NKT subtypes. Obtaining the TCR repertoire will serve two purposes. First, it will enable certify that the isolated NKT cells mostly express the invariant TCRs. Second, we will be able to assess whether TCR signaling induces a bias in the composition of the TCR repertoire, either at the level of gene usage or the antigen binding domain.

３．研究の方法

To characterize the NKT developmental pathways we isolated CD3+CD1d tetramer:αGalCer+ cells from the thymus of BALB/c (WT) and SKG mice. Cells were incubated with a cocktail of 26 Totalseq-C antibodies including proteins important for NKT development and isotype controls. Single cell droplets were obtained with the 10x Genomics Chromium system. RNA (transcriptome), CITE (protein expression) and TCR 5' sequencing libraries were prepared using the manufacturer's instructions, and sequenced in an Illumina sequencer. Sequencing reads were processed with cellranger multi (version 7.1.0). Raw counts were loaded into Python and processed with scanpy, muon and scirpy to enable joint analysis of multiome

datasets[1-3]. Doublets were identified by calculating RNA low dimensional embeddings with scVI and SOLO [4,5]. Cells identified as doublets and clusters with a high percentage of mitochondrial genes were eliminated from downstream analyses. The final low dimensional embedding was calculated with scVI and used to perform clustering of cells into cell populations with Leiden[6]. UMAP was calculated to visualize the cells in 2D scatterplots. Trajectory analysis was performed with diffusion pseudotime[7]. Genes changing along trajectories were identified with singleCellHaystack[8]. For TCR analysis clonotypes were calculated with scirpy. Cell clusters were annotated using the top cluster's markers and by manually inspecting the expression of genes and proteins known as regulators of NKT differentiation.

４．研究成果

A total of 8,848 (WT) and 8,455 (SKG) cells were kept after QC filtering for downstream analyses (Figure 1A). We performed clustering of cells on the latent embedding returned by scVI, followed by annotation using known markers of NKT differentiation (Figure 1B and 1C). The cell clusters included a small population of double positive (Cd4+Cd8a+) cells, which are thought to represent the earliest NKT progenitors. There was also a population of cells named S0 and characterized by expression of Cd24a, Gata3 and Ccr7. A population of proliferating cells was characterized by the expression of Mki67 and Top2a. Finally, three groups of subtype specific NKT cells were named NKT1 (expressing Tbx21, Ifng), NKT2 (expressing Icos, Il4) and NKT17 (expressing Rorc, Il17a). There was a group of pro-apoptotic cells with relatively high (~40%) percentage of mitochondrial genes. These cells could be further divided into subgroups representing NKT1, NKT2 and NKT17 respectively, and showed strongest expression of Zbtb16 (encoding for PLZF), suggesting they represent overactivated progenitor cells selected for apoptosis during negative selection. The division into developmental cells clusters was further validated by looking at the ordering of cell clusters along pseudotime, and by examining the corresponding protein expression patterns. For pseudotime calculation, one cell in the cluster of DP cells was selected as root. The estimated pseudotime returned a pathway suggesting the order DP -> S0 -> Pro for the initial stems of differentiation, followed by a branching into the NKT subtypes (Figure 1D). Geene expression of known differentiation markers showed a smooth transition in gene expression profile along pseudotime (Figure 1E; shown for NKT1 lineage). Protein expression of known differentiation markers followed similar patterns to the corresponding genes and agreed with this trajectory based pathway (Figure 1F). Importantly, clustering and pseudotime estimation was performed using the information from gene expression alone, and no information from the proteome modality was used. This means that protein expression serves as an independent validation of the results (within the limits of the samples used). Overall, our results suggest a smooth transition in gene expression profiles along differentiation trajectory.

We used the alpha and beta chain information to further validate the reliability of our results. Most cells had an alpha chain with TRAV11 and TRAJ18 genes, in agreement with the expected composition of invariant TCR of NKT cells. These cells were annotated as canonical. Interestingly, we found around 2% of cells that had different gene combinations for the variable and join segments of the alpha chain, which we named "Alternative" (Figure 1G). One possibility is that these cells represent contaminating cells. However, non-canonical TCR cells were not accumulated in a single cell cluster but could be found thorough the entire NKT dataset. It is possible that these cells represent doublets of NKT cells with other cells, in particular, other developing thymocytes. But most of these cells had a single full length TCR, suggesting they were not T/NKT doublets. To further clarify the identity of non-canonical TCR cells, were

examined the distribution of beta chain genes in canonical and non-canonical cells (Figure 1G). We found that NKT cells with canonical TCR had a variety of beta chain gene usage with different proportions. Interestingly, cells with alternative TCR used the same beta genes, with very similar proportions. This finding has one implication: it is possible that what defines the ability to identify lipids by the TCR is a particular combination of beta chain genes. Since the beta chain genes are firstly rearranged in the TCR locus during thymocyte development, arrangement of a particular beta chain would give the developing thymocyte the ability to recognize lipids, and then proceed with the NKT developmental pathway. During alpha chain recombination, there is a bias towards using TRAV11/TRAJ18 as genes. Although other genes might be used and may give specificity to bind to other antigens. Overall, these results identify a putative TCR beta chain signature restricting the development of NKT cells in the thymus.
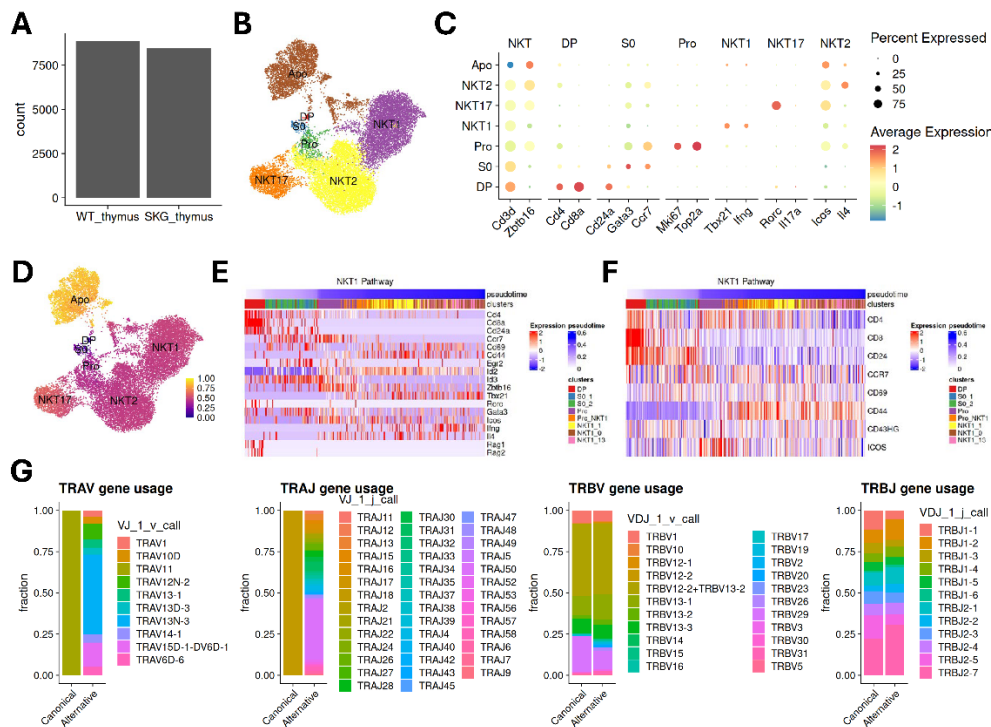

Figure 1. A) Number of cells per sample. B) 2 dimensional scatterplot used for visualization (UMAP). C) Dot plot of gene markers used to annotate cell clusters. D) Pseudotime shown in the UMAP follows the annotation of cells based on developmental markers. E) Known gene markers show a smooth transition along developmental pseudotime. F) Protein markers serve as validation of cell annotation based on gene expression modality. G) Analysis of TCR repertoire shows the existence of a relatively large (~2%) population of NKT cells with non-canonical receptor.

References

1   Wolf, F. A., Angerer, P. & Theis, F. J. SCANPY: large-scale single-cell gene expression data analysis. *Genome Biol* **19**, 15 (2018). https://doi.org:10.1186/s13059-017-1382-0

2   Bredikhin, D., Kats, I. & Stegle, O. MUON: multimodal omics analysis framework. *Genome Biol* **23**, 42 (2022). https://doi.org:10.1186/s13059-021-02577-8

3   Sturm, G., Szabo, T., Fotakis, G., Haider, M., Rieder, D., Trajanoski, Z. & Finotello, F. Scirpy: a Scanpy extension for analyzing single-cell T-cell receptor-sequencing data. *Bioinformatics* **36**, 4817-4818 (2020). https://doi.org:10.1093/bioinformatics/btaa611

4   Bernstein, N. J., Fong, N. L., Lam, I., Roy, M. A., Hendrickson, D. G. & Kelley, D. R. Solo: Doublet Identification in Single-Cell RNA-Seq via Semi-Supervised Deep Learning. *Cell Syst* **11**, 95-101 e105 (2020). https://doi.org:10.1016/j.cels.2020.05.010

5   Lopez, R., Regier, J., Cole, M. B., Jordan, M. I. & Yosef, N. Deep generative modeling for single-cell transcriptomics. *Nat Methods* **15**, 1053-1058 (2018). https://doi.org:10.1038/s41592-018-0229-2

6   Traag, V. A., Waltman, L. & van Eck, N. J. From Louvain to Leiden: guaranteeing well-connected communities. *Sci Rep-uk* **9**, 5233 (2019). https://doi.org:10.1038/s41598-019-41695-z

7   Haghverdi, L., Buttner, M., Wolf, F. A., Buettner, F. & Theis, F. J. Diffusion pseudotime robustly reconstructs lineage branching. *Nat Methods* **13**, 845-848 (2016). https://doi.org:10.1038/nmeth.3971

8   Vandenbon, A. & Diez, D. A universal tool for predicting differentially active features in single-cell and spatial genomics data. *Sci Rep-uk* **13**, 11830 (2023). https://doi.org:10.1038/s41598-023-38965-2

Diego Diez
NKT development pathways in a mouse model of autoimmune disease
GIW XXXII ISCB-ASIA VI
2023

Diego Diez
Analysis of NKT differentiation pathways in a mouse model of autoimmune disease
Bonn Conference on Mathematical Life Sciences
2023

0

0